The University Of Sheffield.

THE ROLE OF SEX-SPECIFIC SELECTION IN GENOME EVOLUTION

THEA FRANCES ROGERS

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Sheffield
School of Biosciences

Submitted November 2021

ABSTRACT

In this thesis, I use transcriptomic and genomic data from multiple bird species to investigate the genetic architecture of sexual dimorphism and to understand how sex-specific selection shapes genome evolution. I consider two routes to sexual dimorphism. Firstly, due to their unequal pattern of inheritance, sex chromosomes are hypothesised to facilitate the evolution of sexual dimorphisms by navigating functional constraints that shared regions of the genome are subject to. However, the sex chromosomes have differences in mutation rate, effective population size and recombination rate relative to the autosomes, which may act to reduce the efficacy of selection acting on them. Consistent with this, in Chapter 2, I show that the avian Z chromosome is not a hotspot of sexual conflict relative to the autosomes. Additionally, in Chapter 3, I reveal that a combination of adaptive and purifying selection are the dominant modes of evolution of the avian W chromosome. Secondly, I examine the role of differential regulation of the parts of the genome that are shared equally between males and females in the evolution of phenotypic dimorphisms. In Chapter 4, I discover an abundance of autosomal genes with sex differences in expression level and alterative splicing and suggest that differential alternative splicing evolves under sex-specific selection and facilitates sex-specific adaptation when differential expression level is limited by pleiotropic constraints. Together, my findings shed light on the role of sex-specific selection on the sex chromosomes and the autosomes in the evolution of intraspecific genetic diversity.

AUTHOR DECLARATION

I, the author, confirm that the thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Chapters 2, 3 and 4 are part of published works which are detailed at the start of each chapter along with the author contributions.

ACKNOWLEDGEMENTS

I am exceptionally grateful to my supervisor, Alison Wright, for her support both professionally and personally throughout my PhD. Her encouragement, insight and guidance have been invaluable, and she has been a brilliant role model to me since I first started in Sheffield, and will continue to be in my future career. I would like to express my gratitude to the past and present members of the Wright Lab, Daniela Palmer and Peter Price, my co-supervisor, Jon Slate, and my collaborators Becky Dean and Tom Pizzari, for making my PhD such an enjoyable experience and for their comments and contribution to this thesis. It is thanks to these people, as well as Alison, that I have been able to work in such a positive and inspiring environment for the last four years. Additionally, I would like to thank my friends in my PhD cohort, who have always been there to support me and cheer me up on the most difficult days throughout this process. Furthermore, I am extremely grateful to my parents and brothers, who have always shared and encouraged my fascination with the natural world. I am incredibly lucky that they have always taken an interest in my work and made time to listen and advise me on it wherever they could. Finally, I'd like to acknowledge my pet rabbit Bramble, for being a wonderful flatmate and a constant source of happiness to me, especially when working from home and in various lockdowns during the COVID-19 pandemic.

CONTENTS

FIGURE LIST

TABLE LIST

CHAPTER 1. INTRODUCTION

1.1 Intraspecific biodiversity

The study of biodiversity presents a way in which we can understand evolutionary processes, ontogenetic development and ecosystem function. Biodiversity is generally quantified as the variety of species in an environment, however, biodiversity also comprises an intraspecific component. Intraspecific variation is typically most pronounced when species express two or more distinct phenotypes or morphs within a panmictic population in the same habitat (Ford, 1965), or between the sexes, where organisms exhibit sexually dimorphic phenotypic traits (Andersson, 1994). Sexual dimorphism is ubiquitous across the natural world and includes differences far beyond reproductive features, such as differences in size, morphology, colouration, physiology and behaviour (Fairbairn, 2013). Therefore, researching sex differences provides a window to understand the processes giving rise to biodiversity, which has many important applications across the biological sciences and beyond.

1.2 Constraints posed by a shared genome

Males and females share the majority of their genomes, yet they often have very different evolutionary interests. Therefore, the sexes are frequently subject to different, even contradicting, selection pressures, often leading to sexual conflict. Intralocus sexual conflict arises when a phenotype that maximises fitness for one sex does not maximise fitness for the other sex and the trait has a shared genetic basis between males and females. It is therefore classified as conflict in selection pressures experienced by alleles at a single locus, resulting in distinct alleles to be favoured in each sex (Bonduriansky & Chenoweth, 2009; Van Doorn, 2009) (examples of this can be found in section 1.3). Conflict can also manifest itself as interlocus sexual conflict, where individuals of one sex interact with the other sex, usually during reproduction, but there is conflict over the outcome of the interaction between males and females (Chapman et al., 2003). One widely recognised example of interlocus sexual conflict is conflict over remating rates in *Drosophila melanogaster.* The ejaculate of *D. melanogaster* males contains seminal fluid proteins (SFPs) that substantially affect female fitness. SFPs can alter processes such as oogenesis, the timing of the onset of ovulation and sperm storage. This in turn affects the success of female fertilisation, delays remating, and

shortens the female's life span, all of which act to decrease female fitness whilst increasing male reproductive success (Hollis et al., 2019).

I focus on intralocus sexual conflict throughout this thesis, and hereafter refer to it as sexual conflict or sexual antagonism. Sexual conflict is predicted to affect a large proportion of loci across the entire genome and has the potential to substantially increase population genetic diversity through balancing selection and thereby act as a key force in adaptation (Chippindale et al., 2001). First, sexual conflict can give rise to balancing selection at sex-linked loci where frequency dependence is generated by the combination of dominance and hemizygosity (Rice et al 1984). Second, sexual antagonism can also result in balancing selection on the autosomes through heterozygote advantage that is generated either by directional selection in both sexes or directional selection in one and heterozygote advantage in the other (Connallon & Clark 2014). In the latter scenario, balancing selection is predicted to be weak and not particularly robust towards drift but can affect loci across the entire genome (Connallon & Clark 2014).

Conversely, there is also evidence that unresolved sexual conflict hinders sexual coevolution. This is because the carrier of any novel sexual characters endures indirect costs that offset its advantages (Chippindale et al., 2001; Kirkpatrick, 2009; Pischedda & Chippindale, 2006). For example, under sexual conflict, the most successful males have been found to sire low-quality daughters, and the benefit of choosing high-fitness males will be diminished even further if several male-advantageous loci are X-linked and are accordingly never passed from father to son (Pischedda & Chippindale, 2006). These circumstances can even cause the counterintuitive evolution of female preference for low-quality males (Albert & Otto, 2005). Nevertheless, sexual antagonism has implications for subjects such as genome organization, sexual selection, population sex ratios and aging (Van Doorn, 2009), although there are still many questions about how it can be resolved and the consequences of this for genome evolution.

1.3 Empirical evidence for intralocus sexual conflict

Sex-specific selection and sex-specific genetic fitness effects were first revealed many years ago (Prout, 1971; Woolf & Church, 1963), however, direct support for sexual conflict is more recent. Experimental evidence is founded on estimates of selection in males and females,

combined with r_{mf}, which is defined as the genetic correlation in phenotypic traits or fitness between the sexes. Trait-based, correlational studies across a range of species have shown that many phenotypes share a similar genetic basis in males and females, setting the stage for intralocus sexual conflict. For instance, positive estimates of r_{mf} have been calculated for locomotory activity in *Drosophila* (Long & Rice, 2007), body size in collared flycatcher (*Ficedula albicollis*) (Merilä et al., 1997, 1998), and bill colour in the zebra finch (*Taeniopygia gutta*) (Price & Burley, 1993, 1994). However, the sexes have very different reproductive strategies that generate divergent, sex-specific selection on many traits with a shared genetic basis (Hedrick & Temeles, 1989). For example, shared traits such as tail feathers, colour spots or behavioural characteristics are frequently exposed to different selection pressures in males and females, and each sex may occupy a distinct ecological niche (Shine, 1989). Consequently, selection in one sex can displace the other sex from its phenotypic optimum (Lande, 1980).

Fitness-based correlational studies have revealed negative r_{mf} for fitness in lab-adapted *Drosophila melanogaster* adults (Chippindale et al., 2001), ground crickets (*Allonemobius socius*) (Fedorka & Mousseau, 2004), natural populations of red deer (*Cervus elaphus*) (Foerster et al., 2007) and the collard flycatcher (Brommer et al., 2007), indicating substantial sexual conflict because alleles that are beneficial when expressed in one sex are harmful when expressed in the other sex. Fitness-based experimental studies in *D. melanogaster* also show increased fitness in one sex when selection is relaxed or halted in the other sex (Morrow et al., 2008; Prasad et al., 2007; Rice, 1996b, 1998), providing further evidence for sexual conflict.

1.4 Resolving sexual conflict

Because intralocus sexual conflict is the result of shared genetic architecture, it is resolved when a locus with sex-specific beneficial effects does not negatively affect fitness in the other sex, whereas interlocus sexual conflict is resolved when there is no longer conflict over the outcome of the interaction between males and females. Resolution of intralocus conflict is predicted to lead to the evolution of sexual dimorphism whereas interlocus conflict is thought to generate an evolutionary arms race between the sexes. In theory, it can be resolved in a multiplicity of ways, all of which involve limiting or biasing expression of alleles to the sex in which their effects are advantageous. For instance, sex chromosomes are predicted to be hotspots of sexual conflict resolution as their unequal inheritance pattern facilitates

evolutionary divergence between the sexes through the decoupling of male and female phenotypes (Rice, 1984; Wright & Mank, 2013). Indeed, it has been suggested that the repeated and independent formation of sex chromosomes across the eukaryotes has occurred in response to selection to resolve sexual conflict (Bachtrog et al., 2014; Roberts et al., 2009; Van Doorn & Kirkpatrick, 2007). However, sex chromosomes are often very small and contain few genes, and some species even lack them altogether (Bachtrog et al., 2011). Because of this, in addition to the evolution of the sex chromosomes, autosomal gene regulation is widely assumed to resolve conflicting sex-specific selection pressures. A growing body of evidence suggests methods of gene regulation, such as chromatin remodelling, DNA methylation, gene expression level, alternative splicing and small RNA regulators differ substantially between the sexes in many species (Bezler et al., 2019; Garcia-Moreno et al., 2019; Gibilisco et al., 2016; Jiang et al., 2011; Mank et al., 2008; Mathers et al., 2019; McCarthy et al., 2014; Tsai et al, 2009). The study of gene regulation may therefore be key to understanding the selection pressures shaping the evolution of complex phenotypes.

However, while we have a rich body of theoretical work outlining how conflict is predicted to be resolved, empirical evidence for some regulatory mechanisms of conflict resolution is lacking and many questions remain. For example, do different mechanisms resolve sexual antagonism to different extents? Do some mechanisms function as evolutionary unstable, temporary resolutions? Are different sex-determination systems responsive to different means of conflict resolution (Bonduriansky & Chenoweth, 2009)? Furthermore, it remains unclear how quickly and fully sexual conflict is resolved. It is probable that sexual conflict is fully resolved at some loci affecting fundamental sexual traits. These loci can experience a consistent pattern of sex-specific selection over numerous generations, granting sufficient time for sexual conflict resolution mechanisms to evolve. Nevertheless, it is unlikely that sexual conflict is ever fully resolved throughout the genome (Fairbairn et al., 2007).

1.4.1 Sex chromosomes as a route to sex-specific adaptation

Sex chromosomes genetically determine the sex of many sexually reproducing organisms. Male heterogamety is observed in all mammals, some insects, some snakes, some fish and some plants. Sex determination is Y-centred in most of these organisms (Fig. 1.1A), i.e. the existence of a Y chromosome determines maleness due to the presence of testis-determining genes on the Y chromosome, for example the *SRY* gene in mammals (Capel & Lovell-Badge,

1993). However, in other organisms such as *Drosophila*, sex determination is dosage dependent, where the presence of two X chromosomes determines femaleness and the presence of one X chromosome determine maleness (Fig. 1.1B). XX/X0 sex chromosome systems are found in many other insects, including cockroaches (order Blattodea), grasshoppers and crickets (order Orthoptera), and a small number of mammals (several rodent species) (Fig. 1.1C). Female heterogamety is found mainly in birds, along with some reptiles and some other insects, including Lepidoptera. In this system, females are the heterogametic sex (ZW) and males are the homogametic sex (ZZ) (Fig. 1.1D). In all birds, sex is determined by *DMRT1* (Smith et al., 2009) in a dosage dependent mechanism i.e. sex is determined by the number of DMRT1 copies (Hirst et al., 2017; Stiglec et al., 2007).

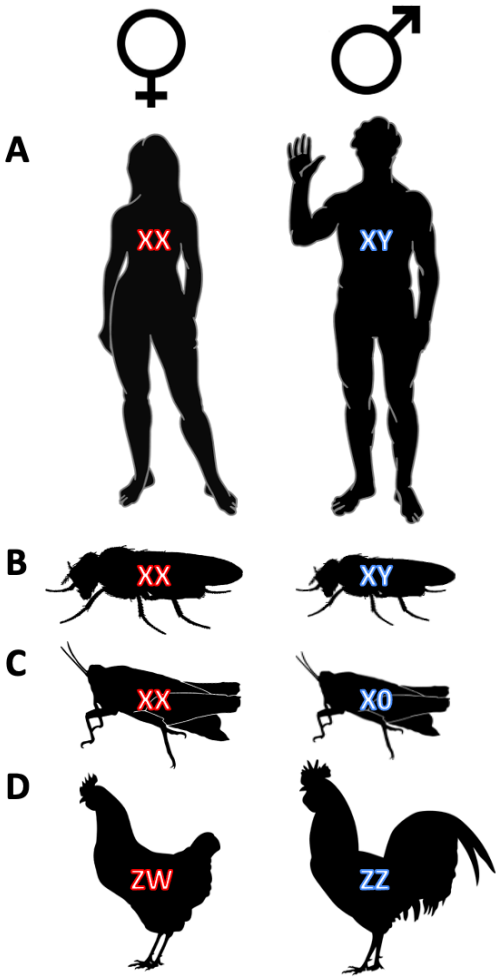

Figure 1.1. Male and female heterogametic sex chromosomes. (**A, B**) The XX/XY sex chromosome system in which males are the heterogametic sex. (**C**) The XX/X0 sex chromosome system, shown here in grasshoppers (*Caelifera*). The 0 denotes the absence of a Y chromosome. (**D**) The ZW/ZZ sex chromosome system, in which females are the heterogametic sex, shown in chickens (*Gallus gallus domesticus*).

Due to the different evolutionary environments they inhabit and depending on whether they are dominant or recessive, alleles on the X, Y, Z and W chromosomes experience different magnitudes of sex-specific selection (Rice, 1984). Male-specific selection is stronger than female-specific selection on dominant Z-linked alleles because the Z is present more often in males than females. However, Z-linked recessive alleles are more exposed to female-specific selection than male-specific selection due to female hemizygosity. Conversely, dominant X-linked alleles are under stronger female-specific than male-specific selection because the X is more often present in females, whereas recessive X-linked alleles experience stronger male-specific selection due to male hemizygosity. Therefore, studying both XX/XY and ZZ/ZW sex chromosome systems presents a way in which we can test predictions of how selection acts differently between the sexes. So far, most research has concentrated on XX/XY systems and ZZ/ZW sex chromosomes are relatively understudied in comparison.

The difference between the selection regimes that alleles on the different sex chromosomes and the autosomes undergo helps to explain why the sex chromosomes might be able to facilitate sex-specific adaption and show unique patterns of gene content and expression. Although not consistent across all species, there is ample evidence for sex-specific adaptation on the sex chromosomes. Genes expressed in male-specific tissues are underrepresented on the X chromosomes of *Caenorhabditis elegans* (Reinke et al., 2000) and mosquitos (Magnusson et al., 2012), well as both the old and neo-X chromosomes in numerous *Drosophila* species (Betrán et al., 2002; Chenoweth et al., 2015; Parisi et al., 2003; Sturgill et al., 2007). These studies suggest that the X has become demasculinised, in some cases over short evolutionary time periods for the species with newer X chromosomes. As well as finding a paucity of male-biased genes, some studies also uncover an overrepresentation of X-linked genes expressed in the ovaries in *Drosophila* (Allen et al., 2013; Zhou & Bachtrog, 2012). Genes expressed in the ovary, the placenta or in early spermatogenesis are also enriched on the X chromosome in mice, while genes expressed during the later stages of spermatogenesis are lacking (Divina et al., 2005; Khil et al., 2004; Wang et al., 2001), most likely as a consequence of early X chromosome inactivation during spermatogenesis (Betrán et al., 2002; Wu & Xu, 2003). These studies indicate that the X chromosome has become both demasculinised and feminised. On the other hand, the avian Z chromosome has been defeminised (Mank & Ellegren, 2009) and masculinised (Wright et al., 2012). The silkworm Z

(*Bombyx mori*) has also been revealed to be masculinised (Arunkumar et al., 2009). These patterns are consistent with sex-specific selection influencing gene content on the X and Z chromosomes. Many unanswered questions still remain however, including, what is the relative role of selection versus drift in creating the gene content and expression patterns we see on the sex chromosomes? Additionally, due to differences in mutation rate, effective population size and recombination rate relative to the autosomes (Wright & Mank, 2013), it remains unclear if sex chromosomes are actually hotspots of sexual conflict, as these features could act to reduce the efficiency of selection operating on the sex chromosomes.

In contrast, the sex-limited Y and W chromosomes are not subject to sexual conflict and are therefore predicted to follow different evolutionary trajectories and act as an important site for sex-specific traits to evolve. Y-linked genes are predicted to be selected for male-specific traits and W-linked genes for female-specific traits. There is some empirical evidence in support of this theory. For instance, the non-recombining part of the human Y chromosome contains few genes, all which have roles in spermatogenesis and sex determination (Lahn et al., 2001). However, because parts of the Y and W do not recombine, they are inclined to accumulate deleterious mutations, ultimately resulting in an almost total degeneration of these chromosomes in some species (Charlesworth, 1991). How these sex-limited chromosomes resist these degenerative forces is still a source of debate.

Gene duplication combined with gene conversion between duplicate pairs is thought to give rise to sex-specific adaptation (Connallon & Clark, 2010). Consistent with this, most remaining, functional, Y-linked genes are members of duplicate gene pairs found within large palindromes, and almost all are expressed exclusively in the testes and have male-specific functions. Unlike many of the single-copy genes, genes in these Y-linked families are not degenerating, but have become fixed and preserved on the Y chromosome over millions of years (Skaletsky et al., 2003). Available data suggests that gene duplication is common on the mammalian and *Drosophila* Y chromosome (Geraldes et al., 2010; Krsticevic et al., 2010; Rozen et al., 2003), and is thought to be driven by sperm competition. Interestingly, evidence for ampliconic gene families on the W chromosome is lacking, with the exception of the HINTW gene family on the avian W (Ceplitis & Ellegren, 2004). This may be consistent with lack of sperm competition on the W chromosome but the role of sex-specific selection in driving gene duplication on the W chromosome is unclear. HINTW is the only known

ampliconic gene on the avian W chromosome and therefore hypothesized to play an important role in female reproduction and oogenesis in birds (Moghadam et al., 2012). However, the extent to which female-specific selection versus other factors such as meiotic drive has driven gene amplification is unknown.

1.4.2. Sex chromosome turnover

Sex chromosomes evolve once recombination is halted between a homologous pair of autosomes. The most widely accepted model of sex chromosome evolution is the sexual conflict model (Fisher, 1931; Bull, 1985; Rice, 1996). This predicts that sex chromosomes evolve when an autosome acquires a sex determining locus. Physical proximity between the sex determining locus and nearby sexually antagonistic alleles selects for recombination suppression between the proto sex chromosomes. This might act to resolve sexual conflict by creating a supergene in which the sex-determining allele and sexually antagonistic alleles are linked. The region over which recombination is supressed is called a stratum. Additional evolutionary strata can be formed when the non-recombining region is expanded to encompass additional sexually antagonistic alleles.

Many clades experience frequent sex chromosome turnover, which has significant implications for adaptation, sexual dimorphism and speciation. A variety of hypotheses have been proposed to explain why transitions to new sex chromosome systems occur (reviewed in Vicoso, 2019). These include sexual conflict (Van Doorn & Kirkpatrick, 2007; 2010), mutation load on the sex-limited chromosomes (Blaser et al., 2013; Blaser et al., 2014), selection on sex ratio (Jaenike, 2001; Werren & Beukeboom, 1998) and genetic drift (Bull & Charnov, 1977; Saunders et al., 2018). Direct empirical evidence supports sexually antagonistic selection driving the evolution of a novel sex determining locus in cichlids (Roberts et al. 2009) as well as a neo sex chromosome in sticklebacks (Kitano et al. 2009) and the Y in guppies (Wright et al., 2017) as well as mutation-load selection driving sex chromosome turnover in frogs (Jeffries et al., 2018). The relative importance of each of these mechanisms remains unclear, however, it will be possible to effectively test these theories as more sex chromosomes are identified.

1.4.3 Autosomal gene regulation as a route to sex-specific adaptation

Gene regulation includes a variety of processes that alter the production of gene product, namely protein or RNA, by cells. Changes in gene regulation may be permanent or transient and each mechanism is commonly used to trigger developmental pathways as well as for environmental acclimation. Furthermore, gene regulation can differ between the sexes and therefore might be involved in the evolution of sex differences. Almost any step of the gene expression pathway can be modified. In this thesis, I focus on the transcriptional mechanism differential gene expression level, and the post-transcriptional mechanism alternative splicing, and their role in facilitating the evolution of sexually dimorphic phenotypes.

In theory, sex-biased gene expression can resolve sexual conflict and lead to sex-specific adaptation through the breakdown in inter-sexual correlations in expression level (Connallon & Knowles, 2005). Sex-specific gene expression level is widespread and well-documented across the animal kingdom, appearing in birds (Mank et al., 2008; Naurin et al., 2011), insects (Jin et al., 2001; Marinotti et al., 2006; Ometto et al., 2011; Ranz et al., 2003), mice (Yang et al., 2006), fish (Wright et al., 2018) and *C. elegans* (Cutter & Ward, 2005) and occurs at both the species (Zhang et al., 2007) and population level (Moghadam et al., 2012; Müller et al., 2011). Much of this variation in gene expression is presumed to be brought about from androgen- or oestrogen-mediated regulation (Zauner et al., 2003). Moreover, some patterns of differential gene expression are known to be condition-dependent, which is expected for some classes of sexually-selected traits (Wyman et al., 2010).

Many studies have uncovered faster rates of coding sequence evolution in sex-biased genes across an array of species due to positive selection (Ellegren & Parsch, 2007; Good & Nachman, 2005; Khaitovich et al., 2005) as well as genetic drift due to relaxed purifying selection (Gershoni & Pietrokovski, 2014; Harrison et al., 2015). Furthermore, accelerated divergence in gene expression level, possibly due to sex-specific selection, has been found in some species (Harrison et al., 2015; Meiklejohn et al., 2003). It should be noted however, that sex-biased genes do not necessarily affect sex-specific fitness and encode sex-specific phenotypes. It is difficult to tie large amounts of gene expression data to particular phenotypes, although a study by Connallon & Clark (2011) does provide direct support for this relationship. Furthermore, it is possible for an allele to attain sex-limited expression without being located on a sex chromosome, or requiring a breakdown of intersexual

correlations in gene regulation, through a process called genomic imprinting. The expression of an imprinted allele is contingent on the parent of the origin. Studies have suggested that imprinting can resolve intralocus sexual conflict because sex-limited genes are able to avoid fitness penalties in the sex where expression is absent (Day & Bonduriansky, 2004; Van Cleve & Feldman, 2007).

Pleiotropy, where a gene influences the development of multiple traits, affects the degree to which selection can facilitate sex-specific adaptation (Harano et al., 2010). It has been suggested that pleiotropy impedes the breakdown of inter-sexual correlations in gene expression, because genes that are more broadly expressed, a proxy for pleiotropy, are less often sex-biased and have slower rates of evolutionary divergence relative to genes with more tissue-specific expression patterns (Mank et al., 2008; Meisel, 2011). This supports the extensively accepted theory that pleiotropy is an evolutionary constraint (Orr, 2000; Snell-Rood et al., 2010).

Importantly, change in expression level is only one of many gene regulation mechanisms and the role of other processes in sex-specific adaptation and sexual conflict resolution have been overlooked. For example, alternative splicing is a mechanism that produces multiple different transcripts from the same locus and, through the generation of separate male and female isoforms, could be a route to resolve sexual antagonism and lead to sex-specific adaptation. So far, only a handful of studies have provided evidence for significant sex differences in alternative splicing (Blekhman et al., 2010; Chang et al., 2011; Gibilisco et al., 2016; Mazin et al., 2021; Naftaly et al., 2021; Prince et al., 2010; Telonis-Scott et al., 2009). Research on the evolution of alternative splicing indicates that splice variants are adaptive, and are advantageous because they enable an increase in proteomic complexity without a corresponding increase in genome size (Barbosa-Morais et al., 2012; Merkin et al., 2012). Sex-specific splicing is a key element of sex determination in *Drosophila* (Telonis-Scott et al., 2009), but other than this, evidence for the exact role of alternative splicing in sexual dimorphic phenotypes is lacking. Moreover, little is known about how sex-specific isoforms evolve in response to sex-specific selection, and how alternative splicing and differential gene expression work together to facilitate the transcriptional architecture of sexual dimorphism is unknown.

1.5 Study system

To test the role of sex-specific selection in genome evolution, I used birds as my study system. Avian species are ideal for studies such as this for several reasons. Firstly, they often exhibit strong sexual dimorphism, and I chose species that are exposed to different magnitudes of sex-specific selection. Secondly, birds can be reared in controlled conditions, which is essential for transcriptomics studies. Minimising environmental variation is crucial to limit transcriptional noise that could mask sex differences in expression. The RNA-seq samples used in this thesis were taken from semi-wild captive populations and all birds were sampled at the same age. Thirdly, the avian genome has high genomic stability with sequence conservation and synteny across broad evolutionary time periods (Ellegren, 2005). As a result, sex-specific selection acts on a relatively static genome which allows for more accurate evolutionary contrasts. Additionally, reference genomes are available for all species used.

1.6 Thesis outline

The following data chapters will address some of outstanding questions discussed above. In Chapter 2 (Wright, Rogers et al., 2019), 'Contrasting patterns of sexual conflict on the avian Z chromosome relative to the autosomes', I address whether sexual conflict is abundant on the Z chromosome, in order to decipher the extent to which this chromosome has the potential to lead to sex-specific adaptation relative to the autosomes. In Chapter 3 (Rogers, Pizzari et al., 2021), 'Multi-copy gene family evolution on the avian W chromosome', I evaluate the number and variability of ampliconic gene families on the avian W and attempt to assess whether it evolves under sex-specific selection, purifying selection or genetic drift. In Chapter 4 (Rogers, Palmer et al., 2021), 'Sex-specific selection drives the evolution of alternative splicing in birds', I quantify sex differences in alternative splicing across three bird species. I assess whether alternative splicing plays a role in producing sexually dimorphic phenotypes and whether it evolves under sex-specific selection. In addition to this, I uncover whether sex-biased alternative splicing and sex-biased gene expression level work together or play distinct roles in sex-differences.

1.7 REFERENCES

Albert, A. Y. K., & Otto, S. P. (2005). Evolution: Sexual selection can resolve sex-linked sexual antagonism. *Science*, *310*(5745), 119–121.

Allen, S. L., Bonduriansky, R., & Chenoweth, S. F. (2013). The genomic distribution of sex-biased genes in *Drosophila serrata*: X chromosome demasculinization, feminization, and hyperexpression in both sexes. *Genome Biology and Evolution*, *5*(10), 1986–1994.

Andersson, M. (1994). *Sexual selection*. Princeton, NJ: Princeton University Press.

Arunkumar, K. P., Mita, K., & Nagaraju, J. (2009). The silkworm Z chromosome is enriched in testis-specific genes. *Genetics*, *182*(2), 493–501.

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W. R., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*(9), 350–357.

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T. L., … Vamosi, J. C. (2014). Sex determination: why so many ways of doing it? *PLoS Biology*, *12*(7), 1–13.

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., Gueroussov, S., Lee, L. J., … Blencowe, B. J. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science*, *338*(6114), 1587–1593.

Betrán, E., Thornton, K., & Long, M. (2002). Retroposed new genes out of the X in *Drosophila*. *Genome Research*, 12(12), 1854–1859.

Bezler, A., Braukmann, F., West, S. M., Duplan, A., Conconi, R., Schütz, F., … Keller, L. (2019). Tissue- and sex-specific small RNAomes reveal sex differences in response to the environment. *PLoS Genetics*, *15*(2), e1007905.

Blaser, O., Grossen, C., Neuenschwander, S., & Perrin, N. (2013). Sex-chromosome turnovers induced by deleterious mutation load. *Evolution*, *67*(3), 635–645.

Blaser, O., Neuenschwander, S., & Perrin, N. (2014). Sex-chromosome turnovers: the hot-potato model. *The American Naturalist*, *183*(1), 140–146.

Blekhman, R., Marioni, J. C., Zumbo, P., Stephens, M., & Gilad, Y. (2010). Sex-specific and lineage-specific alternative splicing in primates. *Genome Research*, *20*(2), 180–189.

Bonduriansky, R., & Chenoweth, S. F. (2009). Intralocus sexual conflict. *Trends in Ecology and Evolution*, 24(5), 280–288.

Brommer, J. E., Kirkpatrick, M., Qvamström, A., & Gustafsson, L. (2007). The intersexual genetic correlation for lifetime fitness in the wild and its implications for sexual selection. *PLoS ONE*, *2*(8), e744.

Bull, J. J. (1985). *Evolution of sex determining mechanisms.* Benjamin-Cummings Publishing Co., Subs. of Addison Wesley Longman, US.

Bull, James J., & Charnov, E. L. (1977). Changes in the heterogametic mechanism of sex determination. *Heredity*, *39*, 1–14.

Capel, B., & Lovell-Badge, R. (1993). The *sry* gene and sex determination in mammals. *Advances in Developmental Biology, 2*, 1–35.

Ceplitis, H., & Ellegren, H. (2004). Adaptive molecular evolution of HINTW, a female-specific gene in birds. *Molecular Biology and Evolution*, *21*(2), 249–254.

Chang, P. L., Dunham, J. P., Nuzhdin, S. V., & Arbeitman, M. N. (2011). Somatic sex-specific transcriptome differences in *Drosophila* revealed by whole transcriptome sequencing. *BMC Genomics*, *12*, 364.

Chapman, T., Arnqvist, G., Bangham, J., & Rowe, L. (2003). Sexual conflict. *Trends in Ecology and Evolution, 18*(1), 41-47.

Charlesworth, B. (1991). The evolution of sex chromosomes. *Science*, *251*(4997), 1030–1033.

Chenoweth, S. F., Appleton, N. C., Allen, S. L., & Rundle, H. D. (2015). Genomic evidence that sexual selection impedes adaptation to a novel environment. *Current Biology*, *25*(14), 1860–1866.

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(4), 1671–1675.

Connallon, T., & Clark, A. G. (2010). Gene duplication, gene conversion and the evolution of the Y chromosome. *Genetics*, *186*(1), 277–286.

Connallon, T., & Clark, A. G. (2011). Association between sex-biased gene expression and mutations with sex-specific phenotypic consequences in *Drosophila. Genome Biology and Evolution*, *3*, 151–155.

Connallon, T. & Clark, A. G. (2014). Balancing selection in species with separate sexes: insights from Fisher's geometric model. *Genetics, 197*(3), 991–1006.

Connallon, T., & Knowles, L. L. (2005). Intergenomic conflict revealed by patterns of sex-

biased gene expression. *Trends in Genetics*, *21*(9), 495–499.

Cutter, A. D., & Ward, S. (2005). Sexual and temporal dynamics of molecular evolution in *C. elegans* development. *Molecular Biology and Evolution*, *22*(1), 178–188.

Day, T., & Bonduriansky, R. (2004). Intralocus sexual conflict can drive the evolution of genomic imprinting. *Genetics*, *167*(4), 1537–1546.

Divina, P., Vlček, Č., Strnad, P., Pačes, V., & Forejt, J. (2005). Global transcriptome analysis of the C57BL/6J mouse testis by SAGE: Evidence for nonrandom gene order. *BMC Genomics*, *6*, 29.

Ellegren, H. (2005). The avian genome uncovered. *Trends in Ecology and Evolution*, *20(*4), 180-186.

Ellegren, H., & Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene expression. *Nature Reviews Genetics*, *8*, 689-698.

Fairbairn, D. J. (2013). *Odd couples: extraordinary differences between the sexes in the animal kingdom*. Princeton, NJ: Princeton University Press.

Fairbairn, Daphne J., Blanckenhorn, W. U., & Székely, T. (2007). *Sex, size and gender roles: evolutionary studies of sexual size dimorphism.* Oxford: Oxford University Press.

Fedorka, K. M., & Mousseau, T. A. (2004). Female mating bias results in conflicting sex-specific offspring fitness. *Nature*, *429*(6987), 65–67.

Fisher, R. A. (1931). The evolution of dominance. *Biological Reviews*, *6*(4), 345–368.

Foerster, K., Coulson, T., Sheldon, B. C., Pemberton, J. M., Clutton-Brock, T. H., & Kruuk, L. E. B. (2007). Sexually antagonistic genetic variation for fitness in red deer. *Nature*, *447*(7148), 1107–1110.

Ford, E. B. (1965). *Genetic polymorphism.* Faber and Faber: London.

Garcia-Moreno, S. A., Futtner, C. R., Salamone, I. M., Gonen, N., Lovell-Badge, R., & Maatouk, D. M. (2019). Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Developmental Biology*, *446*(2), 168–179.

Geraldes, A., Rambo, T., Wing, R. A., Ferrand, N., & Nachman, M. W. (2010). Extensive gene conversion drives the concerted evolution of paralogous copies of the *sry* gene in European rabbits. *Molecular Biology and Evolution*, *27*(11), 2437–2440.

Gershoni, M., & Pietrokovski, S. (2014). Reduced selection and accumulation of deleterious mutations in genes exclusively expressed in men. *Nature Communications*, *5*(1), 4438.

Gibilisco, L., Zhou, Q., Mahajan, S., & Bachtrog, D. (2016). Alternative splicing within and between *Drosophila* species, sexes, tissues, and developmental stages. *PLoS Genetics*, *12*(12), e1006464.

Good, J. M., & Nachman, M. W. (2005). Rates of protein evolution are positively correlated with developmental timing of expression during mouse spermatogenesis. *Molecular Biology and Evolution*, *22*(4), 1044–1052.

Harano, T., Okada, K., Nakayama, S., Miyatake, T., & Hosken, D. J. (2010). Intralocus sexual conflict unresolved by sex-limited trait expression. *Current Biology*, *20*(22), 2036–2039.

Harrison, P. W., Wright, A. E., Zimmer, F., Dean, R., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(14), 4393–4398.

Hedrick, A. V, & Temeles, E. J. (1989). The evolution of sexual dimorphism in animals: hypotheses and tests. *Trends in Ecology & Evolution*, *4*(5), 4–6.

Hirst, C. E., Major, A. T., Ayers, K. L., Brown, R. J., Mariette, M., Sackton, T. B., & Smith, C. A. (2017). Sex reversal and comparative data undermine the W chromosome and support Z-linked DMRT1 as the regulator of gonadal sex differentiation in birds. *Endocrinology*, *158*(9), 2970–2987.

Sexual conflict drives male manipulation of female postmating responses in *Drosophila melanogaster*. Hollis, B., Koppik, M., Wensing, U. K., Ruhmann, H., Genzoni, E., Erkosar, B., … Keller, L. (2019). *Proceedings of the National Academy of Sciences, 116*(17), 8437–8444.

Jaenike, J. (2001). Sex chromosome meiotic drive. *Annual Review of Ecology and Systematics*, 32, 25–49.

Jeffries, D. L., Lavanchy, G., Sermier, R., Sredl, M. J., Miura, I., Borzée, A., … Perrin, N. (2018). A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. *Nature Communications, 9*(1), 4088.

Jiang, Z.-F., Croshaw, D. A., Wang, Y., Hey, J., & Machado, C. A. (2011). Enrichment of mRNA-like noncoding RNAs in the divergence of *Drosophila* males. *Molecular Biology and Evolution*, *28*(4), 1339–1348.

Jin, W., Riley, R. M., Wolfinger, R. D., White, K. P., Passador-Gurgell, G., & Gibson, G. (2001). The contributions of sex, genotype and age to transcriptional variance in *Drosophila*

melanogaster. Nature Genetics, *29*(4), 389–395.

Khaitovich, P., Hellmann, I., Enard, W., Nowick, K., Leinweber, M., Franz, H., … Pääbo, S. (2005). Evolution: Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. *Science*, *309*(5742), 1850–1854.

Khil, P. P., Smirnova, N. A., Romanienko, P. J., & Camerini-Otero, R. D. (2004). The mouse X chromosome is enriched for sex-biased genes not subject to selection by meiotic sex chromosome inactivation. *Nature Genetics*, *36*(6), 642–646.

Kirkpatrick, M. (2009). Patterns of quantitative genetic variation in multiple dimensions. *Genetica*, *136*(2), 271–284.

Krsticevic, F. J., Santos, H. L., Januário, S., Schrago, C. G., & Carvalho, A. B. (2010). Functional copies of the Mst77F gene on the Y chromosome of *Drosophila melanogaster*. *Genetics*, *184*(1), 295–307.

Lahn, B. T., Pearson, N. M., & Jegalian, K. (2001). The human Y chromosome, in the light of evolution. *Nature Reviews Genetics*, *2*, 207–216.

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, *34*(2), 292.

Long, T. A. F., & Rice, W. R. (2007). Adult locomotory activity mediates intralocus sexual conflict in a laboratory-adapted population of *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1629), 3105–3112.

Magnusson, K., Lycett, G. J., Mendes, A. M., Lynd, A., Papathanos, P. A., Crisanti, A., & Windbichler, N. (2012). Demasculinization of the *Anopheles gambiae* X chromosome. *BMC Evolutionary Biology*, *12*, 69.

Mank, J. E., & Ellegren, H. (2009). Sex-linkage of sexually antagonistic genes is predicted by female, but not male, effects in birds. *Evolution*, *63*(6), 1464–1472.

Mank, J. E., Hultin-Rosenberg, L., Webster, M. T., & Ellegren, H. (2008). The unique genomic properties of sex-biased genes: Insights from avian microarray data. *BMC Genomics*, *9*, 148.

Mank, J. E., Hultin-Rosenberg, L., Zwahlen, M., & Ellegren, H. (2008). Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. *American Naturalist*, *171*(1), 35–43.

Marinotti, O., Calvo, E., Nguyen, Q. K., Dissanayake, S., Ribeiro, J. M. C., & James, A. A. (2006). Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect*

Molecular Biology, *15*(1), 1–12.

Mathers, T. C., Mugford, S. T., Percival-Alwyn, L., Chen, Y., Kaithakottil, G., Swarbreck, D., … van Oosterhout, C. (2019). Sex-specific changes in the aphid DNA methylation landscape. *Molecular Ecology*, *28*(18), 4228–4241.

Mazin, P. V., Khaitovich, P., Cardoso-Moreira, M., & Kaessmann, H. (2021). Alternative splicing during mammalian organ development. *Nature Genetics*, *53*(6), 925–934.

McCarthy, N. S., Melton, P. E., Cadby, G., Yazar, S., Franchina, M., Moses, E. K., … Hewitt, A. W. (2014). Meta-analysis of human methylation data for evidence of sex-specific autosomal patterns. *BMC Genomics*, *15*, 981.

Meiklejohn, C. D., Parsch, J., Ranz, J. M., & Hartl, D. L. (2003). Rapid evolution of male-biased gene expression in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9894–9899.

Meisel, R. P. (2011). Towards a more nuanced understanding of the relationship between sex-biased gene expression and rates of protein-coding sequence evolution. *Molecular Biology and Evolution*, *28*(6), 1893–1900.

Merilä, J., Sheldon, B. C., & Ellegren, H. (1997). Antagonistic natural selection revealed by molecular sex identification of nestling collared flycatchers. *Molecular Ecology*, *6*(12), 1167–1175.

Merilä, J., Sheldon, B. C., & Ellegren, H. (1998). Quantitative genetics of sexual size dimorphism in the collared flycatcher, *Ficedula albicollis*. *Evolution*, *52*(3), 870–876.

Merkin, J., Russell, C., Chen, P., & Burge, C. B. (2012). Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science*, *338*(6114), 1593–1599.

Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S., & Mank, J. E. (2012). W chromosome expression responds to female-specific selection. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(21), 8207–8211.

Morrow, E. H., Stewart, A. D., & Rice, W. R. (2008). Assessing the extent of genome-wide intralocus sexual conflict via experimentally enforced gender-limited selection. *Journal of Evolutionary Biology*, *21*(4), 1046–1054.

Müller, L., Hutter, S., Stamboliyska, R., Saminadin-Peter, S. S., Stephan, W., & Parsch, J. (2011). Population transcriptomics of *Drosophila melanogaster* females. *BMC Genomics*, *12*, 81.

Naftaly, A. S., Pau, S., & White, M. A. (2021). Long-read RNA sequencing reveals widespread

sex-specific alternative splicing in threespine stickleback fish. *Genome Research*, *31*(8), 1486–1497.

Naurin, S., Hansson, B., Hasselquist, D., Kim, Y. H., & Bensch, S. (2011). The sex-biased brain: Sexual dimorphism in gene expression in two species of songbirds. *BMC Genomics*, *12*, 37.

Ometto, L., Shoemaker, D., Ross, K. G., & Keller, L. (2011). Evolution of gene expression in fire ants: The effects of developmental stage, caste, and species. *Molecular Biology and Evolution*, *28*(4), 1381–1392.

Orr, H. A. (2000). Adaptation and the cost of complexity. *Evolution*, *54*(1), 13–20.

Parisi, M., Nuttall, R., Naiman, D., Bouffard, G., Malley, J., Andrews, J., … Oliver, B. (2003). Paucity of genes on the *Drosophila* X chromosome showing male-biased expression. *Science*, *299*(5607), 697–700.

Pischedda, A., & Chippindale, A. K. (2006). Intralocus sexual conflict diminishes the benefits of sexual selection. *PLoS Biology*, *4*(11), 2099–2103.

Prasad, N. G., Bedhomme, S., Day, T., & Chippindale, A. K. (2007). An evolutionary cost of separate genders revealed by male-limited evolution. *American Naturalist*, *169*(1), 29–37.

Price, D. K., & Burley, N. T. (1994). Constraints on the evolution of attractive traits: Selection in male and female zebra finches. *American Naturalist*, *144*(6), 908–934.

Price, Donald K., & Burley, N. T. (1993). Constraints on the evolution of attractive traits: Genetic (co)variance of zebra finch bill colour. *Heredity*, *71*(4), 405–412.

Prince, E. G., Kirkland, D., & Demuth, J. P. (2010). Hyperexpression of the X chromosome in both sexes results in extensive female bias of X-linked genes in the flour beetle. *Genome Biology and Evolution*, *2*, 336–346.

Prout, T. (1971). The relation between fitness components and population prediction in *Drosophila*. II: Population prediction. *Genetics*, *68*(1), 151–167.

Ranz, J. M., Castillo-Davis, C. I., Meiklejohn, C. D., & Hartl, D. L. (2003). Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science*, *300*(5626), 1742–1745.

Reinke, V., Smith, H. E., Nance, J., Wang, J., Van Doren, C., Begley, R., … Kim, S. K. (2000). A global profile of germline gene expression in *C. elegans*. *Molecular Cell*, *6*(3), 605–616.

Rice, W. R. (1984). Sex chromosomes and the evolution of sexual dimorphism. *Evolution*,

38(4), 735–742.

Rice, W. R. (1996a). Evolution of the Y sex chromosome in animals. *BioScience*, *46*(5), 331–343.

Rice, W. R. (1996b). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, *381*(6579), 232–234.

Rice, W. R. (1998). Male fitness increases when females are eliminated from gene pool: Implications for the Y chromosome. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11), 6217–6221.

Rice, W. R. and Chippindale, A. K. (2001), Intersexual ontogenetic conflict. *Journal of Evolutionary Biolog*y, *14*, 685–693.

Roberts, R. B., Ser, J. R., & Kocher, T. D. (2009). Sexual conflict resolved by invasion of a novel sex determiner in lake malawi cichlid fishes. *Science*, *326*(5955), 998–1001.

Rogers, T. F., Palmer, D. H., & Wright, A. E. (2021). Sex-specific selection drives the evolution of alternative splicing in birds. *Molecular Biology and Evolution*, *38*(2), 519–530.

Rogers, T. F., Pizzari, T., & Wright, A. E. (2021). Multi-copy gene family evolution on the avian W chromosome. *Journal of Heredity*, *112*(3), 250–259.

Rozen, S., Skaletsky, H., Marszalek, J. D., Minx, P. J., Cordum, H. S., Waterston, R. H., … Page, D. C. (2003). Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. *Nature*, *423*(6942), 873–876.

Saunders, P. A., Neuenschwander, S., & Perrin, N. (2018). Sex chromosome turnovers and genetic drift: a simulation study. *Journal of Evolutionary Biology*, *31*(9), 1413–1419.

Shine, R. (1989). Ecological causes for the evolution of sexual dimorphism: a review of the evidence. *Quarterly Review of Biology*, *64*(4), 419–461.

Skaletsky, H., Kuroda-Kawaguchl, T., Minx, P. J., Cordum, H. S., Hlllier, L. D., Brown, L. G., … Page, D. C. (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, *423*(6942), 825–837.

Smith, C. A., Roeszler, K. N., Ohnesorg, T., Cummins, D. M., Farlie, P. G., Doran, T. J., & Sinclair, A. H. (2009). The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature*, *461*(7261), 267–271.

Snell-Rood, E. C., Van Dyken, J. D., Cruickshank, T., Wade, M. J., & Moczek, A. P. (2010). Toward a population genetic framework of developmental evolution: The costs, limits, and consequences of phenotypic plasticity. *BioEssays*, *21*(1), 71–81.

Stiglec, R., Ezaz, T., & Graves, J. A. M. (2007). A new look at the evolution of avian sex chromosomes. *Cytogenetic and Genome Research*, *117*(1–4), 103–109.

Sturgill, D., Zhang, Y., Parisi, M., & Oliver, B. (2007). Demasculinization of X chromosomes in the *Drosophila* genus. *Nature*, *450*(7167), 238–241.

Telonis-Scott, M., Kopp, A., Wayne, M. L., Nuzhdin, S. V., & McIntyre, L. M. (2009). Sex-specific splicing in *Drosophila*: Widespread occurrence, tissue specificity and evolutionary conservation. *Genetics*, *181*(2), 421–434.

Tsai, H. W., Grant, P. A., & Rissman, E. F. (2009). Sex differences in histone modifications in the neonatal mouse brain. *Epigenetics*, *4*(1), 47–53.

Van Cleve, J., & Feldman, M. W. (2007). Sex-specific viability, sex linkage and dominance in genomic imprinting. *Genetics*, *176*(2), 1101–1118.

Van Doorn, G. S., & Kirkpatrick, M. (2007). Turnover of sex chromosomes induced by sexual conflict. *Nature*, *449*(7164), 909–912.

Van Doorn, G. Sander. (2009). Intralocus sexual conflict. *Annals of the New York Academy of Sciences*, *1168*, 52–71.

Van Doorn, G. Sander, & Kirkpatrick, M. (2010). Transitions between male and female heterogamety caused by sex-antagonistic selection. *Genetics*, *186*(2), 629–645.

Vicoso, B. (2019). Molecular and evolutionary dynamics of animal sex-chromosome turnover. *Nature Ecology and Evolution*, *3*, 1632–1641.

Wang, P. J., McCarrey, J. R., Yang, F., & Page, D. C. (2001). An abundance of X-linked genes expressed in spermatogonia. *Nature Genetics*, *27*(4), 422–426.

Werren, J. H., & Beukeboom, L. W. (1998). Sex determination, sex ratios, and genetic conflict. *Annual Review of Ecology and Systematics*, *29*, 233–261.

Woolf, C. M., & Church, K. (1963). Studies on the advantage of heterokaryotypes in the tumorous-head strain of *Drosophila melanogaster. Evolution*, *17*(4), 486.

Wright, A. E., & Mank, J. E. (2013). The scope and strength of sex-specific selection in genome evolution. *Journal of Evolutionary Biology*, *26*(9), 1841–1853.

Wright, A. E., Darolti, I., Bloch, N. I., Oostra, V., Sandkam, B., Buechel, S. D., … Mank, J. E. (2017). Convergent recombination suppression suggests role of sexual selection in guppy sex chromosome formation. *Nature Communications*, *8*, 14251.

Wright, A. E., Fumagalli, M., Cooney, C. R., Bloch, N. I., Vieira, F. G., Buechel, S. D., … Mank, J. E. (2018). Male-biased gene expression resolves sexual conflict through the evolution

of sex-specific genetic architecture. *Evolution Letters*, *2*(2), 52–61.

Wright, A. E., Moghadam, H. K., & Mank, J. E. (2012). Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*, *192*(4), 1433–1445.

Wright, A. E., Rogers, T. F., Fumagalli, M., Cooney, C. R., & Mank, J. E. (2019). Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Molecular Ecology*, *28*(11), 2860–2871.

Wu, C. I., & Xu, E. Y. (2003). Sexual antagonism and X inactivation - The SAXI hypothesis. *Trends in Genetics*, *19*(5), 243–247.

Wyman, M. J., Agrawal, A. F., & Rowe, L. (2010). Condition-dependence of the sexually dimorphic transcriptome in *Drosophila melanogaster. Evolution*, *64*(6), 1836–1848.

Yang, X., Schadt, E. E., Wang, S., Wang, H., Arnold, A. P., Ingram-Drake, L., … Lusis, A. J. (2006). Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Research*, *16*(8), 995–1004.

Zauner, H., Begemann, G., Marí-Beffa, M., & Meyer, A. (2003). Differential regulation of msx genes in the development of the gonopodium, an intromittent organ, and of the "sword," a sexually selected trait of swordtail fishes (*Xiphophorus*). *Evolution and Development*, *5*(5), 466–477.

Zhang, Y., Sturgill, D., Parisi, M., Kumar, S., & Oliver, B. (2007). Constraint and turnover in sex-biased gene expression in the genus *Drosophila*. *Nature*, *450*(7167), 233–237.

Zhou, Q., & Bachtrog, D. (2012). Sex-specific adaptation drives early sex chromosome evolution in *Drosophila*. *Science*, *337*(6092), 341–345.

CHAPTER 2. CONTRASTING PATTERNS OF SEXUAL CONFLICT ON THE AVIAN Z CHROMOSOME RELATIVE TO THE AUTOSOMES

2.1 AUTHOR CONTRIBUTIONS

This work forms part of the publication "Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict" published in *Molecular Ecology* (2019), the published version of which is included in Chapter 7 of this thesis.

Wright, A. E.**, Rogers, T. F.,** Fumagalli, M., Cooney, C. R. & Mank, J. E. (2019). Phenotypic sexual dimorphism associated with genomic signatures of resolved sexual conflict. *Molecular Ecology 28*(11),2860‐2871.

A.E.W, T.F.R, and J.E.M conceived of the study and designed the experiments. J.E.M. and A.E.W collected tissue samples for sequencing. A.E.W assembled avian transcriptomes. A.E.W and M.F generated population genomic data. T.R.F, A.E.W and C.R.C analyzed the data. All authors contributed to the writing of the published paper. T.F.R is responsible for sections of the published manuscript that focus on the Z chromosome, which analysed population genomic data and T.F.R wrote relevant parts of the manuscript. T.F.R has reformatted the paper specifically for this thesis to focus more directly on the methods, analyses, results and conclusions relevant to sex chromosomes.

2.2 ABSTRACT

Due to their unequal pattern of inheritance, sex chromosomes are predicted to be hotspots of sexual conflict resolution. Sex-specific selection can act on the sex chromosomes, enabling evolutionary divergence between the sexes via the decoupling of male and female phenotypes. On the other hand, the sex chromosomes are subject to distinct evolutionary environments in comparison to the rest of the genome, resulting in differences in mutation rate, effective population size and recombination rate, all of which might act to reduce the ability of selection to operate on the sex chromosomes. We aimed to examine the trade-off between these two evolutionary scenarios and directly test whether the Z chromosome is a hotspot of sexual conflict. Our results indicate that the sex chromosomes may not be hotspots of sexual conflict and their adaptability could be diminished by their pattern of inheritance and the characteristics associated with this. These findings are consistent across varying levels of sexual dimorphism both within the body plan and across species, and are key to understanding the role of the sex chromosomes in sex-specific adaption and sexual dimorphism, as well as how sexual conflict manifests over broad evolutionary timescales.

2.3 INTRODUCTION

Males and females in many species often have divergent evolutionary interests and are subject to conflicting selection pressures (Andersson, 1994). However, with the exception of the sex chromosomes, the sexes share an identical genome, and this can give rise to intralocus sexual conflict, where an allele benefits one sex at the expense of the other (Parker & Partridge, 1998). This shared genomic architecture is thought to hamper males and females simultaneously evolving towards their respective fitness peaks, and in turn acts as a constraint in the evolution of sexual dimorphism (Mank, 2017; Rowe, Chenoweth, & Agrawal, 2018; Stewart & Rice, 2018).

Recently, studies have used population genomic statistics to detect the signature of sexual conflict across the genome (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Lucotte et al., 2016; Mostafavi et al., 2017; Rowe et al., 2018; Wright et al., 2018). Ongoing sexual conflict can arise from several different factors and these leave distinct population genomic signatures in sequence data (Mank, 2017; Wright et al., 2018). Sexual conflict can result over reproduction, where an allele increases the reproductive fitness of one sex at a cost to the other (Barson et al., 2015; Lonn et al., 2017). Alternatively, sexual conflict can result when an allele has

differential effects on survival between males and females (Czorlich et al., 2018). Both of these scenarios are predicted to produce elevated genetic diversity and higher Tajima's *D*, a population genomic statistic that estimates the proportion of polymorphic nucleotide sites in a given sequence within a population. Tajima's *D* is calculated as the difference between the number of segregating sites and the mean number of pairwise nucleotide differences, both scaled such that in neutrally evolving populations of a constant size Tajima's *D* is equal to zero. If there is balancing selection, there is the assumption that intra-specific diversity is elevated.

Population genomic approaches have made it possible to investigate the manifestation of different types of intralocus sexual conflict at the genomic level and the mechanisms by which they can be resolved. In a ZZ/ZW sex chromosome system, male-specific selection is relatively stronger for dominant Z-linked alleles because the Z is present more often in males than females. However, female-specific selection is relatively stronger for recessive Z-linked alleles as they are more often exposed in females to selection due to female hemizygosity (Rice, 1984). Due to this unequal pattern of inheritance, sex chromosomes can facilitate the initial build-up of sexually antagonistic loci within the gene pool. A gene on the autosomes that produces a sexually antagonistic phenotype will only increase in frequency when rare if the disadvantage to one sex is smaller than the advantage to the other sex. If a sexually antagonistic gene is located on the X or Z chromosome, the conditions for increase are less stringent. The Z is therefore predicted to harbour dominant male-benefit, female-detriment alleles and recessive female-benefit, male-detriment alleles. The evolution of modifiers to restrict the expression of sexually antagonistic genes to the sex in which they are selectively favoured is predicted to follow, thereby resolving sexual conflict. (Rice, 1984).

However, the sex chromosomes experience unique evolutionary environments relative to the rest of the genome (Bachtrog et al., 2011), resulting in differences in mutation rate, effective population size and recombination rate, all of which might act to reduce the efficiency of selection acting on the sex chromosomes (Wright & Mank, 2013). This is particularly pronounced for the Z chromosome for several reasons. The effective population size of the Z chromosome (N_{EZ}) is ¾ that of the autosomes (N_{EA}) when there is no difference in the variance of reproductive success between the sexes, like in strictly monogamous breeding systems. Moreover, some types of sexual selection can cause increased variance in male reproductive

success (Andersson, 1994), which decreases N_{EZ}/N_{EA}. In extreme cases for instance, when a single male dominates the reproductive output of multiple females, N_{EZ} approximates ½ N_{EA} (Vicoso & Charlesworth, 2009). This creates a potential for elevated genetic drift to act on the homogametic sex chromosome (Charlesworth et al. 1993; Vicoso & Charlesworth, 2009), particularly in sexually dimorphic species, in turn reducing the power of selection on the Z. It therefore remains unclear whether the Z chromosome is indeed a hotspot of ongoing sexual conflict. We conducted a comparative analysis of Tajima's *D* across a clade of birds that vary in the magnitude of sexual dimorphism and sexual conflict to investigate sexual conflict on the Z chromosome.

2.4 MATERIALS AND METHODS

2.4.1 Tissue collection

We previously extracted RNA from the left gonad and spleen of individuals with the RNeasy Kit (Qiagen), following the manufacturer's instructions, from the following captive avian populations: mallard (*Anas platyrynchos*), wild turkey (*Meleagris gallopavo*), common pheasant (*Phasianus colchicus*), helmeted guinea fowl (*Numida meleagris*), Indian peafowl (*Pavo cristatus*) and swan goose (*Anser cynoides*) (Harrison et al., 2015) (Fig. 2.1). These captive populations are not maintained under sterile or biosafety conditions. Samples were collected during the first breeding season from five males and five females of each species, with the exception of the pheasant, where six male gonad and spleen samples were collected, and turkey where four male and two female spleens were collected.

These six species were deliberately chosen to reflect a full range of sexual dimorphism, ranging from monogamous and sexually monomorphic species such as the swan goose and guineafowl, to polygynous and sexually dimorphic species such as the peafowl and wild turkey. We estimated the intensity of sexual conflict in each species using three proxies of sperm competition and male promiscuity: sexual dichromatism score, sperm number and relative testes size, obtained from Harrison et al. (2015).

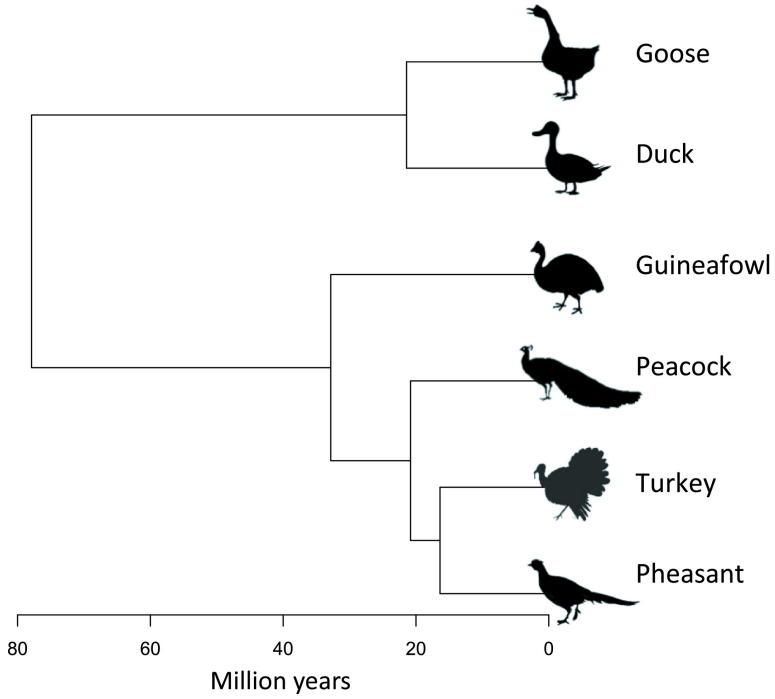

Figure 2.1 Phylogenetic relationships across the six avian species in this study. These species were chosen to reflect the full range of mating system and sexual dimorphism. The intensity of sexual conflict in each species was estimated using three proxies: sexual dichromatism score, sperm number and relative testes size.

2.4.2 Transcriptome assembly

Samples were sequenced on an Illumina HiSeq 2000 device with 100-bp paired-end reads and are available in the NCBI SRA (BioProject ID PRJNA271731). We assembled and filtered transcriptomes for each species using previously implemented approaches (Harrison et al., 2015). Briefly, we quality filtered RNA data using Trimmomatic version 0.36 (Bolger et al., 2014) to filter reads containing adaptor sequences and trim reads if the sliding window average Phred score over four bases was < 15 or if the leading/trailing bases had a Phred score < 3. Reads were removed after filtering if either read pair was < 36 bases in length. We assembled a de novo transcriptome for each species using trinity version 2.4.0 (Grabherr et al., 2011) with default parameters. We then filtered each transcriptome to remove spurious and low-confidence genes. First, we selected the "best isoform" per gene to avoid redundancy. We used the trinity script align_and_estimate_abundance.pl to map RNA-seq reads to transcriptomes using BOWTIE 2 and to quantify expression for each sample using rsem. We suppressed unpaired and discordant alignments for paired reads. We then picked the most highly expressed isoform per gene to obtain a set of "best isoforms" for each

species. RNA-seq reads were remapped to the set of "best isoforms" in each species using the same approach as above to ensure consistency between expression and sequence data. Second, we filtered the transcriptome to remove lowly expressed genes. Specifically, we removed genes with expression < 2 FPKM (fragments per kilobase of transcript per million mapped reads) in half or more of the individuals in either tissue. We assessed the completeness of our transcriptome assembly using eukaryota_odb9 busco version 3.0.2 (Waterhouse et al., 2018) (Table S6.1.1).

2.4.3 Identification of orthologues

We used BLAST (Altschul et al., 1990) to identify orthologous genes across the six species. First, we identified pairwise reciprocal orthologues between the chicken reference genome (Gallus_gallus-5.0) and the wild turkey, common pheasant, helmeted guineafowl and Indian peafowl, and between the duck reference genome (BGI_duck_1.0) and mallard and swan goose (Zerbino et al., 2018). We downloaded cDNA sequences from Ensembl (Zerbino et al., 2018) and selected the longest transcript per gene. We ran reciprocal BLASTN with an e-value cut-off of 1×10^{-10} and selected the best hit reciprocal orthologue using a minimum percentage identity of 30% and the highest bitscore following previous approaches (Harrison et al., 2015; Wright et al., 2018). If two hits shared the same highest bitscore, then the hit with the highest percentage identity was chosen. If both hits had the same highest bitscore and percentage identity, the gene was discarded.

For the wild turkey, common pheasant, helmeted guineafowl and Indian peafowl, we assigned chromosomal location and gene position from the pairwise reciprocal orthologue in the chicken reference genome. Chromosomal positional information is not available in the duck reference genome and so we used a synteny-based approach to obtain chromosomal location using MCScanX (Wang et al., 2012). Briefly, we downloaded chicken and duck protein sequences from Ensembl, selected the longest protein per gene in each species, and then conducted a reciprocal BLASTP with an e-value cut-off of 1×10^{-10}. We restricted the number of BLASTP hits for each gene to the top five, generated gff files, and concatenated the duck and chicken results as recommended by MCScanX. We then identified syntenic regions between the duck and chicken reference genome using MCScanX run with default parameters. For the mallard and swan goose, we assigned chromosomal location and gene position from the syntenic information available for the pairwise reciprocal orthologue in the

duck reference genome. For all species, we split genes into autosomal or Z-linked based on location in the chicken reference genome (Table S6.1.1) as evolutionary forces including sexual conflict act differently across these genomic regions (Rice, 1984; Wright & Mank, 2013).

Second, we identified reciprocal orthologues using the same approach across all species using the chicken and duck reference genomes to assign chromosomal location. This resulted in 1,457 autosomal reciprocal orthologues, which we used to contrast population genetic statistics across species. Finally, potential immune loci were identified from Gene Ontology terms in Biomart in the chicken and duck reference genomes (Zerbino et al., 2018). Specifically, we removed all loci with the terms "immune" or "MHC" in their Gene Ontology annotations from subsequent analyses. This was to reduce any potential confounding effects as heterozygote advantage in immunity can produce patterns of balancing selection independent of sexual conflict (Ghosh et al., 2012; Stahl et al., 1999).

2.4.4 Gene expression analyses

Read counts for autosomal and Z-linked genes were extracted for all gonad and spleen samples and normalized using TMM in EDGER (Robinson et al., 2010). We identified gonad-biased, spleen-biased and non-tissue-biased genes using a standard $\log_2$ fold change value of 2 (Wright et al., 2018) in each species (Table S6.1.2, S6.1.3). The gonad is transcriptionally more sexually dimorphic than the spleen and so we identified tissue-biased genes in each sex separately instead of combining all samples to avoid biasing our analyses against highly sex-biased or sex-limited genes. We report results from tissue-biased genes identified in males in the main text but results based on tissue-biased genes identified from female expression data were qualitatively identical. We identified tissue-biased genes on the Z chromosome separately due to the unique expression profile of the avian Z chromosome arising from incomplete dosage compensation (Itoh et al., 2007; Mank et al., 2008; Wright et al., 2012).

2.4.5 Filtering data for population genomic analyses

Population genomic analyses were conducted on BAM files generated by mapping RNA-seq data to the set of "best isoforms" in each species with RSEM. For each individual, we merged the spleen and gonad BAM files using SAMTOOLS (Li et al., 2009). The exception was the

turkey, where the spleen and gonad were not sequenced for all individuals so we used only gonad data for subsequent analyses.

We used ANGSD (Korneliussen et al., 2014) to estimate population genetic summary statistics, following our previous approach (Wright et al., 2018) as ANGSD implements methods to account for sequencing uncertainty and is appropriate for uneven sequencing depth associated with transcriptome data. We filtered BAM files to discard reads if they did not uniquely map, had a flag ≥ 256, had a mate that was not mapped or had a mapping quality below 20. Bases were filtered if base quality fell below 13 or there was data in fewer than half the individuals. Mapping quality scores were adjusted for excessive mismatches and quality scores were adjusted around indels to rule out false single nucleotide polymorphisms (SNPs).

We identified and removed related individuals (four peacock, two wild turkey and two swan goose individuals) from our analyses using NGSRELATE (Korneliussen & Moltke, 2015) to avoid violating Hardy–Weinberg assumptions, and calculated inbreeding coefficients using an EM algorithm with the NGSF package in NGSTOOLS (Fumagalli et al., 2014) (full details in S6.1.1). For all species, inbreeding coefficients were < 0.03 with the exception of the peacock where we identified two inbred individuals. We incorporated inbreeding coefficients for the peacock in subsequent analyses.

2.4.6 Calculating Tajima's D

ANGSD was used for each species to calculate sample allele frequency likelihoods at each site from genotype likelihoods calculated with the SAMTOOLS model. We calculated allele frequency likelihoods separately for the Z chromosome and the autosomes as they are subject to different evolutionary pressures and differ in ploidy. The Z chromosome is diploid in males yet haploid in females, and therefore we used only male samples to estimate allele frequency to avoid violating Hardy–Weinberg assumptions. Next, we estimated the overall unfolded site frequency spectrum (SFS) for each species (Nielsen et al., 2012) (Fig. S6.1.1). Specifically, at each site we randomly sampled an allele frequency according to its likelihood, as calculated by ANGSD. Finally, we computed genetic diversity indices, including allele frequency posterior probability and Tajima's *D* using the SFS as prior information with ANGSD thetaStat (Korneliussen et al., 2014).

For each species, we calculated a relative measure of Tajima's *D* for spleen-biased and gonad-biased genes. Specifically, we quantified median *D* relative to non-tissue-biased genes, our neutral estimate of *D* for each species. Calculating a relative measure of Tajima's *D* makes it possible to circumvent problems arising from demographic changes in population size that would otherwise bias comparative analyses of population genetic statistics across species.

2.4.7 Calculating intersexual F_{ST}

Intersexual F_{ST} was calculated using the same procedure and filtering criteria as Tajima's *D*, except that RNA-seq data were instead filtered to remove bases where we had data in less than half the individuals in males and females separately. This ensures we do not exclude sex-limited genes from the analysis. Hudson's F_{ST}, which is less sensitive to small sample sizes (Bhatia et al., 2013), was estimated as implemented in ANGSD (Korneliussen et al., 2014). Estimates across loci were obtained using weighted averages (see Fumagalli et al., 2014 equations 4 and 12), where per-gene F_{ST} is the ratio between the sum of the between-populations variance across loci and the sum of the total variance across loci. Given the Z chromosome is haploid in females, we do not have the power to analyze patterns of F_{ST} across the Z chromosome in this study.

2.5 RESULTS

2.5.1 Lower levels of ongoing sexual conflict in reproductive versus somatic tissue

Reproductive tissue, such as the gonad, has many sex-specific functions whereas the function of somatic tissue, such as the spleen, is more aligned between male and female fitness. To test whether phenotypic sexual dimorphism is associated with resolved sexual conflict at the genomic level, we contrasted population genomic statistics between genes expressed in the gonad versus the spleen.

As heterozygote advantage in immunity can produce patterns of balancing selection independent of sexual conflict (Ghosh et al., 2012; Hedrick, 2011; Stahl et al., 1999), we removed all loci with potential immune function from downstream analyses. We found that median Tajima's *D* is lower for gonad-biased genes relative to non-tissue-biased genes in all species across the autosomes, with the exception of the turkey (Fig. 2.2). The results are unchanged if a strict Bonferroni correction for multiple testing is applied. The conclusion of this paper hinges on the fact that Tajima's *D* for autosomal gonad-biased genes and non-

tissue-biased genes differ for the majority of species at $p < 0.001$ (*** in Fig. 2.2) and this is still significant after the Bonferroni correction when α is 0.002 (0.05/24). This result is consistent with lower levels of ongoing sexual antagonism in the gonad. In contrast, we found no significant difference in Tajima's *D* between spleen-biased genes and loci expressed in both tissues in most species. It should be noted however that the power to detect statistically significant differences is reduced due to limited numbers of tissue-biased Z-linked genes.

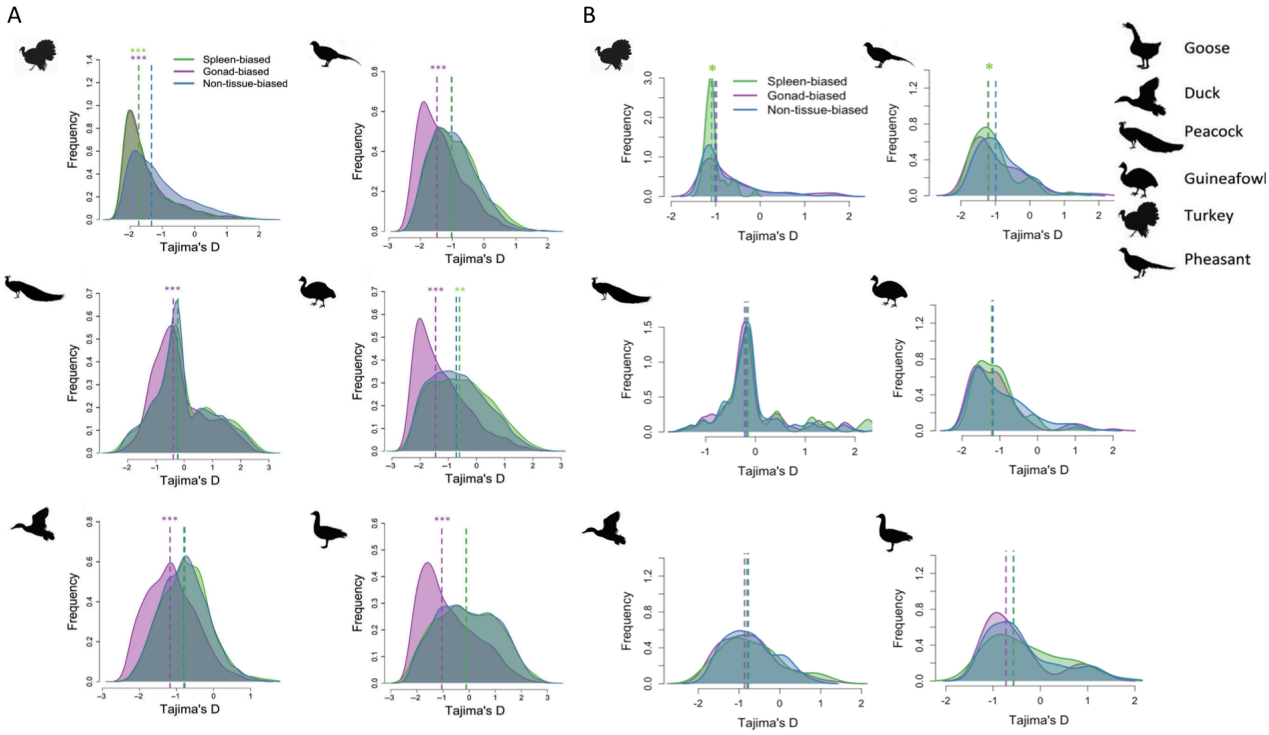

Figure 2.2. Distribution of Tajima's *D* for (A) autosomal genes and (B) Z-linked genes that are spleen-biased, gonad-biased and non-tissue-biased in each species. Dotted lines show median Tajima's *D* for each set of genes and *,**,*** denote a difference relative to non-tissue-biased genes (Wilcoxon test, $p < 0.05$, $p < 0.01$, $p < 0.001$, α after Bonferroni correction = 0.002). Numbers of genes in each species and group can be found in Tables S6.1.2 and S6.1.3.

It is important to note that multiple factors can influence population genetic statistics for any particular locus. Therefore, we tested whether our results could also be attributed to the effect of covariates that might vary across tissue-biased genes. We incorporated measures of gene length, average expression level, GC content and Watterson's theta for autosomal genes into a multiple regression (TD ~ Tissue bias + log(tW) + log(Gene length) + log(GC) + log(Gene expression level)). Tissue-bias remains a significant factor in explaining variation in Tajima's *D* once accounting for these covariates (Table S6.1.4). However, the effect size in some species

is relatively small, indicating that the pattern we detect is subtle and influenced by multiple factors.

Next, we tested the power of intersexual F_{ST} (Lewontin & Krakauer, 1973) to detect sexual conflict arising over survival through contrasts between the spleen and gonad. Intersexual F_{ST} measures divergence in allele frequency between males and females within a generation. As allele frequencies are identical between the sexes at conception, different allele frequencies in male and female adults are assumed to be the result of sexual conflict over survival. We contrasted intersexual F_{ST} for gonad- and spleen-biased genes using three approaches. First, we found no significant difference in median F_{ST} for autosomal unbiased genes expressed primarily in the gonad relative to those expressed broadly across both the gonad and the spleen (Table S6.1.5). We observed the same pattern in the spleen, with the exception of the goose and turkey where F_{ST} was elevated marginally. Second, there was no significant difference in the number of unbiased genes with elevated intersexual F_{ST} that were expressed primarily in the gonad compared to those expressed in both tissues (Table 2.1). We observe the same result in the spleen, with the exception of the turkey. Most importantly, our results are consistent with theoretical work suggesting that intersexual divergence in allele frequency may not always be a reliable indicator of ongoing sexual conflict over viability (Kasimatis et al., 2017; 2019), particularly in studies with low numbers of samples. Therefore, we did not extend this analysis to the Z chromosome.

Table 2.1. Observed and expected number of genes with intersexual $F_{ST} > 0$ across tissue-biased genes.

Species	Gonad-biased			Spleen-biased		
	E	O	*p*-value	E	O	*p*-value
Mallard	116	118	0.875	112	111	0.956
Swan goose	56	65	0.248	56	70	0.056
Wild turkey	166	160	0.644	204	236	**0.026**[a]
Common pheasant	165	163	0.520	187	174	0.532
Guinea fowl	112	124	0.269	151	142	0.461
Indian peafowl	200	209	0.520	217	208	0.532

Only unbiased genes were used in this analysis. Tissue-biased genes were identified from male expression data. Only autosomal genes are included in the analyses. The expected number of genes with intersexual $F_{ST} > 0$ was calculated from observations of F_{ST} in non-tissue-specific genes. *p*-values were calculated using chi-squared tests. [a] *p*-values in bold are significant ($p < 0.05$)

2.5.2 In contrast to the autosomes, the Z chromosome is not a hotspot of sexual conflict

Organisms in polygamous mating systems experience more sex-specific selection and therefore are expected to be under a higher degree of sexual conflict. In monogamous systems however, sexual conflict is lower because male and female interests are more aligned. Therefore, to investigate the target of sexual conflict across the genome, we conducted a phylogenetically controlled comparative analysis of Tajima's *D* across species that vary in mating system and sexual dimorphism. Specifically, we used phylogenetic generalized least squares (PGLS) from the R package caper (Orme, 2013) to test the relationship between Tajima's *D* and measures of sexual dimorphism, while accounting for the observed level of phylogenetic signal in the data. For each species, we quantified median Tajima's *D* for spleen- and gonad-biased genes relative to non-tissue-biased genes. Tajima's *D* cannot be compared directly across species or populations, as demographic history has a major influence on genetic diversity, and therefore on Tajima's *D* estimation. Calculating a relative measure of Tajima's *D* makes it possible to circumvent problems arising from demographic changes in population size. There are a number of phenotypic indices of sexual conflict, including degree of sexual dichromatism, sperm number and residual testes weight, that are widely used indicators of post-copulatory sexual selection and therefore a measure of variance in male mating success in birds (Birkhead & Møller, 1998; Møller, 1991; Pitcher et al., 2005).

We recovered a significant and positive relationship between relative Tajima's *D* in the gonad and sexual dichromatism across the autosomes ($r^2 = 0.890$, $p = 0.003$) after correcting for phylogeny, and marginally nonsignificant positive associations with both sperm number ($r^2 = 0.491$, $p = 0.073$) and residual testes weight ($r^2 = 0.298$, $p = 0.152$). The proportion of sex-biased genes varies with mating system across these species (Harrison et al., 2015), which together with the fact that sex-biased genes have distinct patterns of Tajima's *D* (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Wright et al., 2018) and are subject to different selective pressures relative to unbiased genes (Ellegren & Parsch, 2007; Harrison et al., 2015), may confound the pattern we observe. We therefore repeated the analyses using relative median Tajima's *D* calculated using only unbiased genes in each tissue. In doing so, we found that relative Tajima's *D* in the gonad becomes significantly and positively correlated with sexual dichromatism ($r^2 = 0.788$, $p = 0.011$), and sperm number ($r^2 = 0.679$, $p = 0.027$) after

correcting for phylogenetic relationships (Fig. 2.3A), and marginally nonsignificantly associated with residual testes weight (r^2 = 0.446, p = 0.089). In contrast, there was no significant association with Tajima's *D* in the spleen and measures of sexual dimorphism (Fig. S6.1.2).

Interestingly, we found no significant relationship between Tajima's *D* and phenotypic sexual conflict for Z-linked genes in either tissue (Fig. 2.3B, S6.1.3). Given there are fewer genes on the Z chromosome relative to the autosomes, this pattern might simply be a consequence of smaller sample sizes and therefore greater uncertainty around the median. To assess the role of gene number in our population genetic parameter estimates, we subsampled tissue-biased genes on the autosomes to the equivalent number of the Z-linked genes in each species 1,000 times. The Pearson's correlation coefficients for the relationship between Tajima's *D* and sexual dichromatism, testes weight and sperm number for gonad-biased Z-linked genes are smaller relative to the subsampled data set (p = 0.027, p = 0.048, p = 0.168). The slope of the regression is also smaller than the subsampled data (p = 0.024, p = 0.058, p = 0.121). This indicates that our failure to observe a significant relationship between Tajima's *D* and sexual conflict on the Z chromosome is not a consequence of reduced gene numbers relative to the autosomes. This is in stark contrast to the autosomes, where we found a significant and positive relationship (Fig. 2.3A).

2.6 DISCUSSION

The manifestation, resolution and consequences of intralocus sexual conflict have been the subject of considerable recent debate. To address this, we exploited natural variation in the magnitude of sexual conflict across the body plan within individuals, and across mating systems between species, in a clade of birds that diverged 90 million years ago.

Whereas identifying the mechanisms responsible for the resolution of genomic sexual conflict has received considerable attention, the consequences for phenotypic evolution have been comparatively understudied. This is in part due to the difficulties of identifying specific loci subject to sexual conflict and establishing their phenotypic effects from genome scans alone. Our study adds considerably to this goal by using different levels of dimorphism within the body plan and across related species to determine the relationship between population genetic and phenotypic measures of sexual conflict.

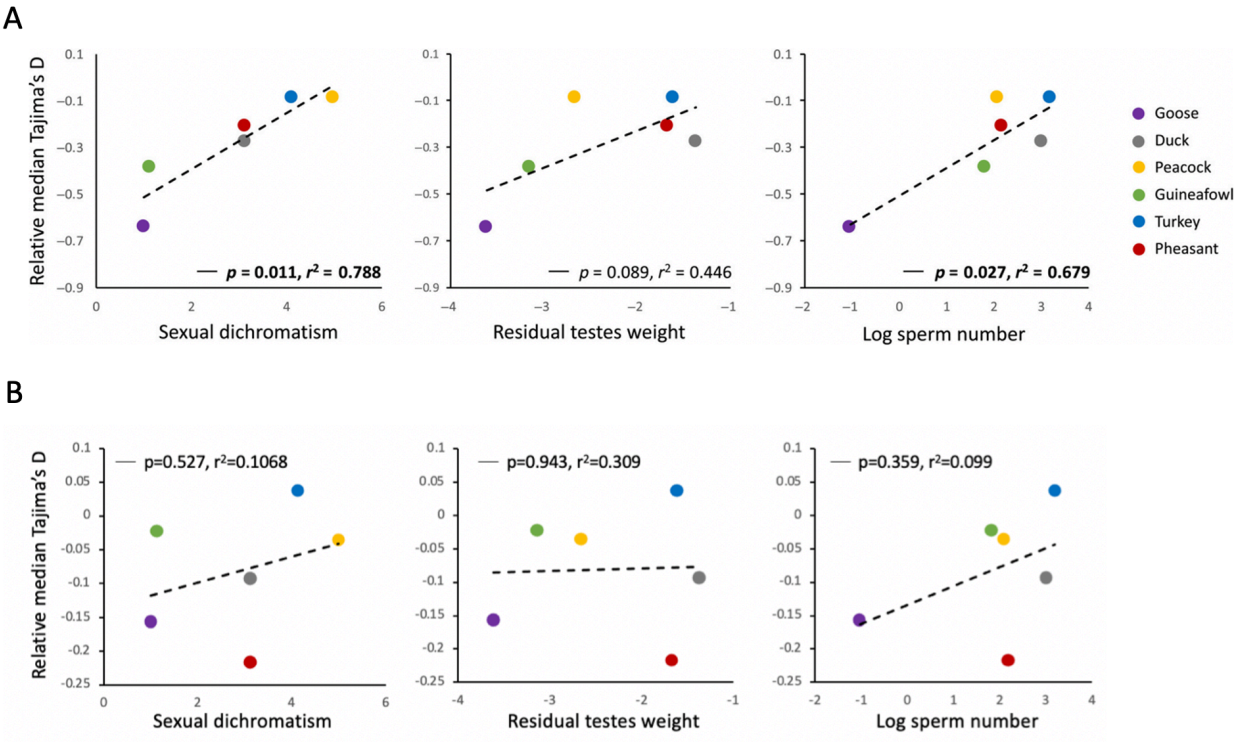

Figure 2.3. Phylogenetically controlled regression between proxies of sperm competition and Tajima's *D* for (A) autosomal genes and (B) Z-linked genes in the gonad. Relative *D* is shown for genes with unbiased expression between males and females in the gonad. Relative *D* is calculated as the difference between median *D* for tissue-biased genes compared to non-tissue-biased genes. Tissue-biased genes were identified from male expression data.

Relative to the spleen, the gonad is more phenotypically sexually dimorphic, has higher levels of sex-biased gene expression, and has evolved many sex-specific functions. If sexual dimorphism represents resolved sexual conflict, we might expect gonad-biased genes to have lower levels of balancing selection than spleen-biased genes and loci expressed similarly in both tissues. Consistent with this prediction, we find reduced balancing selection in the gonad across both the autosomes and Z chromosome, indicative of lower levels of ongoing sexual conflict. This supports the theory that resolved sexual conflict facilitates the evolution of phenotypic sex differences. It is plausible that the large numbers of sex-biased genes in the gonad relative to somatic tissue act to resolve conflict through regulatory decoupling of male and female expression and the evolution of sex-specific architecture.

While we found that intralocus sexual conflict is resolved in the gonad across both the autosomes and Z chromosome, we found a significant and positive correlation between the magnitude of sexual conflict, arising from differences in mating system, and balancing selection in the gonad but not the spleen for autosomal genes. Whilst this may appear initially

contradictory, this relationship is in fact consistent with an ephemeral nature of sexual antagonism and rapid turnover of sexual conflict loci. This is in line with previous work showing that sex-biased genes exhibit rapid rates of evolution and turnover (Harrison et al., 2015; Zhang et al., 2007). Our results suggest that autosomal unbiased genes are the locus of ongoing sexual conflict due to mating system, and that increasing levels of sexual conflict over reproduction result in elevated levels of genetic diversity across a greater proportion of genes. In contrast, relative Tajima's *D* in spleen-biased genes is not associated with any phenotypic measure of sexual conflict, suggesting that sexual conflict over reproduction has the greatest potential to contribute significantly to variation in the maintenance of genetic diversity across species. This has important consequences for understanding the relationship between sexual conflict and adaptation, where higher levels of conflict promote genetic diversity and provide genetic fuel for adaptive opportunities (Candolin & Heuschele, 2008; Chenoweth et al., 2015; Jacomb et al., 2016; Lumley et al., 2015).

In contrast, we observed no significant relationship between mating system and balancing selection on the Z chromosome. These results suggest that the Z chromosome is not a hotspot of ongoing sexual conflict relative to the rest of the genome, and that sexual conflict has less potential to shape patterns of genetic diversity on the Z. Previously, we showed that the adaptive potential of the Z chromosome is compromised by increasing sexual selection, which decreases the relative effective population size of the Z chromosome compared to autosomes (Wright et al., 2015), leading to increased levels of genetic drift. This means that Z-linked genes in sexually dimorphic species are subject to higher levels of genetic drift (Vicoso & Charlesworth, 2009; Wright & Mank, 2013). Our results indicate that the potential for unresolved sexual conflict to shape patterns of genetic diversity on the Z chromosome might be counteracted by the depleting forces of genetic drift, and that sexual conflict may not play a disproportionally greater role in Z chromosome evolution compared to the rest of the genome.

It should be noted that negative Tajima's *D* can be interpreted in the context of positive selection, where selective sweeps can result in lower estimates. A greater frequency of selective sweeps in sex-biased genes could therefore explain our finding that Tajima's *D* is lower in the gonad than in the spleen. Furthermore, the positive correlation between Tajima's *D* and sexual dimorphism we observe in the gonad could also be due to more intense

positive selection in species with less sexual dimorphism. However, elevated positive selection is unlikely to explain our results, as previous research on the same data set found no significant evidence for positive selection acting on sex-biased genes in the gonad, or any evidence for variation in the magnitude of positive selection across species based on mating system (Harrison et al., 2015). Therefore, we conclude that lower Tajima's *D* is indicative of lower levels of balancing selection and resolved intralocus conflict, probably mediated by the evolution of sex-biased gene expression.

Population genomic measures of intersexual F_{ST} and Tajima's *D* can be influenced by a number of demographic events, not just sexual conflict, including sex-biased migration, sex-biased predation and changes in population size (Hartl & Clark, 2016). By conducting comparisons of population genomic statistics within each species, instead of directly comparing across species, we controlled for the effect of population contractions or expansions, and our use of captive populations further minimizes the effects of sex-biased migration or predation. Furthermore, samples were taken from all individuals during their first breeding season, effectively controlling for age differences that can confound measures of intersexual F_{ST} or lead to high levels of regulatory variation. However, we note that due to statistical noise, probably due to low sample sizes, we could not reliably identify specific loci subject to sexual conflict, and instead compare large groups of genes to determine broad trends across tissues and species. Our analyses of intersexual F_{ST} are particularly limited by sample size and therefore we urge caution when interpreting these in the light of sexual conflict. However, while we do find loci with elevated intersexual F_{ST}, which has previously been interpreted as evidence for ongoing sexual conflict (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Lucotte et al., 2016), the number of loci with elevated F_{ST} do not appear to differ between the gonad and spleen, despite the obvious differences in function and role in survival between the two tissues.

Interestingly, our failure to detect differences in conflict over viability between the tissues across the autosomes is consistent with recent theoretical work (Kasimatis et al., 2017) suggesting that the magnitude of sexual conflict, and associated mortality load, required to generate patterns of intersexual F_{ST} across large numbers of loci is implausibly high. This suggests that they may be a result of alternative demographic processes or statistical noise arising from low sample sizes, instead of ongoing sexual conflict. Instead, our previous work

indicates that divergence in allele frequencies between males and females in somatic tissue could instead be indicative of the evolution of sex-specific architectures, which would invoke weaker genetic loads.

In conclusion, our findings suggest that sex chromosomes may not be hotspots of ongoing sexual conflict and their adaptability might be compromised by their unique inheritance pattern. Our results are consistent both across a gradient of sexual dimorphism within the body plan and across species, and have important implications regarding the role of sexual selection in adaptive potential (Candolin & Heuschele, 2008; Chenoweth et al., 2015; Jacomb et al., 2016; Lumley et al., 2015), the persistence of sexual conflict over evolutionary timescales, and the role of dimorphism in facilitating sex-specific fitness optima.

2.7 ACKNOWLEDGEMENTS

This work was supported by a NERC Independent Research Fellowship to A.E.W., and the European Research Council (grant agreements 260233 and 680951) to J.E.M., who also gratefully acknowledges funding from a Canada 150 Research Chair. We thank Thorfinn Korneliussen for help with angsd and ngstools, Ida Koltke with ngsrelate, Jon Slate, Kai Zeng and Toni Gossmann for productive discussions, and four anonymous reviewers for helpful comments. The authors acknowledge the use of the UCL Legion High Performance Computing Facility (Legion@UCL), and associated support services, in the completion of this work.

2.8 DATA AVAILABILITY

RNA-seq data are publicly available in the NCBI SRA (BioProject ID PRJNA271731). Transcriptome assemblies are available via Dryad (https://doi.org/10.5061/dryad.1v2d850).

2.9 REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410.

Andersson, M. (1994). *Sexual Selection*. Princeton, NJ: Princeton University Press.

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W. R., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*(9), 350–357.

Barson, N. J., Aykanat, T., Hindar, K., Baranski, M., Bolstad, G. H., Fiske, P., … Primmer, C. R. (2015). Sex-dependent dominance at a single locus maintains variation in age at

maturity in salmon. *Nature*, *528*(7582), 405–408.

Bhatia, G., Patterson, N., Sankararaman, S., & Price, A. L. (2013). Estimating and interpreting F. *Genome Research*, (2), 1–9.

Birkhead, T., & Møller, A. (1998). *Sperm Competition and Sexual Selection.* New York: Academic Press.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120.

Candolin, U., & Heuschele, J. (2008). Is sexual selection beneficial during adaptation to environmental change? *Trends in Ecology and Evolution*, *23*(8), 446–452.

Charlesworth, B., Coyne, J. A., & Orr, H. A. (1993). Letters to the editor meiotic drive and unisexual hybrid sterility: A comment. *Genetics*, 133(2), 421–424.

Cheng, C., & Kirkpatrick, M. (2016). Sex-specific selection and sex-biased gene expression in humans and flies. *PLOS Genetics*, *12*(9), e1006170.

Chenoweth, S. F., Appleton, N. C., Allen, S. L., & Rundle, H. D. (2015). Genomic evidence that sexual selection impedes adaptation to a novel environment. *Current Biology*, *25*(14), 1860–1866.

Connallon, T., & Knowles, L. L. (2005). Intergenomic conflict revealed by patterns of sex-biased gene expression. *Trends in Genetics*, *21*(9), 495–499.

Czorlich, Y., Aykanat, T., Erkinaro, J., Orell, P., & Primmer, C. R. (2018). Rapid sex-specific evolution of age at maturity is shaped by genetic architecture in Atlantic salmon. *Nature Ecology and Evolution*, *2*(11), 1800–1807.

Dapper, A. L., & Wade, M. J. (2016). The evolution of sperm competition genes: The effect of mating system on levels of genetic variation within and between species. *Evolution*, *70*(2), 502–511.

Dutoit, L., Mugal, C. F., Bolívar, P., Wang, M., Nadachowska-Brzyska, K., Smeds, L., … Ellegren, H. (2018). Sex-biased gene expression, sexual antagonism and levels of genetic diversity in the collared flycatcher (*Ficedula albicollis*) genome. *Molecular Ecology*, *27*(18), 3572–3581.

Ellegren, H., & Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene expression. *Nature Reviews Genetics, 8*, 698–698.

Fumagalli, M., Vieira, F. G., Linderoth, T., & Nielsen, R. (2014). NgsTools: Methods for population genetics analyses from next-generation sequencing data. *Bioinformatics*,

30(10), 1486–1487.

Gershoni, M., & Pietrokovski, S. (2014). Reduced selection and accumulation of deleterious mutations in genes exclusively expressed in men. *Nature Communications*, 5, 4438

Ghosh, R., Andersen, E. C., Shapiro, J. A., Gerke, J. P., & Kruglyak, L. (2012). Natural variation in a chloride channel subunit confers avermectin resistance in *C. elegans*. *Science*, *335*(6068), 574–578.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–652.

Harrison, P. W., Wright, A. E., Zimmer, F., Dean, R., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(14), 4393–4398.

Hartl, D. L., & Clark, A. G. (2016). *Principles of population genetics*. Sunderland, Massachusetts: Oxford University Press USA.

Hedrick, P. W. (2011). Population genetics of malaria resistance in humans. *Heredity*, *107*(4), 283–304.

Innocenti, P., & Morrow, E. H. (2010). The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS Biology*, *8*(3), e1000335.

Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., … Arnold, A. P. (2007). Dosage compensation is less effective in birds than in mammals. *Journal of Biology*, *6*(1), 2.

Jacomb, F., Marsh, J., & Holman, L. (2016). Sexual selection expedites the evolution of pesticide resistance. *Evolution*, *70*(12), 2746–2751.

Kasimatis, K. R., Nelson, T. C., & Phillips, P. C. (2017). Genomic signatures of sexual conflict. *Journal of Heredity, 108*(7), 780–790.

Kasimatis, K. R., Ralph, P. L., & Phillips, P. C. (2019). Limits to genomic divergence under sexually antagonistic selection. *G3: Genes, Genomes, Genetics*, *9*(11), 3813–3824.

Korneliussen, T. S., Albrechtsen, A., & Nielsen, R. (2014). ANGSD: Analysis of next generation sequencing data. *BMC Bioinformatics*, *15*, 356.

Korneliussen, T. S., & Moltke, I. (2015). NgsRelate: A software tool for estimating pairwise relatedness from next-generation sequencing data. *Bioinformatics*, *31*(24), 4009–4011.

Lewontin, R. C., & Krakauer, J. (1973). Distribution of gene frequency as a test of the theory

of the selective neutrality of polymorphisms. *Genetics*, *74*(1), 175–195.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079.

Lonn, E., Koskela, E., Mappes, T., Mokkonen, M., Sims, A. M., & Watts, P. C. (2017). Balancing selection maintains polymorphisms at neurogenetic loci in field experiments. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(14), 3690–3695.

Lucotte, E. A., Laurent, R., Heyer, E., Ségurel, L., & Toupance, B. (2016). Detection of allelic frequency differences between the sexes in humans: A signature of sexually antagonistic selection. *Genome Biology and Evolution*, *8*(5), 1489–1500.

Lumley, A. J., Michalczyk, Ł., Kitson, J. J. N., Spurgin, L. G., Morrison, C. A., Godwin, J. L., … Gage, M. J. G. (2015). Sexual selection protects against extinction. *Nature*, *522*(7557), 470–473.

Mank, J. E. (2009). Sex chromosomes and the evolution of sexual dimorphism: Lessons from the genome. *American Naturalist, 171*(2), 141–150.

Mank, J. E. (2017). Population genetics of sexual conflict in the genomic era. *Nature Reviews Genetics*, *18*, 721–730.

Mank, J. E., Hultin-Rosenberg, L., Zwahlen, M., & Ellegren, H. (2008). Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. *American Naturalist*, *171*(1), 35–43.

Meiklejohn, C. D., Parsch, J., Ranz, J. M., & Hartl, D. L. (2003). Rapid evolution of male-biased gene expression in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9894–9899.

Moller, A. P. (1991). Sperm competition, sperm depletion, paternal care, and relative testis size in birds. *American Naturalist*, *137*(6), 882–906.

Mostafavi, H., Berisa, T., Day, F. R., Perry, J. R. B., Przeworski, M., & Pickrell, J. K. (2017). Identifying genetic variants that affect viability in large cohorts. *PLoS Biology*, *15*(9), e2002458.

Nielsen, R., Korneliussen, T., Albrechtsen, A., Li, Y., & Wang, J. (2012). SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data. *PLoS ONE*, *7*(7), e37558.

Orme, D. (2013). The caper package : comparative analysis of phylogenetics and evolution in

R. *R Package Version 0.5.*

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *353*(13660), 261–274.

Parsch, J., & Ellegren, H. (2013). The evolutionary causes and consequences of sex-biased gene expression. *Nature Reviews Genetics*, *14*(2), 83–87.

Pitcher, T. E., Dunn, P. O., & Whittingham, L. A. (2005). Sperm competition and the evolution of testes size in birds. *Journal of Evolutionary Biology*, *18*(3), 557–567.

Pröschel, M., Zhang, Z., & Parsch, J. (2006). Widespread adaptive evolution of *Drosophila* genes with sex-biased expression. *Genetics*, *174*(2), 893–900.

Rice, W. R. (1984). Sex chromosomes and the evolution of sexual dimorphism. *Evolution*, *38*(4), 735–742.

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140.

Rowe, L., Chenoweth, S. F., & Agrawal, A. F. (2018). The genomics of sexual conflict. *American Naturalist*, *192*(2), 274–286.

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., & Bergelson, J. (1999). Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. *Nature*, *400*(6745), 667–671.

Stewart, A. D., & Rice, W. R. (2018). Arrest of sex-specific adaptation during the evolution of sexual dimorphism in *Drosophila. Nature Ecology and Evolution*, *2*(9), 1507–1513.

Vicoso, B., & Charlesworth, B. (2009). Effective population size and the faster-X effect: An extended model. *Evolution*, *63*(9), 2413–2426.

Wang, Y., Tang, H., Debarry, J. D., Tan, X., Li, J., Wang, X., … Paterson, A. H. (2012). MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Research*, *40*(7), e49–e49.

Waterhouse, R. M., Seppey, M., Simao, F. A., Manni, M., Ioannidis, P., Klioutchnikov, G., … Zdobnov, E. M. (2018). BUSCO applications from quality assessments to gene prediction and phylogenomics. *Molecular Biology and Evolution*, *35*(3), 543–548.

Wright, A. E., & Mank, J. E. (2013). The scope and strength of sex-specific selection in genome evolution. *Journal of Evolutionary Biology*, *26*(9), 1841–1853.

Wright, A. E., Fumagalli, M., Cooney, C. R., Bloch, N. I., Vieira, F. G., Buechel, S. D., … Mank, J.

E. (2018). Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evolution Letters*, *2*(2), 52–61.

Wright, A. E., Harrison, P. W., Zimmer, F., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Variation in promiscuity and sexual selection drives avian rate of Faster-Z evolution. *Molecular Ecology*, *24*(6), 1218–1235.

Wright, A. E., Moghadam, H. K., & Mank, J. E. (2012). Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*, *192*(4), 1433–1445.

Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., … Flicek, P. (2018). Ensembl 2018. *Nucleic Acids Research*, *46*(D1), D754–D761.

Zhang, Y., Sturgill, D., Parisi, M., Kumar, S., & Oliver, B. (2007). Constraint and turnover in sex-biased gene expression in the genus *Drosophila*. *Nature*, *450*(7167), 233–237.

CHAPTER 3. MULTI-COPY GENE FAMILY EVOLUTION ON THE AVIAN W CHROMOSOME

3.1 AUTHOR CONTRIBUTIONS

This works forms the publication "Multi-copy gene family evolution on the avian W chromosome" published in *Journal of Heredity* (2021), the published version of which is included in Chapter 7 of this thesis.

Rogers, T. F. Pizzari, T. & Wright, A. E. (2021). Multi-copy gene family evolution on the avian W chromosome. *Journal of Heredity 112*(3), 250–259.

T.P. provided Red Junglefowl samples. T.F.R and A.E.W designed the research. T.F.R and A.E.W. conducted the analyses. T.F.R wrote the manuscript, with input from all authors.

3.2 ABSTRACT

The sex chromosomes often follow unusual evolutionary trajectories. In particular, the sex-limited Y and W chromosomes frequently exhibit a small but unusual gene content in numerous species, where many genes have undergone massive gene amplification. The reasons for this remain elusive with a number of recent studies implicating meiotic drive, sperm competition, genetic drift and gene conversion in the expansion of gene families. However, our understanding is primarily based on Y chromosome studies as few studies have systematically tested for copy number variation on W chromosomes. Here, we conduct a comprehensive investigation into the abundance, variability, and evolution of ampliconic genes on the avian W. First, we quantified gene copy number and variability across the duck W chromosome. We find a limited number of gene families as well as conservation in W-linked gene copy number across duck breeds, indicating that gene amplification may not be such a general feature of sex chromosome evolution as Y studies would initially suggest. Next, we investigate the evolution of HINTW, a prominent ampliconic gene family hypothesized to play a role in female reproduction and oogenesis. In particular, we investigate the factors driving the expansion of HINTW using contrasts between modern chicken and duck breeds selected for different female-specific selection regimes and their wild ancestors. Although we find the potential for selection related to fecundity in explaining small-scale gene amplification of HINTW in the chicken, purifying selection seems to be the dominant mode of evolution in the duck. Together, this challenges the assumption that HINTW is key for female fecundity across the avian phylogeny.

3.3 INTRODUCTION

Sex chromosomes are subject to unique evolutionary pressures due to their sex-limited inheritance and exhibit many unusual characteristics compared to the rest of the genome (Furman et al., 2020). They evolve when an autosome acquires a sex determining locus followed by halting of recombination between the sex chromosome pairs (Bergero & Charlesworth, 2009; Charlesworth, 1991). This recombination suppression triggers a cascade of neutral and adaptive processes that cause the once identical chromosomes to diverge from each other, often leading to the evolution of heteromorphic sex chromosomes (Bachtrog, 2013). These effects are most pronounced for the sex-limited Y and W chromosomes, which experience a reduction in the efficacy of selection, often resulting in rapid decay of gene

content and activity due to processes such as Muller's ratchet, the Hill-Robertson effect and genetic hitchhiking (Bachtrog, 2008; Bachtrog & Charlesworth, 2002; Charlesworth, 1978; Charlesworth & Charlesworth, 2000; Rice, 1996). In addition, because the Y and W chromosomes are haploid and only present in one sex, their effective population size is a fraction of that of the autosomes (Bachtrog & Charlesworth, 2002; Haddrill et al., 2007), making them more susceptible to genetic drift. Indeed, many Y chromosomes often consist of very few functional genes (Mank, 2012), however, intriguingly many of these genes have undergone massive gene amplification and persist as members of multi-copy gene families. For instance, the human Y chromosome harbours nine multi-copy ampliconic gene families which constitute the majority of protein-coding genes present on the Y (Skaletsky et al., 2003). Why these ampliconic gene families have evolved on heteromorphic sex chromosomes is an open question and their phenotypic consequences remain debated. It is also becoming increasingly apparent that copy number of these gene families can vary substantially, not only across closely related species but also individuals of the same species (Brashear et al., 2018; Lucotte et al., 2018; Poznik et al., 2016; Vegesna et al., 2019; Vegesna et al., 2020; Ye et al., 2018). Understanding the factors driving this variability can provide insight into the adaptability and functional importance of sex chromosomes more broadly.

It is widely assumed that the expansion of multi-copy ampliconic gene families is an adaptive response to lack of recombination between the sex chromosomes, where non-allelic homologous gene conversion between copies can escape Muller's ratchet and the accumulation of deleterious mutations (Betrán et al., 2012; Charlesworth & Charlesworth, 2000; Connallon & Clark, 2010). Indeed, gene conversion appears to be a common feature of amplicons on both the Y and W chromosome across multiple species (Backström et al., 2005; Davis et al., 2010; Geraldes et al., 2010; Rozen et al., 2003; Skov et al., 2017). Furthermore, many Y amplicons are expressed exclusively within the testes (Mueller et al., 2008; Skaletsky et al., 2003; Vegesna et al., 2020) and often implicated in spermatogenesis and male fertility in humans (Kuroda-Kawaguchi et al., 2001; Lahn & Page, 1997; Vogt et al., 1996), leading to the hypothesis that selection on male fertility, often as a consequence of sperm competition, drives the expansion of multi-copy gene families. While there appears to be a positive relationship between copy number and expression level across some gene families (Vegesna et al., 2019), as well as with sperm mobility in humans (Yan et al., 2017), comparative

approaches across species have failed to detect a significant correlation between copy number and intensity of sperm competition (Vegesna et al., 2020), although this may be due to the small number of species examined to date. Intriguingly, in several species there has been rapid co-amplification of genes on both sex chromosomes, suggestive of genomic conflict during gametogenesis to bias the transmission of the X versus Y (Bachtrog et al., 2019; Hughes et al. 2020; Soh et al., 2014). Detailed molecular analysis of the Sly and Slx gene families in the mouse provides strong support for antagonistic interactions and segregation distortion as a major force in driving gene amplification (Cocquet et al., 2012; Larson et al., 2018). Similarly, meiotic drive has been implicated in the evolution of gene families on the *Drosophila* Y chromosome (Bachtrog et al., 2019). Finally, many amplicons appear to be evolving under relaxed purifying selection, consistent with the reduced efficacy of selection on the non-recombining Y (Ghenu et al., 2016; Vegesna et al., 2020). Thus, while a myriad of forces have been implicated in the amplification of gene families on the Y and W chromosomes, the relative importance of each remains unclear.

To date, our understanding of multi-copy ampliconic gene families is primarily based on Y chromosome studies across mammals and *Drosophila*, and the W chromosome has been largely overlooked. Although the W is in many ways comparable to the Y chromosome, as both are sex-limited and do not recombine, the W is only present in females and the Y is only present in males. Therefore, the W chromosome, unlike the Y chromosome, does not experience sperm competition and might be subject to weaker sexual selection than the Y (Bachtrog et al., 2011). Additionally, in polygynous mating systems where a small proportion of males in the population mate with multiple females, the effective population size of the Y relative to the autosomes is smaller than that of the W (Mank, 2012; Wright & Mank, 2013) As a result, the W chromosome may be less susceptible to genetic drift than the Y. Therefore, if multi-copy gene families are a consequence of random gene amplification due to genetic drift, they should be more pronounced on the Y chromosome rather than represent a general feature of heteromorphic sex chromosomes. It remains unclear whether W-linked amplicons have followed similar patterns of evolution to ampliconic genes on Y chromosomes, and whether gene amplification always occurs in parallel with sex chromosome degeneration.

A limited number of W-linked multi-copy gene families have been documented in a handful of species, primarily avian (Backström et al., 2005; Davis et al., 2010; Moghadam et al., 2012;

Smeds et al., 2015; Zhou et al., 2020). The best studied is HINTW, an ampliconic gene family present on the avian W chromosome that is hypothesized to play a role in female reproduction and oogenesis (Ceplitis & Ellegren, 2004; O'Neill et al., 2000), and was originally proposed as the avian sex determining gene (Moriyama et al., 2006; Pace & Brenner, 2003; Parks et al., 2005). While an initial study of HINTW indicated that large scale amplification of copy number is conserved across avian non-ratites (Hori et al., 2000), a recent study suggested that HINTW is single-copy in the Pekin duck (Li et al., 2021). To date, there has been no comprehensive investigation into the abundance, variability, and evolution of multi-copy ampliconic gene families on the W chromosome both across and within species.

Here, we conduct a comparative analysis of copy number variation of W-linked genes across chicken and duck breeds. Multi-copy gene families are notoriously challenging to study due to their highly repetitive nature (Tomaszkiewicz et al., 2016). This problem is confounded on the sex-limited Y and W chromosomes where amplicons are often located in repeat-rich regions that are poorly annotated in reference genomes. We employ NanoString technology, which is based on fluorescent probes, to provide high-throughput fine-scale estimates of gene copy number and variability (Ahn et al., 2016; Cui et al., 2014). First, we quantify the frequency and variability of multi-copy gene families on the W across duck breeds, and find a limited number of amplicons on the duck W as well as conservation in copy number of W-linked genes. Next, we investigate the role of selection for fecundity in driving the amplification of HINTW using contrasts between chicken and duck breeds selected for either egg laying, male meat production or male plumage. We find that although large scale amplification of HINTW is ancestral to land and waterfowl species, smaller scale gene duplications have occurred independently across chicken breeds. Our results support a potential role of female-specific selection in driving amplification of the HINTW gene family in the chicken but not the duck, challenging the assumption that HINTW is key for female fecundity across the avian phylogeny.

3.4 MATERIALS & METHODS

3.4.1 Samples and DNA extraction

Our workflow is summarised in Fig. S6.2.1. We obtained tissue samples from Khaki Campbell, Indian Runner, Aylesbury and Cayuga duck breeds and their modern ancestor, the Mallard duck (*Anas platyrhynchos*) (Zhang et al., 2018). In addition, we sampled the White Leghorn,

Black Minorca, Oxford Old English and Black Sumatra chicken breeds and their main modern ancestor, the Red Junglefowl (*Gallus gallus*) (Frisby et al., 1979; Fumihito et al., 1996).

Samples were collected in accordance with national and ethical guidelines. Specifically, we obtained feathers from White Leghorn and Black Minorca. We also obtained 50 microlitres of Red Junglefowl blood in 1ml of absolute ethanol from a captive population at Oxford University (PPL P50402706). We obtained fertilised eggs from the following duck breeds; Mallard, Khaki Campbell, Cayuga, Aylesbury, Indian Runner, and the following chicken breeds; Oxford Old English and Black Sumatra. All eggs were kept under standard incubation conditions at The University of Sheffield. Samples were collected according to national and ethical guidelines and the liver was dissected at embryonic day 19 and 24 in chicken and duck breeds respectively, then stored in 95% ethanol.

DNA was extracted from feather and embryonic liver samples using DNeasy blood and tissue kit (QIAGEN) using standard protocols. DNA was extracted from blood samples using the ammonium acetate precipitation method. In total, DNA was obtained for three female and two male samples from each of the domesticated breeds, and two female and two male samples from each of the modern ancestor breeds. Embryonic birds were sexed visually and feather and blood samples were sexed using published sexing primers (Fridolfsson & Ellegren, 1999).

The majority of modern chicken breeds originated at the start of the 20th century (Rubin et al., 2010). Most modern chicken breeds are descended from the Red Junglefowl (Frisby et al., 1979; Fumihito et al., 1996) with some genes introgressed from the Grey Junglefowl and possibly other Junglefowl species (Eriksson et al., 2008). The Black Minorca and White Leghorn are layer breeds, which have been selected for female reproductive traits (e.g. fecundity), and the Oxford Old English and Black Sumatra chickens have been selected for male traits such as plumage for ornamentation purposes and aggression for cockfighting. The Oxford Old English and Black Sumatra lay fewer eggs than the two layer breeds and experience numerous female fecundity problems (Ekarius, 2007; Lewis, 2010). Importantly, the chicken breeds used in this study have independent origins (Moghadam et al., 2012) and so we can treat them as independent replicates of increased or relaxed female-specific selection. Most modern duck breeds are descended from the Mallard duck (Zhang et al., 2018). The Indian Runner and Khaki Campbell duck breeds have been subject to strong

female-specific selection for egg laying, and the Aylesbury and the Cayuga for meat production (Ashton et al., 1999). Selection for meat- and egg-purpose breeds occurred at the early stages of duck domestication (Zhang et al., 2018) and so it is unclear whether the two layer breeds in our study can be considered independent replicates of increased female-specific selection.

3.4.2 Identification of W-linked genes

Previously, we identified 26 W-linked genes in the duck reference genome (Wright et al., 2014) using a combination of phylogenetic analyses and PCR validation in females. Some of these W genes share the same Z-linked ortholog, indicating they are either paralogs of a multi-copy gene family or fragments of the same gene, which have been assembled into separate genic sequences in the reference genome. Genome assemblies of sex chromosomes can be unreliable due to their repetitive nature and low sequencing coverage (Tomaszkiewicz et al., 2017) and so the latter scenario is plausible. To distinguish between these two scenarios, we aligned W-linked coding sequences with their Z-linked ortholog using PRANK (Löytynoja, 2014) and calculated pairwise distances. For the majority of cases, W-linked sequences shared no sequence similarity with each other, indicating they are fragments of the same gene that have been incorrectly assembled and annotated into separate genes. For subsequent analyses, we averaged data across fragments for these genes. Our results are quantitatively identical whether fragments are analysed separately or combined (see Supplementary tables). The exception was KCMF1 in which the two annotated W sequences in the reference align and have a low pairwise distance, where the proportion of nucleotide differences was 0.091, suggesting these are paralogs of the same multigene family.

However, HINTW is not annotated in the duck reference genome and a previous study only identified a short fragment of sequence (Hori et al., 2000). Therefore, we sequenced a 702 bp fragment of HINTW in the Mallard using Sanger sequencing at the Core Genomic Facility, University of Sheffield with primers designed for the black oystercatcher (*Haematopus bachmani*) (Guzzetti et al., 2008). Primers are listed in Table S6.2.1.

For each PCR reaction the following volumes and concentrations of reagents were used: 4 ul multiplex PCR Master Mix (QIAGEN), 2 ul forward primer, 2 ul reverse primer (initial conc of each 0.2 uM) and 1 ul DNA (initial conc 15 ng/ul). In addition to this, 1 ul of nuclease free H_2O was added to reach a total volume of 10 ul per reaction. The PCR conditions were: initial

denaturing stage of 95°C for 15 minutes, then 35 cycles of the following three steps; 94°C for 30 seconds, an annealing step at 57°C for 90 seconds, and an extension at 72°C for 90 seconds. This was then followed by a final extension at 72°C for 10 minutes.

3.4.3 Identification of autosomal invariant genes

The NanoString pipeline relies on the identification of invariant genes, autosomal single copy genes in that do not vary in copy number, as internal controls. We identified invariant genes in the duck and chicken separately using a genomic coverage approach. SOLiD DNA-seq data from nine chicken breeds were obtained from Rubin et al. (2010) and reads were aligned to the chicken reference genome (Gallus_Gallus-5.0, Zerbino et al., 2018) using SHRiMP v. 2.2.2 (Rumble et al., 2009). Mapped reads with a quality score of 10 or above were retained using SAMtools v. 1.8 (Li et al., 2009). Illumina DNA-seq reads from seven duck breeds (Zhang et al. 2018) were aligned to the duck reference genome (BGI_duck_1.0, Zerbino et al., 2018) using BWA v. 0.5.7 (Li & Durbin, 2009) with the 'mem' algorithm. Read depth for each gene was calculated for both the chicken and the duck using the depth function in SAMtools. For each species, we conducted pairwise regressions of read depth per gene across every breed. We ranked residuals and identified genes in the lowest 35% quantile across all pairwise comparisons, indicative of limited or no copy number variation. We then used SNP data to test for nucleotide polymorphism across these genes, and we only called SNPs if the minor allele was present in one than one read. We chose genes with an absence of nucleotide polymorphism, and therefore an absence of multiple copies, as our invariant genes.

3.4.4 Quantification of gene copy number using NanoString

Copy number was quantified using the NanoString nCounter platform at the NERC Biomolecular Analysis facility (NBAF), University of Liverpool. NanoString nCounter technology uses fluorescent probes to estimate fine scale variation in gene copy number across samples (Ahn et al., 2016; Cui et al., 2014). Probes were designed for W-linked genes and invariant genes in the Red Jungle Fowl and Mallard duck separately in accordance with NanoString protocol (Table S6.2.2). Specifically, two or three probes were designed for HINTW in the chicken and 26 W-linked genes in the duck. One or two probes were designed for each invariant gene.

We implemented a number of controls to ensure copy number was quantified for only W-linked and not their Z-linked orthologs. Genome assemblies of sex chromosomes are often unreliable due to their repetitive nature and low sequencing coverage (Tomaszkiewicz et al., 2017) and therefore accurately identifying W-specific regions can be problematic. Furthermore, given that the Z and W chromosome evolved from the same pair of autosomes, certain regions of W-linked genes have high sequence similarity to their Z-linked gametolog (Wright et al., 2012). First, we designed probes to W-linked exons with low sequence similarity to Z-linked orthologs. Second, we included male samples in the CNV CodeSet analysis, making it possible to identify and exclude probes that bind to the Z chromosome.

The NanoString nCounter assay was performed according to standard protocol. Briefly, at least 300ng of DNA per sample was fragmented via AluI digestion and then hybridized to the custom CNV CodeSet. Samples included three females and two males from each of the selectively bred breeds, and two female and two male samples from each of the modern ancestor breeds. Samples were distributed randomly over the CNV CodeSets to avoid batch effects. The nCounter Digital Analyzer was used to count and quantify signals of reporter probes. Data analysis was performed using the nSolver Analysis Software.

We implemented a number of sanity checks as recommended by NanoString. First, we removed probes with count data above background noise in males and therefore affinity to the Z chromosome (Table S6.2.2). Background noise was calculated for each sample according to NanoString protocol as the average plus two standard deviations of the count number in the negative controls. We also removed one probe with count data below background noise in females, indicating low binding affinity. Second, as multiple probes were designed per W-linked gene, we calculated the coefficient of variation for copy number across probes. A high coefficient of variation is indicative of a probe that is not binding as predicted. As recommended by NanoString, we removed two probes from two different genes where the sum of the coefficient of variation across samples was >= 100 (Table S6.2.2). We averaged count data across all remaining probes of each gene in every individual.

3.4.5 Quantification of gene copy number from SNP data

We used polymorphism estimates from publicly available DNA-seq data to independently verify the results obtained from the NanoString nCounter assay in the Mallard duck. Given

that we expect many gene copies to share identical sequences due to gene conversion (Backström et al., 2005), we can only use SNPs to estimate a minimum copy number.

Illumina data from nine unsexed Mallard ducks (Zhang et al., 2018) were quality trimmed to a minimum of 34 bp using Trimmomatic v. 0.36 (Bolger et al., 2014). Data were then aligned to the duck reference genome (BGI_duck_1.0, Zerbino et al., 2018), with the 702 bp sequenced fragment of HINTW added, using BWA v. 0.7.17 (Li & Durbin, 2009) with the 'aln' algorithm. Alignments were filtered for uniquely mapped reads by keeping only lines of the BAM files that matched the flag 'XT:A:U'. We used read coverage to sex individuals, where Z-linked genes should show half the number of reads in females relative to males. Read depth per gene was calculated using the depth function in SAMtools. To control for differences in overall sequencing depth between individuals we divided read depth on the Z chromosome by average autosomal read depth in each sample. Six females were identified and used in subsequent analyses.

BCFtools v. 1.9 (Narasimhan et al., 2016) was used to call SNPs at sites with a mapping quality > 20. In order to classify a SNP that indicated copy number variation, both the major and minor allele had to be supported by at least four reads and be present in more than half the individuals. Minor allele read depth was also required to be supported by at least 10% the number of reads that supported the major allele.

3.5 RESULTS

3.5.1 Copy number of genes on the Mallard W chromosome

We surveyed copy number of 26 genes on the Mallard duck W chromosome using count data obtained from NanoString nCounter. First, count data for W genes were normalised to invariant genes, autosomal genes present in a single copy, following NanoString protocol to account for any differences across samples in genomic DNA input arising from pipetting error or inaccuracies in DNA quantitation. Specifically, in each individual separately, we calculated average counts across all 10 invariant genes and bootstrapped with 1000 replicates to obtain the 95% confidence intervals. We divided the confidence intervals by two to account for comparisons between autosomal genes, which are present in two copies, and W-linked genes, which are present at a minimum of one copy. We then divided count number for each W gene

by invariant count values to obtain estimates of W copy number in each individual and 95% confidence intervals.

In the Mallard duck, most W genes are present in a single copy. We found that HINTW is ampliconic, present in approximately 18 copies. This is in contrast to recent work suggesting that HINTW is single-copy in the Pekin duck (Li et al., 2021; Xu & Zhou, 2020). Furthermore, we found that KCMF1W is a multi-copy gene family present in 2 to 3 copies (Tables 3.1 & S3.3).

We independently verified copy number estimates using publicly available sequence data from Mallard individuals and nucleotide polymorphism analyses. No SNPs were found in any of the W genes with the exception of KCMF1W (ENSAPLG00000003106), where a single SNP was identified. This supports our finding that the majority of W-linked genes are present in a single copy in the Mallard. Although we verified that HINTW is ampliconic using NanoString data, we did not identify any nucleotide polymorphism across copies. This instead may indicate the occurrence of gene conversion across HINTW in the duck, which acts to homogenise gene sequence among variants, and is consistent with previous results in galliform birds (Backström et al., 2005).

3.5.2 Copy number variation across duck breeds

We used the same approach to estimate copy number of W-linked genes across the four duck breeds, with the exception of HINTW which we discuss separately below. Copy number was broadly conserved, as the majority of genes are present in a single copy across all breeds (Tables 3.1 & S3.3), with the exception of KCMF1W. This multi-copy gene family varies from 2 to 3 copies in some breeds to 3 to 4 copies in others, suggesting there may have been lineage-specific duplications in certain breeds (Tables S3.3 & S3.4).

In order to verify these results using a separate approach, we next estimated copy number in each breed relative to the Mallard duck. For each W-linked gene, normalised count data in each individual were divided by the average normalised count data for the Mallard to estimate relative copy number. We found that every W gene had a copy number ranging from 0.88 to 1.21 relative to the Mallard in all individuals, supporting our finding that there is limited copy number variation across duck breeds.

Table 3.1. Copy number of W-linked genes across duck breeds.

Gene name	Duck Ensembl ID	Average copy number					Δ Copy number	Coefficient of variation	Stratum^
		Mallard	Caguya	Aylesbury	Indian Runner	Khaki Campbell			
HINTW+	NA	18.03	16.35	16.57	17.22	16.83	1.68	0.04	1
CHD1W*	ENSAPLG05191 ENSAPLG02506	0.94	0.97	0.98	0.99	0.98	0.05	0.09	1
KCMF1W	ENSAPLG03026 ENSAPLG03106	2.43	2.59	2.65	2.63	2.63	0.20	0.10	2
RASA1W	ENSAPLG05611 ENSAPLG10611	0.64	0.69	0.70	0.69	0.70	0.06	0.11	2
ATP5A1W+	ENSAPLG10611 ENSAPLG09007	0.82	0.79	0.84	0.85	0.83	0.06	0.07	3
BTF3W	ENSAPLG04652	0.65	0.60	0.64	0.65	0.65	0.05	0.06	3
HNRPKW*+	ENSAPLG10986	0.97	1.00	1.09	1.02	1.05	0.12	0.11	3
MIER3W+	ENSAPLG10850	0.62	0.61	0.65	0.62	0.63	0.04	0.08	3
NIPBLW	ENSAPLG02953 ENSAPLG03022 ENSAPLG05315 ENSAPLG10290 ENSAPLG10560	0.67	0.69	0.71	0.69	0.70	0.04	0.09	3
SMAD2W	ENSAPLG04964	0.69	0.72	0.74	0.71	0.71	0.05	0.09	3
SPIN1W*	ENSAPLG02923	0.63	0.61	0.64	0.66	0.64	0.05	0.08	3
UBAP2W	ENSAPLG16004 ENSAPLG16155	0.61	0.58	0.59	0.60	0.60	0.03	0.06	3
UBE2R2W	ENSAPLG16000	0.76	0.74	0.76	0.78	0.76	0.04	0.07	3
VCPW+	ENSAPLG05806	0.91	0.84	0.90	0.91	0.90	0.07	0.06	3
ZFRW*	ENSAPLG15519	0.67	0.68	0.69	0.70	0.68	0.03	0.08	3
ZSWIM6W	ENSAPLG13555 ENSAPLG14338	0.77	0.80	0.80	0.83	0.80	0.06	0.09	3

* q-PCR analysis showed variation in copy number of ortholog across chicken breeds (Moghadam *et al.* 2012)
+ SNP analysis showed chicken ortholog is multicopy (Moghadam *et al.* 2012)
^ Anseriform strata as defined by Wright *et al.* 2014 Evolution. Strata 1 & 2 are conserved in chicken and duck but Stratum 3 evolved independently.
Note: six zeros have been removed from start of the digits in the Ensembl IDs.

Finally, we estimated variation in copy numbers by calculating the coefficient of variance of raw count data across all individuals and breeds for each W-linked gene. Coefficient estimates ranged from 0.078 to 0.112 across individuals (Tables S3.5 & S3.6), and importantly no value exceeded the maximum coefficient of variation for invariant genes (mean COV = 0.131, max COV = 0.416), indicating limited variation in W-linked copy number. We repeated the analysis across breeds using average copy number in each breed and found a similar pattern, whereby coefficients of variation ranged from 0.043 to 0.106. No W gene exhibited higher variation across breeds than that observed across invariant genes (mean COV = 0.111, max COV = 0.356).

3.5.3 Copy number variation of ampliconic HINTW across duck and chicken breeds

Next, using contrasts between modern chicken and duck breeds selected for different female-specific selection regimes and their wild ancestors, we investigated the factors driving the expansion of HINTW. First, we estimated the size of the ampliconic HINTW gene family across duck breeds and found limited differences, where the number of copies ranged from 15 to 18 across individuals (Fig. 3.1A, Tables S3.3 & S3.4). In addition, the coefficient of variance of HINTW count data across individuals (mean COV = 0.080) and breeds (mean COV = 0.043) was not higher than variation across invariant genes (Tables S3.5 & S3.6). Importantly, there is no significant difference in average copy number between breeds (ANOVA; $p = 0.312$). This suggests that copy number of HINTW is broadly conserved across duck individuals and breeds (Table S6.2.7), consistent with our predictions for purifying selection.

In contrast, we found notable variation in the size of the HINTW gene family across chicken breeds and individuals, ranging from 7 to 17 copies. The coefficient of variance for the chicken was 0.213 across individuals and 0.221 across breeds, both of which are higher than mean variation exhibited across invariant genes (mean COV = 0.151, max COV = 0.244 across individuals and mean COV = 0.116, max COV = 0.166 across breeds). Importantly, we found that the average size of HINTW gene family varied significantly between breeds (ANOVA; $p = 0.001$). Interestingly, all breeds have higher copy number of HINTW than the Red Junglefowl, and this was significant for three breeds (Fig. 3.1B), indicating that the early domestication of chicken breeds may have been associated with a period of female-specific selection, presumably for egg laying. We find a general trend that breeds which have been selected for egg production via artificial female-specific selection (Kerje et al., 2003), had on average higher number of copies relative to breeds which have been bred for male fighting and plumage and subject to relaxed female-specific selection (Ekarius, 2007; Lewis, 2010) (Fig. 3.1B). However, this relationship was only significant for the Black Minorca and not the White Leghorn (Table S6.2.8).

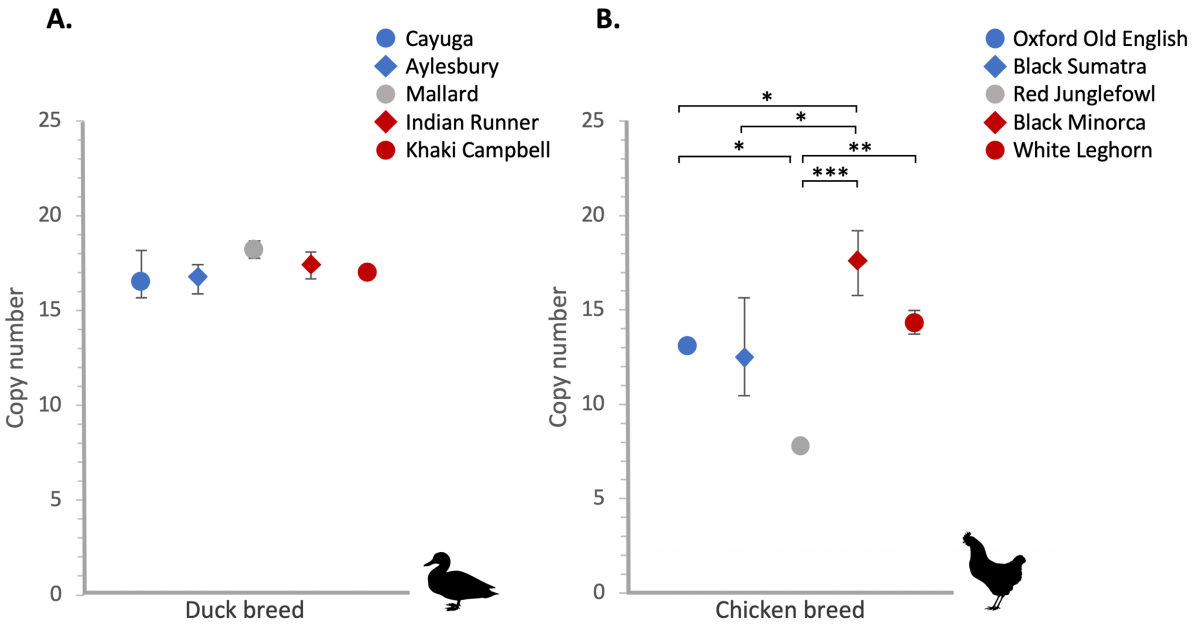

Figure 3.1. Copy number variation of HINTW across (A) duck and (B) chicken breeds. Copy number was estimated using the NanoString nCounter platform. Each circle or diamond represents the mean HINTW copy number per breed, and bars show the range of HINTW copy number across individuals. Blue markers represent breeds subject to relaxed female-specific selection, red markers represent female-selected breeds, and grey markers denote the modern ancestor for each bird species. Stars indicate pairwise significance values from Tukey multiple comparisons of means where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.6 DISCUSSION

The sex-limited Y and W chromosomes exhibit a small but unusual gene content in many species compared to the rest of the genome. One striking feature is the existence of ampliconic gene families, arising from massive gene amplification of distinct classes of genes. Our understanding of how and why these ampliconic regions have evolved is primarily based on detailed Y chromosome studies across mammals and *Drosophila*, which have implicated a multitude of factors in the expansion of gene families, including meiotic drive, sperm competition, genetic drift and gene conversion (Bachtrog et al., 2019; Cocquet et al., 2012; Ellis et al., 2011; Ghenu et al., 2016; Good, 2012; Larson et al., 2018; Skaletsky et al., 2003; Soh et al., 2014; Vegesna et al., 2020). However, the evolution of multi-copy gene families on the W chromosome has been largely overlooked, with the exception of a handful of studies (Backström et al., 2005; Davis et al., 2010; Hori et al., 2000; Moghadam et al., 2012; Zhou et al., 2020). As a result, it remains unclear whether ampliconic genes are a fundamental feature

of heteromorphic sex chromosome evolution or a peculiar quirk of Y chromosomes. Here, we conduct a comparative analysis to examine the abundance, variability, and evolution of ampliconic gene families on the avian W chromosome both across and within two avian species.

Our results show little evidence for gene amplification on the duck W chromosome. Of the 26 W-linked genes we studied, only two are present in multiple copies. One of these is HINTW, a large well-known ampliconic gene family, that has previously been characterized across a wide range of avian species (Backström et al., 2005; Hori et al., 2000). The fact that HINTW is ampliconic in the Mallard and four duck breeds is in contrast to recent work in the Pekin duck (Li et al., 2021; Xu & Zhou, 2020). Moreover, our finding that the W chromosome in the Mallard and domesticated duck breeds is generally depauperate in multi-copy gene families is consistent with a growing body of avian literature, including studies in the chicken (Moghadam et al., 2012), flycatcher (Smeds et al., 2015), sparrow (Davis et al., 2010), songbirds (Xu et al., 2019) and Pekin duck (Li et al., 2021). Outside of birds, to our knowledge, there is only one report of a W-linked ampliconic gene family in the willow *Salix purpurea* (Zhou et al., 2020), though few W chromosomes have been studied in sufficient detail. This deficit of gene families on the W is in stark contrast to the Y chromosome in mammals and *Drosophila*, where there has been massive amplification of gene sets.

This emerging pattern is consistent with theoretical predictions for how we expect the W to evolve differently to the Y due to their contrasting inheritance patterns (Bachtrog et al., 2014; Mank, 2012). First, as the W chromosome is maternally inherited it is not subject to sperm competition, a factor which has been hypothesised, with mixed empirical support, to drive the expansion of Y-linked gene families (Hughes et al., 2010; Vegesna et al., 2020). It should be noted that the lack of support Vegesna et al. (2020) find for this hypothesis could be due to the small number of species examined in their study. Second, genetic drift is predicted to be weaker on the W in comparison to the Y chromosome. In polygynous mating systems, where a small proportion of males in the population mate with several females, the effective population size of the Y relative to the autosomes is smaller than that of the W (Mank, 2012; Wright & Mank, 2013). Relaxed purifying selection has been invoked to explain amplification of certain gene families on the primate and human Y chromosome, and the large variability in copy number across individuals and populations (Ghenu et al., 2016; Vegesna et al., 2020;

Ye et al., 2018). Under drift, we expect variance in copy number to be approximately proportional to gene family size, where larger gene families will have a greater chance of gene duplication. Interestingly, we do not observe this pattern on the duck W chromosome where variability in the size of the HINTW gene family, present in ~18 copies, was similar to KFMC1, present in ~2 copies, across individuals and breeds. This is consistent with previous work showing evidence for purifying selection on the Mallard W (Wright et al., 2014).

Lastly, Y and W chromosomes are exposed to different types of gametogenesis, where the W is subject to oogenesis and the Y to spermatogenesis. Importantly, these contrasting environments likely lead to differences in the potential for antagonistic coevolution between the sex chromosomes. Antagonistic coevolution is predicted to drive the co-amplification of X and Y-linked genes (Bachtrog, 2020), but should be weaker during oogenesis than spermatogenesis. This is because the window for intragenomic conflict between chromosomes is restricted to the first meiotic division during oogenesis as only a single oocyte is produced containing either the Z or W (Bellott et al., 2017). Therefore, antagonistic coevolution between the Z and W will be limited to the first meiotic division. In contrast, competition between the X and Y can occur during meiosis I and II of spermatogenesis as both of these cell divisions produce viable gametes. As a result, we expect meiotic drive to play a less prominent role in the evolution of the W compared to the Y, and might explain why meiotic drive has been heavily implicated in the amplification of gene families on the mouse and *Drosophila* Y chromosomes (Bachtrog et al., 2019; Cocquet et al., 2012; Ellis et al., 2011; Good, 2012; Larson et al., 2018; Soh et al., 2014)

In addition, expression of the sex chromosomes is repressed during the post meiotic stages of spermatogenesis, leading to intragenomic conflict between X- and Y-linked genes over the transcriptional machinery and selection for gene amplification to maintain gene expression (Moretti et al., 2020). In contrast, no corresponding mechanism of sex chromosome repression in oogenesis has been reported thus far, and so we expect less co-amplification due to antagonistic coevolution in ZW systems. In support of these predictions, there is no evidence for co-amplification of HINTZ or KCMF1 on the avian Z chromosome (Bellott et al., 2010), indicating that antagonistic coevolution is unlikely to be a major factor influencing gene amplification on the W. Together, our results indicate that large scale expansions of gene families does not always occur in parallel with sex chromosome degeneration and so

may not be such a general feature of sex chromosome evolution as Y studies would initially suggest.

Finally, as the W chromosome is maternally inherited it is not subject to sperm competition, a factor which has been hypothesised, with mixed empirical support, to drive the expansion of Y-linked gene families (Hughes et al., 2010; Vegesna et al., 2020). However, in theory, sex-specific selection for increased expression of genes associated with fecundity could drive amplification of gene families on the W chromosome, analogous to the hypothesised role of sperm competition on the Y chromosome (Hughes et al., 2005). In order to examine the factors driving the evolution of multi-copy gene families, we contrasted copy number of HINTW across breeds of the duck and chicken. Specifically, we chose breeds that have been subject to stronger or relaxed female-specific selection. In theory, sex-specific selection for increased expression of genes associated with fecundity could drive amplification of gene families. This seems particularly relevant for HINTW, which is expressed in the developing ovaries (O'Neill et al., 2000) and hypothesized to play a role in female reproduction (Ceplitis & Ellegren, 2004; O'Neill et al., 2000). Furthermore, increased copy number of Y-linked genes has been shown to result in greater gene expression level across primates, although this pattern is not universal across all gene families (Vegesna et al., 2019; Yan et al., 2017). However, in general there is uncertainty over whether the W chromosome is subject to female-specific selection, and is enriched for female reproductive functions (Moghadam et al., 2012), or subject to purifying selection for dosage effects (Bellott et al., 2017; Smeds et al., 2015; Xu et al., 2019; Xu & Zhou, 2020).

We find that HINTW copy number across duck breeds and individuals is remarkably conserved, in contrast to ampliconic gene families of equivalent size on the mammalian and *Drosophila* Y chromosomes (Bachtrog, 2013). We were unable to identify any sequence polymorphism across copies of HINTW, indicative of persistent gene conversion. While gene conversion is unlikely to explain the origin of multi-copy gene families, because it acts at a scale of a few hundreds of bases as opposed to a much larger scale of whole gene duplicates (Chen et al., 2007; Connallon & Clark, 2010; Marais et al., 2010), it has been proposed to select for the maintenance of ampliconic gene families and has been shown to operate across HINTW copies in a number of avian species (Backström et al., 2005). However, it is worth noting that the duck HINTW fragment in our study was only 702 bp, lowering the probability

of finding a SNP in this gene and increasing our chances of inferring the action of gene conversion. Together, our results are inconsistent with the role of female-specific selection in driving the evolution of HINTW copy number in the duck. Instead, the conservation in copy number we observe across breeds suggests that HINTW copy number is under strong purifying selection. This is consistent with a number of recent studies showing that the avian W chromosome evolves predominantly under purifying selection to maintain ancestral gene dosage (Bellott et al. 2017; Bellott & Page, 2021; Smeds et al., 2015; Wright et al., 2014).

In contrast, in the chicken, we find notable variation in HINTW copy number across breeds. Breeds subject to female-specific selection tend to exhibit a greater number of HINTW copies This is consistent with the prediction that the chicken HINTW plays a role in female fecundity (Ceplitis & Ellegren, 2004; O'Neill et al., 2000). However, there is considerable variation in this trend, potentially indicating that female-limited selection is not the dominant force driving the evolution of HINTW.

The discrepancy between levels of variation in the size of the HINTW gene family in the chicken and duck is intriguing, particularly as large-scale gene amplification likely occurred in the ancestor of non-ratite birds (Hori et al., 2000). While evidence from the chicken indicates that HINTW plays a role in oogenesis (Ceplitis & Ellegren, 2004; O'Neill et al., 2000), evidence for functionality of HINTW in the duck is lacking. In fact, HINTW in the duck has been shown to lack the C-terminal 14 residues (Hori et al., 2000). HINTW forms a heterodimer with, and inhibits HINTZ in the chicken (Hori et al., 2000), and it is possible that the deletion in the duck has altered its ancestral functionality. Alternatively, it is possible that HINTW may have evolved differential gene expression across duck breeds without a corresponding increase in copy number. Consistent with this explanation, many W-linked genes have evolved increased expression in the chicken embryonic gonad in response to female-specific selection relative to the modern ancestor Red Junglefowl in the absence of copy number variation (Moghadam et al., 2012). It is also possible that the chicken has been subjected to stronger or more consistent sex-specific selection regimes than the duck, although evidence for this is currently lacking. Similarly, it is possible that the timing of domestication differs between the duck and chicken breeds in our study, or that there are differences in the extent of interbreeding. Although the exact breed history of chicken and ducks is obscure, evidence indicates that duck breeds selected for egg laying and meat production form two monophyletic groups that

split early in duck domestication approximately 2200 years ago (Zhang et al., 2018). Therefore, we think that the lack of inter-breed copy number variation in the duck is unlikely to be a consequence of more recent origin or greater levels of interbreeding, although we cannot rule out this possibility.

In addition, we find that gene amplification has proceeded independently on the chicken or duck W chromosome (Van Tuinen & Hedges, 2001). When we contrast copy number estimates from previous work for the chicken (Moghadam et al., 2012) with our study, we find that W genes tend to duplicate independently, albeit at low copy number, in each species separately (Table 3.1). This suggests that the W is not an inert genetic wasteland but seems to evolve dynamically even after recombination was halted between the sex chromosomes.

Lastly, it is worth discussing the difficulties and limitations associated with studying copy number variation in ampliconic gene families. First, while our NanoString probe-based approach offers high-throughput fine-scale estimates of gene copy number and variability, we were not able to distinguish between functional and non-functional gene copies. This is particularly relevant for our conclusions surrounding the evolution of HINTW in the duck. Furthermore, it is not possible to detect gene copies with sequences that are substantially divergent from the probe sequences used. However, gene conversion should homogenise the sequence of gene copies, limiting the potential for this to confound our results. Finally, there is evidence that certain ampliconic genes on the Y are lineage-specific, for instance Sly and Slx are specific to the mouse lineage (Moretti et al., 2020). The list of W-linked genes we included in our analyses is not exhaustive (Wright et al., 2014) due to the challenges of sequencing sex chromosomes. Expanding the scope of this work to test whether lineage-specific loci are more likely to undergo massive scale amplification would be an interesting future avenue.

3.7 CONCLUDING REMARKS

Massive gene amplification is a characteristic feature of Y chromosome evolution. However, until now, it has remained unclear whether gene duplication is as prevalent on the W chromosome. We reveal that on the duck W chromosome, only two out of 26 W-linked genes show evidence of gene duplication. We hypothesise that this may be because genetic drift is reduced on the W relative to Y chromosomes, and we find limited variation of within-species gene copy number consistent with purifying selection. Contrary to this, we find some

evidence that expansion of the HINTW gene family has evolved in response to female-specific selection for egg laying in the chicken but not the duck, calling into question the broad functionality of this prominent gene family. Taken together, our results suggest that in terms of gene duplication, the W chromosome follows a different evolutionary trajectory to that of the Y.

3.8 FUNDING

This work was supported by a Natural Environment Research Council (NERC) Independent Research Fellowship to A.E.W. (NE/N013948/1), a NERC ACCE PhD studentship to T.F.R., and an EECG Research Award from the American Genetics Association to A.E.W.

3.9 ACKNOWLEDGEMENTS

We thank the following for providing avian samples; Edward Boothman (White Leghorn), Richard Windsor (Black Minorca), David Hackett (Oxford Old English), Charles Hardcastle (Mallard), Graham Mortimer (Khaki Campbell, Indian Runner), and Richard Hedges and Paul Hayes (Cayuga, Aylesbury, Black Sumatra). We thank Nicola Hemmings for helpful discussions on staging and incubating avian eggs, Geoff Parker for advice on chicken breeds and chicken breeders, The British Waterfowl Association and Rare Poultry Club for helping finding breeders, Judith Mank, Daniela Palmer and Peter Price for productive discussions. The authors acknowledge the use of the Sheffield HPC Cluster, and associated support services, in the completion of this work. Data generation was carried out by the Centre for Genomic Research, which is based at the University of Liverpool.

3.10 DATA AVAILABILITY

The raw data underlying these analyses is available in Dryad, DOI: https://doi.org/10.5061/dryad.18931zcw9

3.11 REFERENCES

Ahn, S., Hong, M., Van Vrancken, M., Lyou, Y. J., Kim, S. T., Park, S. H., … Kim, K. M. (2016). A nCounter CNV assay to detect HER2 amplification: a correlation study with immunohistochemistry and in situ hybridization in advanced gastric cancer. *Molecular Diagnosis and Therapy*, *20*(4), 375–383.

Ashton, C., Ashton, M., & Donner, C. (1999). *British waterfowl standards*. British Waterfowl

Association.

Bachtrog, D. (2008). The temporal dynamics of processes underlying Y chromosome degeneration. *Genetics*, *179*(3), 1513–1525.

Bachtrog, D. (2013). Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nature Reviews Genetics*, *14*(2), 113–124.

Bachtrog, D. (2020). The Y chromosome as a battleground for intragenomic conflict. *Trends in Genetics*, *36*(7), 510–522.

Bachtrog, D., & Charlesworth, B. (2002). Reduced adaptation of a non-recombining neo-Y chromosome. *Nature*, *416*(6878), 323–326.

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W. R., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*(9), 350–357.

Bachtrog, D., Mahajan, S., & Bracewell, R. (2019). Massive gene amplification on a recently formed *Drosophila* Y chromosome. *Nature Ecology and Evolution*, *3*(11), 1587–1597.

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T. L., … Vamosi, J. C. (2014). Sex determination: why so many ways of doing it? *PLoS Biology*, *12*(7), 1–13.

Backström, N., Ceplitis, H., Berlin, S., & Ellegren, H. (2005). Gene conversion drives the evolution of HINTW, an ampliconic gene on the female-specific avian W chromosome. *Molecular Biology and Evolution*, *22*(10), 1992–1999.

Bellott, D. W., & Page, D. C. (2021). Dosage-sensitive functions in embryonic development drove the survival of genes on sex-specific chromosomes in snakes, birds, and mammals. *Genome Research*, *31*(2), 198–210.

Bellott, D. W., Skaletsky, H., Cho, T. J., Brown, L., Locke, D., Chen, N., … Page, D. C. (2017). Avian W and mammalian Y chromosomes convergently retained dosage-sensitive regulators. *Nature Genetics*, *49*(3), 387–394.

Bergero, R., & Charlesworth, D. (2009). The evolution of restricted recombination in sex chromosomes. *Trends in Ecology and Evolution*. 24: 94-102

Betrán, E., Demuth, J. P., & Williford, A. (2012). Why chromosome palindromes? *International Journal of Evolutionary Biology*, 1–14.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120.

Brashear, W. A., Raudsepp, T., & Murphy, W. J. (2018). Evolutionary conservation of Y chromosome ampliconic gene families despite extensive structural variation. *Genome Research*, *28*(12), 1826–1840.

Ceplitis, H., & Ellegren, H. (2004). Adaptive molecular evolution of HINTW, a female-specific gene in birds. *Molecular Biology and Evolution*, *21*(2), 249–254.

Charlesworth, B. (1978). Model for evolution of Y chromosomes and dosage compensation. *Proceedings of the National Academy of Sciences of the United States of America*, *75*(11), 5618–5622.

Charlesworth, B., & Charlesworth, D. (2000). The degeneration of Y chromosomes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *355*(1403), 1563–

Charlesworth, B. (1991). The evolution of sex chromosomes. *Science*, *251*(4997), 1030–1033.

Chen, J.-M., Cooper, D. N., Chuzhanova, N., Férec, C., & Patrinos, G. P. (2007). Gene conversion: mechanisms, evolution and human disease. *Nature Reviews Genetics*, *8*(10), 762–775.

Cocquet, J., Ellis, P. J. I., Mahadevaiah, S. K., Affara, N. A., Vaiman, D., & Burgoyne, P. S. (2012). A genetic basis for a postmeiotic X versus Y chromosome intragenomic conflict in the Mouse. *PLoS Genetics*, *8*(9), e1002900.

Connallon, T., & Clark, A. G. (2010). Gene duplication, gene conversion and the evolution of the Y chromosome. *Genetics*, *186*(1), 277–286.

Cui, W., Cai, Y., Wang, W., Liu, Z., Wei, P., Bi, R., … Zhou, X. (2014). Frequent copy number variations of PI3K/AKT pathway and aberrant protein expressions of PI3K subunits are associated with inferior survival in diffuse large B cell lymphoma. *Journal of Translational Medicine*, *12*, 10.

Davis, J. K., Thomas, P. J., & Thomas, J. W. (2010). A W-linked palindrome and gene conversion in New World sparrows and blackbirds. *Chromosome Research*, *18*(5), 543–553.

Ekarius, C. (2007). *Storey's Illustrated Guide to Poultry Breeds*. Marceline, MO: Walsworth Publishing Company.

Ellis, P. J. I., Bacon, J., & Affara, N. A. (2011). Association of Sly with sex-linked gene amplification during mouse evolution: A side effect of genomic conflict in spermatids? *Human Molecular Genetics*, *20*(15), 3010–3021.

Eriksson, J., Larson, G., Gunnarsson, U., Bed'hom, B., Tixier-Boichard, M., Strömstedt, L., … Andersson, L. (2008). Identification of the yellow skin gene reveals a hybrid origin of the domestic chicken. *PLoS Genetics*, *4*(2).

Fridolfsson, A.-K., & Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, *30*(1), 116.

Frisby, D. P., Weiss, R. A., Roussel, M., & Stehelin, D. (1979). The distribution of endogenous chicken retrovirus sequences in the DNA of galliform birds does not coincide with avian phylogenetic relationships. *Cell*, *17*(3), 623–634.

Fumihito, A., Miyake, T., Takada, M., Shingu, R., Endo, T., Gojobori, T., … Ohno, S. (1996). Monophyletic origin and unique dispersal patterns of domestic fowls. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(13), 6792–6795.

Furman, B. L. S., Metzger, D. C. H., Darolti, I., Wright, A. E., Sandkam, B. A., Almeida, P., … Fraser, B. (2020). Sex chromosome evolution: so many exceptions to the rules. *Genome Biology and Evolution*, *12*(6), 750–763.

Geraldes, A., Rambo, T., Wing, R. A., Ferrand, N., & Nachman, M. W. (2010). Extensive gene conversion drives the concerted evolution of paralogous copies of the SRY gene in European rabbits. *Molecular Biology and Evolution*, *27*(11), 2437–2440.

Ghenu, A. H., Bolker, B. M., Melnick, D. J., & Evans, B. J. (2016). Multicopy gene family evolution on primate Y chromosomes. *BMC Genomics*, *17*, 157.

Good, J. M. (2012). The conflict within and the escalating war between the sex chromosomes. *PLoS Genetics*, *8*(9), e1002955.

Guzzetti, B. M., Talbot, S. L., Tessler, D. F., Gill, V. A., & Murphy, E. C. (2008). Secrets in the eyes of Black Oystercatchers: a new sexing technique. *Journal of Field Ornithology*, *79*(2), 215–223.

Haddrill, P. R., Halligan, D. L., Tomaras, D., & Charlesworth, B. (2007). Reduced efficacy of selection in regions of the *Drosophila* genome that lack crossing over. *Genome Biology*, *8*(2), 1–9.

Hori, T., Asakawa, S., Itoh, Y., Shimizu, N., & Mizuno, S. (2000). *Wpkci* , encoding an altered form of *PKCI*, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. *Molecular Biology of the Cell*, *11*(10), 3645–3660.

Hughes, J. F., Skaletsky, H., Pyntikova, T., Minx, P. J., Graves, T., Rozen, S., …Page, D. C.

(2005). Conservation of Y-linked genes during human evolution revealed by comparative sequencing in chimpanzee. *Nature*, *437*(7055), 100-3.

Hughes, J. F., Skaletsky, H., Pyntikova, T., Graves, T. A., Van Daalen, S. K. M., Minx, P. J., … Page, D. C. (2010). Chimpanzee and human Y chromosomes are remarkably divergent in structure and gene content. *Nature*, *463*(7280), 536–539.

Hughes, J. F., Skaletsky, H,. Pyntikova, T., Koutseva, N., Raudsepp, T., Brown, L. G., ... Page, D. C. (2020). Sequence analysis in *Bos taurus* reveals pervasiveness of X-Y arms races in mammalian lineages. *Genome Research, 30*, 1716–1726.

Kerje, S., Carlborg, Ö., Jacobsson, L., Schütz, K., Hartmann, C., Jensen, P., & Andersson, L. (2003). The twofold difference in adult size between the Red Junglefowl and White Leghorn chickens is largely explained by a limited number of QTLs. *Animal Genetics*, *34*(4), 264–274.

Kuroda-Kawaguchi, T., Skaletsky, H., Brown, L. G., Minx, P. J., Cordum, H. S., Waterston, R. H., … Page, D. C. (2001). The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nature Genetics*, *29*(3), 279–286.

Lahn, B. T., & Page, D. C. (1997). Functional coherence of the human Y chromosome. *Science*, *278*(5338), 675–680.

Larson, E. L., Kopania, E. E. K., & Good, J. M. (2018). Spermatogenesis and the evolution of mammalian sex chromosomes. *Trends in Genetics*, *34*(9), 722–732.

Lewis, C. (2010). *The illustrated guide to chickens: how to choose them - how to keep them*. Oswestry, UK: Scotprint.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, *25*(14), 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079.

Li, J., Zhang, J., Liu, J., Zhou, Y., Cai, C., Xu, L., … Zhou, Q. (2021). A new duck genome reveals conserved and convergently evolved chromosome architectures of birds and mammals. *GigaScience*, *10*(1), 1-15.

Löytynoja, A. (2014). Phylogeny-aware alignment with PRANK. *Methods in Molecular Biology*, *1079*, 155–170.

Lucotte, E. A., Skov, L., Jensen, J. M., Macià, M. C., Munch, K., & Schierup, M. H. (2018).

Dynamic copy number evolution of X-and Y-linked ampliconic genes in human populations. *Genetics*, *209*(3), 907–920.

Mank, J. E. (2012). Small but mighty: The evolutionary dynamics of W and Y sex chromosomes. *Chromosome Research*, *20*(1), 21–33.

Marais, G. A. B., Campos, P. R. A., & Gordo, I. (2010). Can intra-Y gene conversion oppose the degeneration of the human Y chromosome? A simulation study. *Genome Biology and Evolution*, *2*, 347–357.

Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S., & Mank, J. E. (2012). W chromosome expression responds to female-specific selection. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(21), 8207–8211.

Moretti, C., Blanco, M., Ialy-Radio, C., Serrentino, M., Gobé, C., Friedman, R., … Cocquet, J. (2020). Battle of the sex chromosomes: competition between X and Y chromosome-encoded proteins for partner interaction and chromatin occupancy drives multicopy gene expression and evolution in muroid rodents. *Molecular Biology and Evolution*, *37*(12), 3453-3468.

Moriyama, S., Ogihara, J., Kato, J., Hori, T., & Mizuno, S. (2006). PKCI-W forms a heterodimer with PKCI-Z and inhibits the biological activities of PKCI-Z in vitro, supporting the predicted role of PKCI-W in sex determination in birds. *Journal of Biochemistry*, *139*(1), 91–97.

Mueller, J. L., Mahadevaiah, S. K., Park, P. J., Warburton, P. E., Page, D. C., & Turner, J. M. A. (2008). The mouse X chromosome is enriched for multicopy testis genes showing postmeiotic expression. *Nature Genetics*, *40*(6), 794–799.

Narasimhan, V., Danecek, P., Scally, A., Xue, Y., Tyler-Smith, C., & Durbin, R. (2016). BCFtools/RoH: A hidden Markov model approach for detecting autozygosity from next-generation sequencing data. *Bioinformatics*, *32*(11), 1749–1751.

O'Neill, M., Binder, M., Smith, C., Andrews, J., Reed, K., Smith, M., … Sinclair, A. (2000). ASW: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. *Development Genes and Evolution*, *210*(5), 243–249.

Pace, H. C., & Brenner, C. (2003). Feminizing chicks: a model for avian sex determination based on titration of Hint enzyme activity and the predicted structure of an Asw-Hint heterodimer. *Genome Biology*, *4*(3), R18.

Parks, K. P., Seidle, H., Wright, N., Sperry, J. B., Bieganowski, P., Howitz, K., … Brenner, C.

(2005). Altered specificity of Hint-W123Q supports a role for Hint inhibition by ASW in avian sex determination. *Physiological Genomics*, *20*(1), 12–14.

Poznik, G. D., Xue, Y., Mendez, F. L., Willems, T. F., Massaia, A., Wilson Sayres, M. A., … Tyler-Smith, C. (2016). Punctuated bursts in human male demography inferred from 1,244 worldwide Y-chromosome sequences. *Nature Genetics*, *48*(6), 593–599.

Rice, W. R. (1996). Evolution of the Y sex chromosome in animals. *BioScience*, *46*(5), 331–343

Rozen, S., Skaletsky, H., Marszalek, J. D., Minx, P. J., Cordum, H. S., Waterston, R. H., … Page, D. C. (2003). Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. *Nature*, *423*(6942), 873–876.

Rubin, C. J., Zody, M. C., Eriksson, J., Meadows, J. R. S., Sherwood, E., Webster, M. T., … Andersson, L. (2010). Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature*, *464*(7288), 587–591.

Rumble, S. M., Lacroute, P., Dalca, A. V., Fiume, M., Sidow, A., & Brudno, M. (2009). SHRiMP: accurate mapping of short color-space reads. *PLoS Computational Biology*, *5*(5).

Skaletsky, H., Kuroda-Kawaguchl, T., Minx, P. J., Cordum, H. S., Hlllier, L. D., Brown, L. G., … Page, D. C. (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, *423*(6942), 825–837.

Skov, L., Schierup, M. H., & Schierup, M. H. (2017). Analysis of 62 hybrid assembled human Y chromosomes exposes rapid structural changes and high rates of gene conversion. *PLoS Genetics*, *13*(8), e1006834.

Smeds, L., Warmuth, V., Bolivar, P., Uebbing, S., Burri, R., Suh, A., … Ellegren, H. (2015). Evolutionary analysis of the female-specific avian W chromosome. *Nature Communications*, *6*, 7330.

Soh, Y. Q. S., Alföldi, J., Pyntikova, T., Brown, L. G., Graves, T., Minx, P. J., … Page, D. C. (2014). Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. *Cell*, *159*(4), 800–813.

Tomaszkiewicz, M., Medvedev, P., & Makova, K. D. (2017). Y and W chromosome assemblies: approaches and discoveries. *Trends in Genetics*. 33: 266-282

Tomaszkiewicz, M., Rangavittal, S., Cechova, M., Sanchez, R. C., Fescemyer, H. W., Harris, R., … Makova, K. D. (2016). A time- and cost-effective strategy to sequence mammalian Y chromosomes: An application to the de novo assembly of gorilla Y. *Genome Research*,

26(4), 530–540.

Van Tuinen, M., & Hedges, S. B. (2001). Calibration of avian molecular clocks. *Molecular Biology and Evolution*, *18*(2), 206–213.

Vegesna, R., Tomaszkiewicz, M., Medvedev, P., & Makova, K. D. (2019). Dosage regulation, and variation in gene expression and copy number of human Y chromosome ampliconic genes. *PLoS Genetics*, *15*(9), e1008369.

Vegesna, R., Tomaszkiewicz, M., Ryder, O. A., Campos-Sánchez, R., Medvedev, P., DeGiorgio, M., & Makova, K. D. (2020). Ampliconic genes on the great ape Y chromosomes: Rapid evolution of copy number but conservation of expression levels. *Genome Biology and Evolution*, *12*(6), 842–859.

Vogt, P. H., Edelmann, A., Kirsch, S., Henegariu, O., Hirschmann, P., Kiesewetter, F., … Haidl, G. (1996). Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Human Molecular Genetics*, *5*(7), 933–943.

Wright, A. E., & Mank, J. E. (2013). The scope and strength of sex-specific selection in genome evolution. *Journal of Evolutionary Biology*, *26*(9), 1841–1853.

Wright, A. E., Harrison, P. W., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2014). Independent stratum formation on the avian sex chromosomes reveals inter-chromosomal gene conversion and predominance of purifying selection on the W chromosome. *Evolution*, *68*(11), 3281–3295.

Wright, A.E., Moghadam, H. K., & Mank, J. E. (2012). Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*, *192*(4), 1433–1445.

Xu, L., Auer, G., Peona, V., Suh, A., Deng, Y., Feng, S., … Zhou, Q. (2019). Dynamic evolutionary history and gene content of sex chromosomes across diverse songbirds. *Nature Ecology and Evolution*, *3*(5), 834–844.

Xu, L., & Zhou, Q. (2020). The female-specific W chromosomes of birds have conserved gene contents but are not feminized. *Genes*, *11*(10), 1–14.

Yan, Y., Yang, X., Liu, Y., Shen, Y., Tu, W., Dong, Q., … Yang, Y. (2017). Copy number variation of functional RBMY1 is associated with sperm motility: an azoospermia factor-linked candidate for asthenozoospermia. *Human Reproduction*, *32*(7), 1521–1531.

Ye, D., Zaidi, A. A., Tomaszkiewicz, M., Anthony, K., Liebowitz, C., DeGiorgio, M., … Makova, K. D. (2018). High levels of copy number variation of ampliconic genes across major

human Y haplogroups. *Genome Biology and Evolution*, *10*(5), 1333–1350.

Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., … Flicek, P. (2018). Ensembl 2018. *Nucleic Acids Research*, *46*(D1), D754–D761.

Zhang, Z., Jia, Y., Almeida, P., Mank, J. E., van Tuinen, M., Wang, Q., … Qu, L. (2018). Whole-genome resequencing reveals signatures of selection and timing of duck domestication. *GigaScience*, *7*(4), 1–11.

Zhou, R., Macaya-Sanz, D., Carlson, C. H., Schmutz, J., Jenkins, J. W., Kudrna, D., … Difazio, S. P. (2020). A willow sex chromosome reveals convergent evolution of complex palindromic repeats. *Genome Biology*, *21*, 38.

CHAPTER 4. SEX-SPECIFIC SELECTION DRIVES THE EVOLUTION OF ALTERNATIVE SPLICING IN BIRDS

4.1 AUTHOR CONTRIBUTIONS

This works forms the publication “Sex-specific selection drives the evolution of alternative splicing in birds” published in *Molecular Biology and Evolution* (2021), the published version of which is included in Chapter 7 of this thesis.

Rogers, T. F. Palmer, D. H. & Wright, A. E. (2021). Sex-Specific selection drives the evolution of alternative splicing in birds. *Molecular Biology and Evolution 38*(2), 519–530.

T.F.R and A.E.W designed the research. T.F.R and D.H.P conducted the analyses. T.F.R and A.E.W wrote the manuscript, with input from all authors.

4.2 ABSTRACT

Males and females of the same species share the majority of their genomes, yet they are frequently exposed to conflicting selection pressures. Gene regulation is widely assumed to resolve these conflicting sex-specific selection pressures, and although there has been considerable focus on elucidating the role of gene expression level in sex-specific adaptation, other regulatory mechanisms have been overlooked. Alternative splicing enables different transcripts to be generated from the same gene, meaning that exons which have sex-specific beneficial effects can in theory be retained in the gene product, while exons with detrimental effects can be skipped. However, at present, little is known about how sex-specific selection acts on broad patterns of alternative splicing. Here we investigate alternative splicing across males and females of multiple bird species. We identify hundreds of genes that have sex-specific patterns of splicing, and establish that sex differences in splicing are correlated with phenotypic sex differences. Additionally, we find that alternatively spliced genes have evolved rapidly as a result of sex-specific selection, and suggest that sex differences in splicing offer another route to sex-specific adaptation when gene expression level changes are limited by functional constraints. Overall, our results shed light on how a diverse transcriptional framework can give rise to the evolution of phenotypic sexual dimorphism.

4.3 INTRODUCTION

Males and females of many species can have divergent evolutionary optima, and are often subject to conflicting selection pressures (Andersson, 1994), yet they share an almost identical set of genes. As a result, when contradictory sex-specific selection pressures act on traits that have a shared genetic basis, significant amounts of sexual conflict can occur (Bonduriansky & Chenoweth, 2009; Parker & Partridge, 1998). Despite this, sex differences are common across a broad range of phenotypes, including morphology, physiology, behaviour and life history, and it is widely assumed that transcriptional dimorphism encodes these sexually dimorphic traits by breaking down intersexual correlations and facilitating sex-specific adaptation (Connallon & Clark, 2010; Connallon & Knowles, 2005; Innocenti & Morrow, 2010; Mank, 2017a). Genes with differences in expression level between males and females are pervasive across many species, and exhibit unique evolutionary properties, including faster rates of sequence and expression evolution (Ellegren & Parsch, 2007; Harrison et al., 2015; Khaitovich et al., 2005; Ranz et al., 2003). Indeed, these genes have been the

subject of considerable focus in understanding how selection can navigate the constraints imposed by a shared genome, and the consequences for sex-specific adaptation (Mank, 2017a; 2017b).

Sex differences in alternative splicing, where different exons are spliced or shuffled in males and females to create distinct sex-specific sequences (Blekhman et al., 2010; Nilsen & Graveley, 2010), have the potential to play key roles in sex-specific adaptation, yet they have been largely overlooked with the exception of a few studies (Blekhman et al., 2010; Brown et al., 2014; Gibilisco et al., 2016; Grantham & Brisson, 2018). In particular, alternative splicing enables multiple transcripts to be generated from a single gene, increasing sex-specific proteome diversity (Matlin et al., 2005; Nilsen & Graveley, 2010). In theory, this could act so that certain exons (e.g. those with sex-specific beneficial functions) are retained in one sex, and certain other exons (e.g. those that have sex-specific detrimental effects) are excluded in the other sex, generating distinct sex-specific isoforms. There is mounting evidence that splicing varies substantially across species, sexes, and tissues (Gibilisco et al., 2016; Su et al., 2008), and has important phenotypic consequences for sex determination, disease, physiology and development (Cline & Meyer, 1996; Gerstein et al., 2014; Kalsotra & Cooper, 2011; McIntyre. et al., 2006; Schütt & Nöthiger, 2000). Despite this, while certain isoforms have key cellular roles and mediate important phenotypes, the extent to which global patterns of splicing are functionally relevant is an important point of discussion (Blencowe, 2017; Tress et al., 2017a, 2017b; Wan & Larson, 2018). Many alternative splicing events are highly tissue-specific and patterns of splicing shift rapidly across species over evolutionary time (Barbosa-Morais et al., 2012; Melé et al., 2015; Merkin et al., 2012; Pan et al., 2005) but whether this reflects stochastic transcriptional noise, relaxed selection or lineage-specific innovations remains unclear (Blencowe, 2017; Tress et al., 2017a, 2017b; Wan & Larson, 2018). Importantly, the contribution of sex-specific selection to the rapid turnover of sex differences in splicing across species has yet to be tested, as most studies exploring the link between transcriptional variation and sexual selection have not accounted for sex-specific patterns of alternative splicing.

Furthermore, the factors constraining the evolution of alternative splicing have yet to be investigated. There is growing evidence that pleiotropy, where a gene performs several functions and affects multiple traits, hinders the evolution of gene expression level and limits

the response to sex-specific selection (Chen & Dokholyan, 2006; Mank et al., 2008; Papakostas et al., 2014). Indeed, genes with broad expression patterns, a proxy for pleiotropy, are less likely to be differentially expressed between males and females (Mank et al., 2008). Alternative splicing could avoid these pleiotropic and functional constraints acting on expression level through the generation of distinct male and female isoforms, thereby acting as an alternate or complementary route to sex-specific adaptation.

Here, we characterize patterns of alternative splicing across males and females of three avian species in order to test the role of sex-specific selection in the evolution of alternative splicing and establish its role in sex-specific adaptation and sexual dimorphism. We identify hundreds of genes that exhibit significant sex-biased alternative splicing and show that sex differences in splicing are correlated with phenotypic sex differences. We find that patterns of sex-specific alternative splicing have evolved rapidly, likely as a product of sex-specific selection, and that genes that are differentially spliced exhibit genomic signatures consistent with sex-specific fitness effects. Broadly, our results provide insight into how, via a diverse transcriptional architecture, the same genome is selected to encode multiple phenotypes, and demonstrates the role of alternative splicing in the evolution of phenotypic complexity.

4.4 RESULTS AND DISCUSSION

4.4.1 Alternative splicing is widespread and common across birds

We quantified alternative splicing in males and females across multiple tissues in three avian species that diverged around 90 million years ago (Fig. S6.3.1). Splicing was estimated as the relative proportion of two alternative isoforms at each splice site, otherwise referred to as percent spliced-in (PSI). A PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. Alternative splicing is common and widespread across all individuals, with an average of 21%, 17%, and 24% autosomal genes undergoing at least one splice event in the duck, turkey and guineafowl respectively (Table S6.3.1). We categorized alternative splicing events as one of five splice types (Fig. S6.3.2); skipped exons (SE), where an entire exon is either included or excluded from the final transcript, mutually exclusive exons (MXE), where one exon is skipped and the other is retained or vice versa, alternative 5′ and 3′ splice sites (A5′SS and A3′SS), which either extend or shorten exons on either the 5′ or 3′ end of the intron respectively, and retained intron (RI) events, where a whole intron is kept, transforming two exons into one

larger exon. Each type of alternative splicing event can occur multiple times within the same gene, and the same gene can exhibit more than one type. Skipped exon and mutually exclusive exon splicing events are the most common type of splicing across the three species, with the other types of splicing occurring at very low frequency (Table S6.3.1). Additionally, skipped exon and mutually exclusive exon events are also more commonly associated with the generation of functional isoforms than other types of splicing (Weatheritt et al., 2016), and so we focus solely on these in subsequent analyses.

4.4.2 Tissues exhibit distinct transcriptional profiles

Next, we examined patterns of sex differences in splicing across tissues. Males and females undergo very similar rates of splicing (Table S6.3.1) in both the spleen and gonad across the autosomes in each of the three species, and this finding is consistent across multiple filtering thresholds (Table S6.3.2). However, despite similarities in the total proportion of alternatively spliced genes, patterns of splicing vary substantially between the sexes (Tables 4.1 & S4.4).

Table 4.1. Differential alternative splicing between males and females across autosomal splice sites and genes.

		Sex-biased alternative splicing events			Sex-biased alternatively spliced genes			
Species	**Tissue**	**MXE**[1]	**SE**[2]	**Total**	**MXE**[1]	**SE**[2]	**Total**	**Proportion of genes**
Duck	Gonad	181	677	886	148	551	640	7.6%
Duck	Spleen	7	27	31	6	26	34	0.4%
Turkey	Gonad	91	481	579	78	421	475	5.2%
Turkey	Spleen	2	39	41	2	38	40	0.5%
Guineafowl	Gonad	219	720	977	174	596	701	7.4%
Guineafowl	Spleen	1	13	14	1	13	13	0.1%

[1] MXE denotes mutually exclusive exon events. [2] SE denotes skipped exon events.

Using hierarchical clustering, we found that both gonad and spleen samples cluster first by phylogenetic relatedness, where splicing is more similar between turkey and guineafowl, which diverged ~30 MYA, than with the duck which diverged ~90 MYA (Fig. S6.3.1). However, in each species, ovary and testes tissue cluster separately whereas the spleen shows no clustering among males and females (Fig. 4.1A, B). Across all three species, we consistently identified far fewer genes with significant differential alternative splicing in the spleen relative to the gonad (Tables 4.1 & S4.4), consistent with results from *Drosophila* (Gibilisco et al., 2016)

Our finding that ovaries and testes exhibit distinct transcriptional profiles mirrors patterns of sex differences in expression level (hereafter termed differential expression) across many species (Uebbing et al., 2015), where the gonad often exhibits significant differential expression between males and females for more than half of all expressed genes (Mank et al., 2010; Zhang et al., 2007) but somatic tissues show less differential expression (Harrison et al., 2015; Mank et al., 2007; Yang et al., 2006). This suggests that ovaries and testes are regulated by distinct sex-specific gene regulatory networks, and that sex-specific splice variants plays a role in the construction of sex-specific genetic architecture (Mank et al., 2017; Wright et al., 2018). Interestingly, we observe far fewer genes exhibiting differential alternative splicing (3.3%, 1.1%, 2.8% of autosomal genes in the duck, turkey and guineafowl gonad respectively; Table S6.3.4) relative to differential expression (45.3%, 45.7%, 44.3% in the duck, turkey and guineafowl gonad respectively), calling into question the relative effect of splicing versus expression in sex-specific regulatory networks.

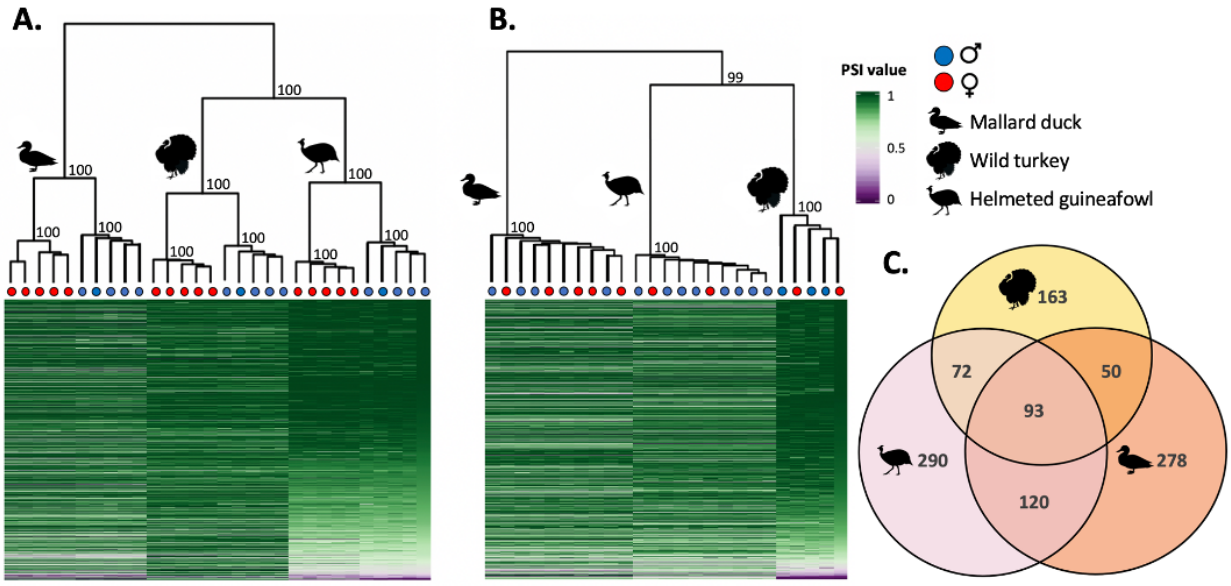

Figure 4.1. Global patterns of alternative splicing across reciprocal orthologs of the three species. Panels A and B show heatmaps and hierarchical clustering of alternative splicing level in the gonad and spleen respectively. Percent spliced-in values (PSI) refer to the proportion of alternative isoforms at a splice site, where a PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. If a gene undergoes multiple splice events, the average PSI is shown. Numbers on each branch represent the bootstrap probability values. Panel C shows orthologous genes with significant differential splicing in the gonad that are shared among the duck (pink), turkey (yellow) and guineafowl (orange). We observe significant overlap (p < 0.0001, Super Exact Test) of differentially spliced orthologs across the three species.

4.4.3 Sex differences in alternative splicing are associated with phenotypic sexual dimorphism

We have shown that patterns of splicing vary substantially between the sexes and across tissues (Tables 4.1 & S4.4). To test whether this sex-biased transcriptional variation (hereafter termed differential splicing) is associated with phenotypic sex differences, we contrasted patterns of splicing across a gradient of sexual dimorphism. Specifically, we employed contrasts across wild turkey individuals that represent a gradient in male secondary sexual characteristics. The wild turkey exhibits two male phenotypes in the forms of dominant and subordinate males. The species is strongly sexually dimorphic, with dominant males showing greater body size than females, along with a range of sexually selected traits including distinct plumage and mating behaviours (Buchholz, 1995, 1997; Hill, et al., 2005). Subordinate males develop fewer and less exaggerated sexually selected traits than dominant males, but are clearly male in phenotype, occupying an intermediate position on the continuum of sexual dimorphism.

Hierarchical clustering of autosomal genes showed that in the gonad, subordinate and dominant males cluster together with high confidence (Fig. S6.3.3), and are distinct from females, as opposed to being intersex. However, there were subtle differences in patterns of alternative splicing between dominant and subordinate males (Fig. 4.2). For exons with significant differences in splicing between dominant males and females (Table 4.1), we classified the alternative isoforms as either male- or female-biased depending on whether they were expressed more highly in dominant males or females. We focused our analyses on the gonad as it exhibits the greatest magnitude of differential splicing, making it the tissue most likely to be influenced by sex-specific selection. Subordinate males express male-biased isoforms in the gonad at significantly lower levels than dominant males (paired Wilcoxon signed-rank test, p = < 0.001), indicating that patterns of splicing are demasculinized in subordinate males (Fig. 4.2A). Subordinate males also express female-biased isoforms at significantly higher levels than dominant males (paired Wilcoxon signed-rank test, $p < 0.001$) (Fig. 4.2B), consistent with feminized splicing. Importantly, subordinate males exhibit intermediate patterns of splicing for all genes that exhibit differential splicing between dominant males and females (Fig. 4.2C). These patterns are consistent with the phenotypic

sex differences observed across morphs, where subordinate males occupy an intermediate position on the continuum of sexual dimorphism.

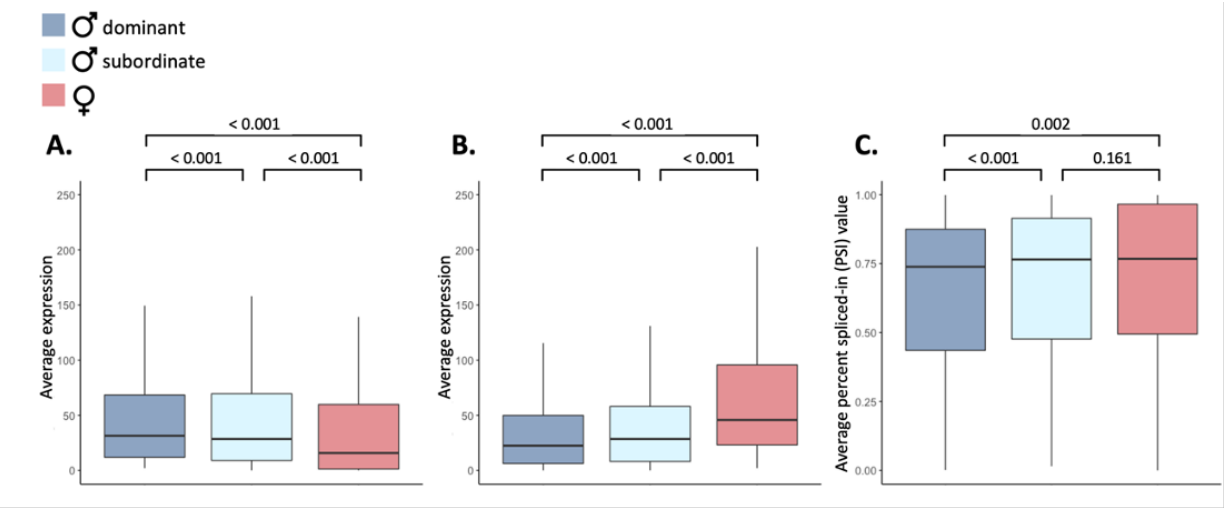

Figure 4.2. Expression of sex-biased isoforms in dominant male turkeys (dark blue), subordinate male turkeys (light blue) and female turkeys (red). Panel A and B show average expression (read counts) of male- and female-biased isoforms and Panel C is the average percent spliced-in value (PSI) of all sex-biased isoforms. Significance values were calculated using a paired Wilcoxon's signed-rank test.

We tested whether this pattern was a result of regression towards the mean by randomising samples 100 times. Each time, we randomly picked three dominant male and three female samples, identified genes with differential splicing, and then assessed the remaining dominant males, females and subordinate males for the magnitude of splicing (PSI). We found that subordinate males had significantly higher PSI than dominant males for all 100 sample comparisons, and significantly lower PSI than females for the majority of the 100 sample combinations (79 significant comparisons). In contrast, significant differences were observed much less frequently between the randomly chosen dominant male samples (34 significant comparisons) or between female samples (6 significant comparisons), indicating that regression towards the mean is unlikely to explain our results. Gene expression level across turkey morphs has previously been shown to exhibit similar patterns of demasculinization and feminization (Pointer et al., 2013), consistent with a role of transcriptional dimorphism in encoding phenotypic sex differences. Our results suggest a previously overlooked link between genomic and phenotypic dimorphism, where differential alternative splicing works concurrently with differential expression level to produce the diverse transcriptional framework underpinning complex phenotypic sexual dimorphisms.

4.4.4 Sex-specific selection acts on isoforms that are differentially expressed between males and females

We find that patterns of alternative splicing cluster strongly by species (Fig. 4.1A, B), consistent with rapid rates of regulatory evolution within lineages. This pattern of clustering is contrary to that observed for gene expression level, including the ones in this study, which clusters first by sex in the gonad, then species (Harrison et al., 2015; Mank, 2017a). Our finding that patterns of differentially expression are more conserved than patterns of alternative splicing is a broad taxonomic trend (Barbosa-Morais et al., 2012; Gibilisco et al., 2016; Merkin et al., 2012), indicative of rapid turnover of alternative splicing across species. However, we observe significant overlap ($p < 0.001$, Super Exact Test) of differentially alternatively spliced orthologs across the three species (Fig. 4.1C, Table S6.3.3), indicating that although patterns of splicing evolve quickly, significant sex differences in splicing are limited to a core set of avian genes. To test whether this conserved set of genes are enriched for specific functions, we conducted a Gene Ontology analysis (Mi et al., 2019), but failed to find any significantly enriched terms ($p < 0.05$).

We implemented an evolutionary framework, using regulatory variation as a proxy for selection, to test whether the rapid rate of regulatory evolution we observe is a product of sexual selection. Studies of regulatory variation have recently been implemented as a powerful approach to infer selection (Brawand et al., 2011; Dean et al., 2015; Moghadam et al., 2012; Gallego Romero et al., 2012), where selection on loci increases with expression level (Drummond et al., 2006; Duret & Mouchiroud, 1999; Gout et al., 2010; Pál et al., 2001).

Applying this framework to alternative splicing, if purifying selection is the dominant evolutionary force acting on splice variants, we predict highly expressed genes to express fewer isoforms than lowly expressed genes, which might be spuriously transcribed and subject to weaker constraints. Furthermore, when expression level differs between the sexes, purifying selection would be strongest in the sex with the higher expression, resulting in the expression of fewer isoforms in that sex. For example, for male-biased genes, we would predict that males tend to have fewer isoforms than females.

However, if there is sexual selection for sex-specific isoforms, we expect the opposite relationship between isoform diversity and sex. Here, we predict the evolution of novel isoforms to be analogous to gene duplication with neofunctionalization, where the ancestral

paralog retains its original function and expression pattern but the newly duplicated paralog evolves sex-specific functions and sex-biased expression (Connallon & Clark, 2011). Applying this to splicing, we expect the ancestral splice variant to retain its ancestral expression pattern and function, but the novel sex-specific isoform to evolve sex-specific functions and expression. As a result, we expect a greater diversity of isoforms in the sex with higher expression, where selection for sex-specific isoforms is the greatest. Specifically, males should express more isoforms than females for male-biased genes, where novel male-specific isoforms are free to evolve male-specific functions while isoforms expressed in both sexes are retained to perform their original function. We predict the opposite pattern for female-biased genes, which under sex-specific selection should exhibit a greater diversity of isoforms expressed in females.

These two scenarios generate opposing predictions for the expected patterns of isoform diversity in males and females. To distinguish between these selective regimes, we developed an isoform specificity index (τ_{AS}) to quantify variation in isoform abundance per gene. This metric is adapted from the tissue specificity index (Yanai et al., 2005), where high values show that a single isoform is always expressed and low values indicate an even representation of multiple isoforms.

We found a significant relationship between isoform specificity (τ_{AS}) and expression level across all genes, where highly expressed genes tend to express fewer isoforms than lowly expressed genes (Fig S6.3.5, Table S6.3.6). This indicates that purifying selection acts on broad patterns of splicing across the genome, suggesting that global patterns of splicing are functionally relevant (Blencowe, 2017; Tress et al., 2017a, 2017b; Wan & Larson, 2018). However, we also recovered a significant association with sex, where isoform specificity (τ_{AS}) differs significantly between males and females for genes that are differentially expressed between the sexes, but not for those with similar expression levels (Fig. 4.3 & S4.5). Importantly, this association is reversed between male-biased and female-biased genes, as we predicted. Specifically, males show significantly greater isoform diversity for male-biased genes, and females show more isoform diversity for female-biased genes. There are no significant differences in isoform diversity between males and females for unbiased genes. This is consistent with our predictions of selection for sex-specific splice variants, and opposite to what we would expect if purifying selection were the dominant evolutionary force

acting on splicing in males and females. These patterns are observed across all three species, which diverged 90 million years ago, indicating that the role of sex-specific selection in splicing evolution is a broad taxonomic trend across birds.

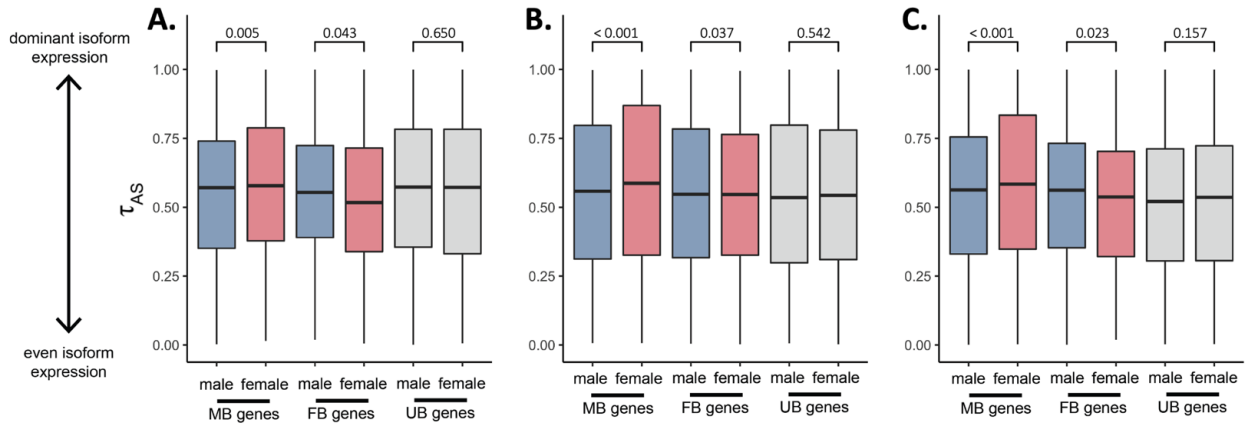

Figure 4.3. Average male and female isoform specificity (τ_{AS}) across genes. τ_{AS} for genes with male-biased expression level, female-biased expression level, and unbiased expression between the sexes for the (A) duck, (B) turkey and (C) guineafowl. Significance values were calculated using a paired Wilcoxon's signed-rank test.

If sex-specific isoforms are indeed under selection for sex-specific functions, then we expect these loci to affect fitness differently in males relative to females. To test whether differential splicing has sex-specific effects, we used a population genomic approach across the three avian species, contrasting patterns of intersexual sequence differentiation and balancing selection (Wright et al., 2018). Recent theoretical work has indicated that patterns of elevated intersexual differentiation previously observed in the literature that have been attributed to ongoing sexual conflict would require implausibly large selective pressures and mortality loads (Kasimatis et al., 2017; 2019; 2021; Ruzicka et al., 2020). However, we do not use this approach to infer ongoing conflict, rather, sex-specific genetic architecture which invokes relatively lower genetic loads. Under sex-specific architecture, where loci exhibit sex differences in their phenotypic effects, we predict elevated intersexual differentiation but relaxed balancing selection (Mank, 2017b).

Consistent with this prediction, we found that differentially alternatively spliced genes exhibited elevated intersexual F_{ST} and low Tajima's *D* in the duck gonad and guineafowl gonad (Chi-squared test, p = 0.003 and p = 0.059 respectively; Table S6.3.5), consistent with differentially spliced genes affecting viability or survival in one sex but having little or no effect in the other. This pattern was not significant in the turkey gonad (Chi-squared test, p = 0.266;

Table S6.3.5), however, there are much fewer sex-biased genes in turkey (Table 4.1) which likely limits our power to test for any relationship in this species. Genes that were significantly differentially expressed between males and females were removed from this analysis as they have been shown previously to have sex-specific phenotypic effects (Wright et al., 2018). To confirm that these sex-specific effects are driven by sex-specifically expressed parts of genes, we extracted intersexual F_{ST} for sex-biased and unbiased exons. We found that F_{ST} was higher across sequences from sex-biased exons relative to unbiased exons in both the turkey and guineafowl (p = 0.014, p = 0.083, turkey and guineafowl respectively, paired Wilcoxon signed-rank test) but there was no significant difference in the duck (p = 0.543). This is the first statistical evidence, to our knowledge, that sex-specific selection acts on broad patterns of alternative splicing and that differentially spliced genes across the genome exhibit genomic signatures consistent with sex-specific effects.

4.4.5 Genes with sex differences in splicing are subject to greater functional constraints

Pleiotropy is thought to hinder the evolution of differential gene expression level and limit the response to sex-specific selection (Mank et al., 2008; Meisel, 2011). Indeed, genes with broad expression patterns, a proxy for pleiotropy, are less likely to be differentially expressed (Mank et al., 2008). Alternative splicing might avoid pleiotropy and other constraints acting on expression level through the generation of distinct male and female isoforms. If so, we expect differential alternative splicing to be more common in genes with similar expression patterns between males and females. In line with our prediction, we found that whilst non-significant (duck p = 0.06, turkey p = 0.55, guineafowl p = 0.49, hypergeometric tests with Benjamini-Hochberg correction), there is less overlap than expected between differentially expressed and differentially spliced genes in the gonad (RF < 1; duck RF = 0.83, turkey RF = 0.86, guineafowl RF = 0.94, Fig. 4.4A-C, Tables S4.7 & S4.8). These results are consistent across multiple filtering thresholds and types of splicing events (Table S6.3.8).

Next, we explicitly tested whether genes under functional constraints are more predisposed to evolve differential splicing. First, we calculated a measure of tissue specificity (τ), a proxy for pleiotropy, where lower values indicate even expression distribution across tissues and larger values equate to greater levels of tissue specificity (Yanai et al., 2005). Measurements of τ were derived from the chicken UniGene database (Mank et al., 2008) and encompass expression patterns from nine tissues. Across all three species, we found that differentially

spliced genes have significantly broader expression patterns relative to genes that are unbiased in expression, consistent with greater functional constraint (Fig. 4.4D-F). This is in stark contrast to genes with differential expression level which, as previously observed (Mank et al., 2008; Meisel, 2011), have greater tissue specificity than unbiased genes. Second, we employed contrasts of coding sequence evolution between genes that are unbiased, are exclusively differentially spliced or exclusively differentially expressed. Previously, differentially expressed genes have been shown to exhibit elevated rates of coding sequence evolution in a wide range of species as a consequence of relaxed evolutionary constraint and genetic drift (Gershoni & Pietrokovski, 2014; Harrison et al., 2015). In contrast, we find that genes with differential splicing do not exhibit significantly elevated rates of sequence evolution in comparison to unbiased genes or genes that are differentially expressed between the sexes (Fig. 4.4G-I), consistent with stronger purifying selection acting on coding sequences. This pattern is conserved when accounting for gene length and expression level, although the pattern then becomes non-significant in the duck (Table S6.3.9). Taken together, these results suggest that when genes are subject to functional constraints, the evolution of sex-specific isoforms may offer a more viable mechanism than changes in expression level to achieve sex-specific functions.

4.5 CONCLUDING REMARKS

Our results indicate that sex-specific selection acts on broad patterns of alternative splicing across the genome, which in turn may facilitate the evolution of sexually dimorphic phenotypes. Sex differences in alternative splicing and gene expression level are restricted to distinct sets of genes, where differential alternative splicing is limited to genes subject to strong purifying selection and functional constraint, indicating that splicing may function as an alternate route to sex-specific adaptation. However, it remains unclear whether dimorphism is a consequence of aggregate patterns of sex-biased splicing or large effect loci, or how the magnitude of splicing scales with phenotypic differences. Taken as a whole, our findings demonstrate how diverse patterns of transcriptional regulation can play an important role in phenotypic complexity.

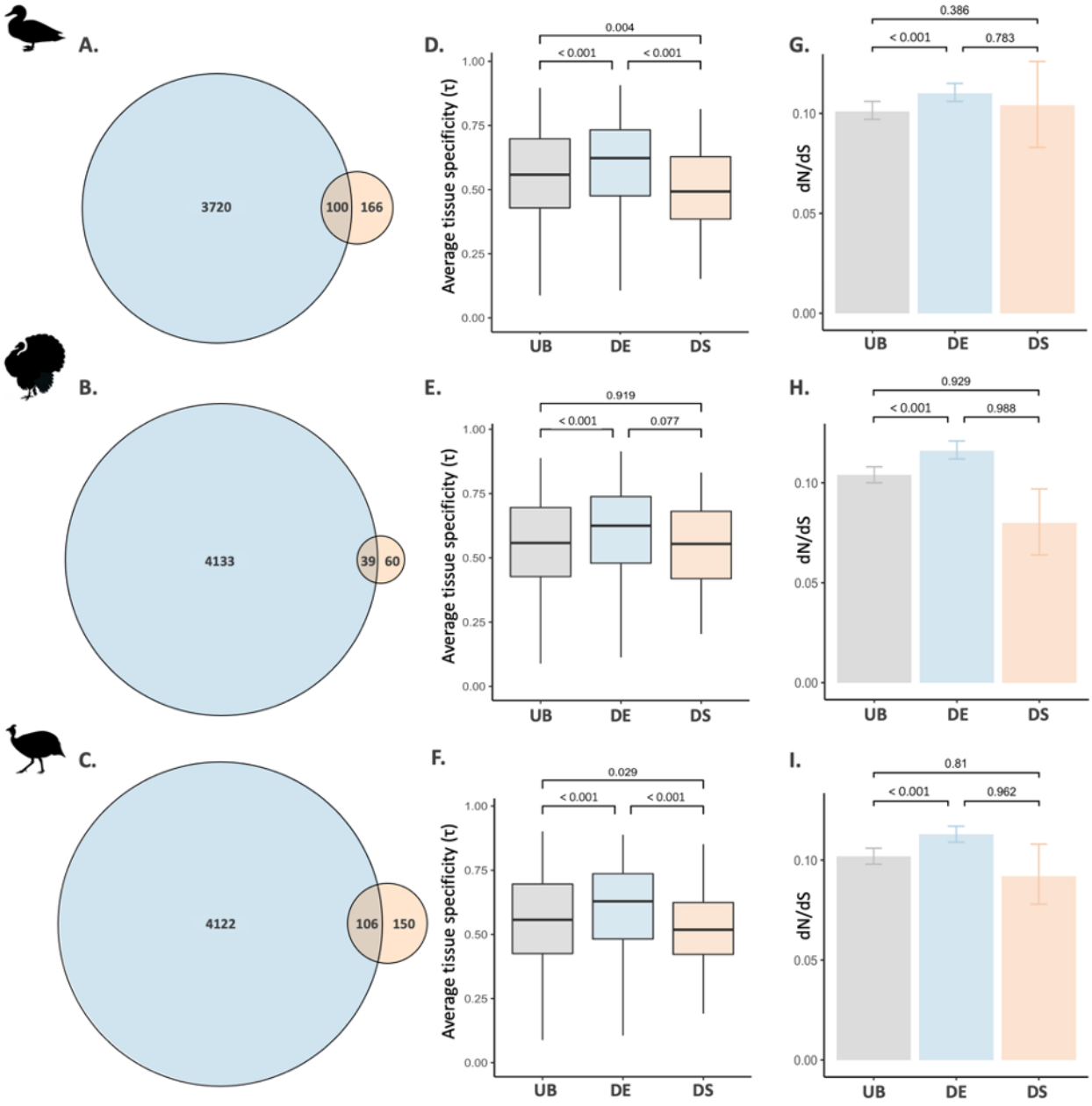

Figure 4.4. Overlap, tissue specificity, and evolutionary rates of genes with sex differences in splicing and expression level in the duck (A, D, G), turkey (B, E, H) and guineafowl (C, F, I) gonad. Panels A, B and C show the overlap between differentially spliced (orange) and differentially expressed (blue) genes. Panels D, E and F show the average tissue specificity (τ), where 0 denotes genes that are expressed ubiquitously and 1 means genes have tissue-specific expression. Panels G, H and I show the average ratio of nonsynonymous (dN) to synonymous (dS) substitutions for genes that are exclusively differentially spliced (orange), exclusively differentially expressed (blue) or unbiased (grey). In D, E, and F, significance values were calculated with Wilcoxon's rank-sum test. In G, H, and I, 95% confidence intervals and significance values were obtained from 1,000 bootstrap replicates.

4.6 MATERIALS AND METHODS

4.6.1 Quality filtering and mapping

Previously, we obtained tissue samples, extracted and sequenced RNA from semi-captive populations of the mallard duck (*Anas platyrhynchos*), wild turkey (*Meleagris gallopavo*) and helmeted guineafowl (*Numida meleagris*) (BioProject ID PRJNA271731, Harrison et al., 2015). The duck diverged from the guineafowl and turkey approximately 90 million years ago, and the turkey and guineafowl diverged 30 million years ago, providing medium- and long-term evolutionary comparison points for assessing divergence in splicing (Fig. S6.3.1). This includes RNA-seq data from five male and five female birds of each species except for the turkey, where five dominant male, two subordinate male, and five female gonad samples were taken along with three dominant male and two female spleen samples. RNA data were quality filtered using Trimmomatic v0.36 (Bolger et al., 2014). We filtered reads containing adapter sequences and trimmed reads if the sliding window average had a Phred score over four bases was < 15 or if the leading/trailing bases had a Phred score < 3. The program used to quantify alternative splice events, rMATS (Shen et al., 2014), requires all reads to be equal length so reads were removed post filtering if either read pair was < 95 bp in length and all remaining reads were trimmed to 95 bp.

RNA-seq reads were mapped to respective reference genomes obtained from Ensembl (mallard duck; CAU_duck1.0; GCA_002743455.1, wild turkey; Turkey_2.01; GCA_000146605.1, helmeted guineafowl; NumMel1.0; GCA_002078875.2), using HISAT2 v.2.10 (Kim, et al., 2015). We suppressed discordant and unpaired alignments for paired reads and excluded reads from the SAM output that failed to align. Reported alignments were tailored for transcript assemblers including StringTie. These alignments were used in downstream analyses to quantify both alternative splicing and gene expression level to ensure accurate comparisons between patterns of splicing and gene expression level.

4.6.2 Quantifying alternative splicing

We quantified alternative splicing in males and females in each species using rMATs v.4.0.3. Specifically, rMATs assesses annotated splice junctions in the reference genome for alternative splicing and detects differential splicing between two groups of samples. Splicing at each splice site is measured as the percent spliced-in (PSI), which indicates the proportion

of two alternative isoforms at each splice site. PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. We detected alternative splicing events using 0 < PSI < 1 in more than half of the individuals in each sample group. To compare splicing between groups of samples, rMATs calculates an inclusion difference (ΔPSI) (average PSI of male samples – average PSI of female samples), which ranges from 1 (the one isoform is only expressed in males) to -1 (the alternative isoform is only expressed in females). Therefore, ΔPSI of zero means that patterns of splicing do not differ between males and females (i.e. the proportion of alternative isoforms for that splice site is the same between the sexes). rMATS uses a likelihood-ratio test to identify significant differences in ΔPSI between males and females. We identified differential splicing events using an FDR *p*-value < 0.05 and ΔPSI threshold of 0.1 following Grantham & Brisson (2018). The only exception was for analyses comparing patterns of differential splicing to differential expression where we used an FDR p-value < 0.05 and male:female $\log_2$ fold change PSI value of 1 to ensure equivalent thresholds were implemented. We calculated the significance of the overlap between differentially spliced orthologs using the SuperExactTest package (Wang et al., 2015) in R. Patterns of splicing were only quantified for autosomal genes as the Z chromosome is subject to unusual patterns of sex-specific selection due to its unequal inheritance pattern between males and females (Rice, 1984). This workflow is summarised in Fig. S6.3.4.

It has been suggested that many of the splicing events detected through next-generation sequencing approaches reflect stochastic transcriptional noise, however, this has been the subject of considerable recent debate (Melamud & Moult, 2009; Tress et al., 2017a, 2017b; Wan & Larson, 2018). We implemented a number of stringent filters to remove alternative splicing events that are likely non-functional noise. First, we evaluated splicing using only reads mapping to exon-exon boundaries that span splicing junctions to quantify splicing. Second, following Grantham & Brisson (2018), splicing sites were excluded if the number of reads supporting the inclusion and spliced exon junction was < 20 in at least half the samples of both sexes in each tissue separately. Finally, while rMATS analyses different types of alternative splicing events, skipped exon (SE) and mutually exclusive exons (MXE) events are more commonly known to translate into functional isoforms (Weatheritt et al. 2016). These types of splicing comprise the majority of splice events we identified (Table S6.3.1) and so

subsequent analyses were only performed on skipped exon and mutually exclusive exon splicing events.

4.6.3 Cluster analysis of alternative splicing data

We assessed transcriptional similarity of splicing across samples, as measured by percent spliced-in (PSI), using the R package Pvclust (Suzuki & Shimodaira, 2006). Hierarchical clustering with Euclidean distance was performed and the reliability of each of the trees produced was tested by bootstrap resampling (1000 replicates).

4.6.4 Quantifying gene expression level

SAM files generated from HISAT2 were coordinate sorted using SAMtools v1.9 (Li et al., 2009) and converted to BAM format. For each species, StringTie v1.3.5 (Pertea et al., 2015) was used to estimate gene expression level only for transcripts in the reference genome, ignoring novel transcripts, to ensure that expression was quantified for the same set of loci across all samples. We then extracted read count information directly from the StringTie output to generate count matrices for genes and transcripts as recommended by the StringTie pipeline. To ensure that our estimates of expression level are not biased by differences in alternative splicing across samples, we calculated gene expression level using only constitutively expressed exons (i.e. removing exons that are alternatively spliced or differentially spliced between males and females, fdr < 0.05).

In each species, a minimum expression level threshold of 1 log CPM in at least half of the individuals of both sexes was imposed to remove lowly expressed genes in the gonad and spleen separately. Expression level was normalized using TMM (trimmed mean of m-values) in EdgeR (Robinson et al., 2010). Genes were excluded from the analysis if they were single-exon or not located on annotated autosomal chromosomes. Sex-biased genes were identified using a standard $\log_2$ fold change value of 1 and FDR *p*-value < 0.05 (Assis et al., 2012; Harrison et al., 2015). This workflow is summarised in Fig. S6.3.4.

4.6.5 Estimating isoform specificity (τ_{AS})

We developed an isoform specificity index to quantify variation in isoform abundance per gene. This is adapted from the tissue specificity index (τ) (Yanai et al., 2005), a commonly used metric that calculates whether expression is broadly expressed or localized in one tissue. Here, we instead use expression of each isoform to calculate isoform specificity, where a

value of 0 indicates an even representation of isoform abundance and a value of 1 shows that a single isoform is always expressed. We call this measure τ_{AS}. For a given gene, τ_{AS} is defined as:

$$\tau_{AS} = \frac{\sum_{i=1}^{n}(1-\hat{x}_i)+(1-\hat{y}_i)}{n-1};\ \hat{x}_i = \frac{x_i}{\max\limits_{1\leq i\leq n}(x_i,y_i)}, \hat{y}_i = \frac{y_i}{\max\limits_{1\leq i\leq n}(x_i,y_i)},$$

where n is the total number of isoforms (assuming each splice site produces two isoforms), x_i is the read count supporting the inclusion of the exon in the gene product, and y_i is the read count supporting the exclusion of the exon from the gene product. We excluded splice sites that did not pass the coverage thresholds described above, and we excluded any exon that did not have a minimum read count of 20 in at least half of the individuals (within or between sexes) supporting both inclusion and exclusion of the exon. We then calculated male and female τ_{AS} for each gene. Importantly, power to detect isoform variation is limited by expression level so we reduced read counts in the sex with higher expression before calculating τ_{AS}. Specifically, read counts in the more highly expressed sex were scaled to the sex with the lower expression for each gene. This accounts for reduced power to detect splice events in samples with lower expression. In addition to this, to check our results are not biased by variation in sequencing depth across samples, we normalised τ_{AS}, where read counts were divided by total library size in each sample. We tested for statistical differences between male and female τ_{AS} using a paired Wilcoxon's signed-rank test.

4.6.6 Estimating population genomic statistics

For each individual, we merged spleen and gonad BAM files using SAMtools v1.9 (Li et al., 2009) with the exception of the turkey, where both tissues were not sequenced for all individuals so we used only gonad data for subsequent analyses. We used ANGSD (Korneliussen et al., 2014) to estimate population genetic summary statistics, following our previous approach (Wright et al., 2018; Wright et al., 2019) as ANGSD implements methods to account for uneven sequencing depth and is therefore appropriate for transcriptome data. We filtered BAM files to discard reads if they did not uniquely map, had a flag ≥ 256, had a mate that was not mapped, or had a mapping quality below 20. Bases were filtered if base quality fell below 13 or there were data in fewer than half the individuals. Mapping quality scores were adjusted for excessive mismatches and quality scores were adjusted around indels to rule out false single nucleotide polymorphisms (SNPs). We identified and removed

related individuals (two wild turkey samples) from our analyses using NGSRELATE (Korneliussen & Moltke, 2015) to avoid violating Hardy Weinberg assumptions.

We calculated sample allele frequency likelihoods at each site from genotype likelihoods with the SAMtools model in ANGSD. Next, we estimated the overall unfolded site frequency spectrum (SFS) for each species (Nielsen et al., 2012). Specifically, at each site we randomly sampled an allele frequency according to its likelihood, as calculated by ANGSD. Finally, we computed genetic diversity indices, including allele frequency posterior probability and Tajima's *D* using the SFS as prior information with ANGSD thetaStat (Korneliussen et al., 2014).

Intersexual F_{ST} was calculated using the same procedure and filtering criteria as above except that we filtered out bases where we had data in fewer than half the individuals in males and females separately. We quantified Hudson's F_{ST}, which is less sensitive to small sample sizes (Bhatia et al., 2013, Gammerdinger et al., 2020). Estimates across coding regions of autosomal loci were obtained using weighted averages, where per-gene F_{ST} is the ratio between the sum of the between-populations variance across loci and the sum of the total variance across loci.

Immune genes can generate patterns of balancing selection via mechanisms such as heterozygote advantage, (Hedrick, 2011; Rockman et al., 2010; Stahl et al., 1999) and negative-frequency dependent selection (Croze et al., 2016). Therefore, genes with potential immune function were excluded from the population genomic analyses. Specifically, we removed all loci with the terms “immune” or “MHC” in their Gene Ontology annotations from population genomic analyses. Furthermore, we applied a strict minimum expression level threshold of 2 log CPM in at least half of the individuals of both sexes to remove lowly expressed genes that may bias population genomic analyses.

4.6.7 Testing the overlap between differentially spliced and expressed genes

We tested whether differentially spliced genes are also differentially expressed. First, we estimated the expected number of genes that are both differentially spliced (DSG) and differentially expressed (DEG) as [total no. DSG * total no. DEG]/ total no. expressed genes. Next, we calculated the representation factor (RF), which is the observed number of overlapping genes divided by the expected number. If RF < 1, there is less overlap between differentially spliced and expressed genes than expected and RF > 1, there is more overlap than expected. We tested whether the overlap was significantly less than expected using

the hypergeometric test with the phyper function in R. We calculated adjusted p-values using the Benjamini–Hochberg (FDR) correction.

4.6.8 Identifying orthologous genes across species

Coding sequences were downloaded from Ensembl v98 (Zerbino et al., 2018) for the mallard duck (*Anas platyrhynchos*; CAU_duck1.0; GCA_002743455.1), wild turkey (*Meleagris gallopavo*; Turkey_2.01; GCA_000146605.1), helmeted guineafowl (*Numida meleagris*; NumMel1.0; GCA_002078875.2), and zebra finch (*Taeniopygia guttata*; taeGut3.2.4). The longest isoform was retained for each species, and reciprocal orthologs across the four taxa were identified using BLASTn v2.9.0+ (Altschul et al., 1990) with an e-value cutoff of 1×10^{-10} and minimum percentage identity of 30%. Across the duck, turkey, guineafowl and zebra finch, 10,622 reciprocal orthologs were identified. We also identified pairwise reciprocal orthologs with the chicken (*Gallus gallus*) for the duck, turkey and guineafowl using the same approach. This resulted in 13,425, 12,764 and 13,942 orthologs in the duck, turkey and guineafowl respectively.

4.6.9 Estimating isoform specificity (τ)

Tissue specificity (Yanai et al., 2005) as calculated from the chicken UniGene database, as previously described (Mank et al., 2008), and encompasses expression level patterns from nine tissues. Lower values indicate even expression level distribution across tissues and larger values equate to greater levels of tissue specificity. For each species, we extracted tissue specificity for genes with pairwise reciprocal orthologs in the chicken, resulting in τ values for 4,747, 5,131 and 5,200 genes in the duck, turkey and guineafowl respectively.

4.6.10 Estimating rates of coding sequence evolution

Orthologous sequences were aligned with PRANK v.140603 (Löytynoja & Goldman, 2008), using a previously published phylogeny (Harrison et al., 2015). The sequence alignments were then checked for gaps, and for poorly aligned regions using SWAMP v.31-03-14 (Harrison et al., 2014) with a threshold of 4 in a window size of 5 bases and a minimum sequence length of 75 bp. Evolutionary parameters were estimated using the branch model in PAML v.4.8a (Z. Yang, 2007) Orthologous genes with dS > 2 were filtered from subsequent analyses as this represents the point of mutational saturation in avian sequence data (Axelsson et al., 2008; Harrison et al., 2015). We extracted the number of nonsynonymous sites (N), the number of

nonsynonymous substitutions (NdN), the number of synonymous sites (S), and the number of synonymous substitutions (SdS) for each taxon in order to calculate dN/dS weighted by alignment length (Harrison et al., 2015; Mank et al., 2010). We then generated 1,000 bootstrap replicates to obtain 95% confidence intervals and tested for significant differences between gene categories using 1,000 permutations. We tested if the pattern of dN/dS was conserved after controlling for gene length and gene expression level using multiple regression and an ANOVA test implemented in R.

4.7 ACKNOWLEDGEMENTS

This work was supported by a NERC DTP grant to T. F. R., a NERC Independent Research Fellowship to A.E.W (NE/N013948/1), a Royal Society Research Grant to A.E.W, and an NSF Postdoctoral Research Fellowship in Biology to D.H.P (Award #1812164). We thank Judith Mank, Jon Slate and Peter Price for productive discussions, Judith Mank for providing tissue specificity values, and two anonymous reviewers for constructive feedback.

4.8 DATA AVAILABILITY

The RNA-seq data in this study is available in the SRA (BioProject ID PRJNA271731).

4.9 REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Molecular Biology, 215*(3), 403–410.

Andersson, M. (1994). *Sexual Selection*. Princeton, NJ: Princeton University Press.

Assis, R., Zhou, Q., & Bachtrog, D. (2012). Sex-biased transcriptome evolution in *Drosophila. Genome Biology and Evolution, 4*(11), 1189–1200.

Axelsson, E., Hultin-Rosenberg, L., Brandström, M., Zwahlén, M., Clayton, D. F., & Ellegren, H. (2008). Natural selection in avian protein-coding genes expressed in brain. *Molecular Ecology, 17*(12), 3008–3017.

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., Gueroussov, S., Lee, L. J., ... Blencowe, B. J. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science, 338*(6114), 1587–1593.

Bhatia, G., Patterson, N., Sankararaman, S., & Price, A. L. (2013). Estimating and interpreting FST: the impact of rare variants. *Genome Research, 23*(9), 1514–1521.

Blekhman, R., Marioni, J. C., Zumbo, P., Stephens, M., & Gilad, Y. (2010). Sex-specific and

lineage-specific alternative splicing in primates. *Genome Research, 20*(2), 180–189.

Blencowe, B. J. (2017). The relationship between alternative splicing and proteomic complexity. *Trends in Biochemical Sciences, 42*(6), 407–408.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Journal of Bioinformatics, 30*(15), 2114–2120.

Bonduriansky, R., & Chenoweth, S. (2009). Intralocus sexual conflict. *Trends in Ecology and Evolution, 23*(9), 1514–1521.

Brawand, D., Soumillon, M., Necsulea, A., Julien, P., Csárdi, G., Harrigan, P., ... Kaessmann, H. (2011). The evolution of gene expression levels in mammalian organs. *Nature, 478*(7369), 343–348.

Brown, J. B., Boley, N., Eisman, R., May, G. E., Stoiber, M. H., Duff, M. O., … Celniker, S. E. (2014). Diversity and dynamics of the *Drosophila* transcriptome. *Nature, 512*(7515), 393–399.

Buchholz, R. (1995). Female choice, parasite load and male ornamentation in wild turkeys. *Animal Behaviour, 50*(4), 929–943.

Buchholz, R. (1997). Male dominance and variation in fleshy head ornamentation in wild turkeys. *Journal of Avian Biology, 28*(3), 223.

Chen, Y., & Dokholyan, N. V. (2006). The coordinated evolution of yeast proteins is constrained by functional modularity. *Trends in Genetics, 22*(8), 416–419.

Cline and, T. W., & Meyer, B. J. (1996). Vive la difference: Males vs females in flies vs worms. *Annual Review of Genetics, 30*, 637–702.

Connallon, T., & Clark, A. G. (2010). Sex linkage, sex-specific selection, and the role of recombination in the evolution of sexually dimorphic gene expression. *Evolution, 64(*12), 3417–3442.

Connallon T., & Clark, A. G. (2011). The resolution of sexual antagonism by gene duplication. *Genetics, 187*(3), 919–937.

Connallon, T., & Knowles, L. L. (2005). Intergenomic conflict revealed by patterns of sex-biased gene expression. *Trends in Genetics, 21*(9), 495–9.

Croze, M., Živković, D., Stephan, W., & Hutter, S. (2016). Balancing selection on immunity genes: review of the current literature and new analysis in *Drosophila melanogaster*. *Zoology, 119*(4), 322–329.

Dean, R., Harrison, P. W., Wright, A. E., Zimmer, F., & Mank, J. E. (2015). Positive selection

underlies faster-Z evolution of gene expression in birds. *Molecular Biology and Evolution, 32*(10), 2646–2656.

Dean, R., & Mank, J. E. (2016). Tissue specificity and sex-specific regulatory variation permit the evolution of sex-biased gene expression. *American Naturalist, 188*(3), E74–E84.

Drummond, D. A., Raval, A., & Wilke, C. O. (2006). A single determinant dominates the rate of yeast protein evolution. *Molecular Biology and Evolution, 23*(2), 327–337.

Duret, L., & Mouchiroud, D. (1999). Expression pattern and, surprisingly, gene length shape codon usage in *Caenorhabditis*, *Drosophila*, and *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America, 96*(8), 4482–4487.

Ellegren, H., & Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene expression. *Nature Reviews Genetics, 8*(9), 689–698.

Gallego Romero, I., Ruvinsky, I., & Gilad, Y. (2012). Comparative studies of gene expression and the evolution of gene regulation. *Nature Reviews Genetics, 13*(7), 505–516.

Gammerdinger, W. J., Toups, M. A., & Vicoso, B. (2020). Disagreement in F_{st} estimators: A case study from sex chromosomes. *Molecular Ecology Resources, 20*(6), 1517–1525.

Gershoni, M., & Pietrokovski, S. (2014). Reduced selection and accumulation of deleterious mutations in genes exclusively expressed in men. *Nature Communications, 5*,4438.

Gerstein, M. B., Rozowsky, J., Yan, K. K., Wang, D., Cheng, C., Brown, L., … Waterston, R. (2014). Comparative analysis of the transcriptome across distant species. *Nature, 512*(7515), 445–448.

Gibilisco, L., Zhou, Q., Mahajan, S., & Bachtrog, D. (2016). Alternative splicing within and between *Drosophila* species, sexes, tissues, and developmental stages. *PLoS Genetics, 12*(12), e1006464.

Gout, J. F., Kahn, D., & Duret, L. (2010). The relationship among gene expression, the evolution of gene dosage, and the rate of protein evolution. *PLoS Genetics, 6*(5), 20.

Grantham, M. E., & Brisson, J. A. (2018). Extensive differential splicing underlies phenotypically plastic aphid morphs. *Molecular Biology and Evolution, 35*(8), 1934–1946.

Harrison, P. W., Jordan, G. E., & Montgomery, S. H. (2014). SWAMP: Sliding window alignment masker for PAML. *Evolutionary Bioinformatics, 10*, 197–204.

Harrison, P. W., Wright, A. E., & Mank, J. E. (2012). The evolution of gene expression and the transcriptome–phenotype relationship. *Seminars in Cell and Developmental Biology,*

23(2), 222–9.

Harrison, P. W., Wright, A. E., Zimmer, F., Dean, R., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences of the United States of America, 112*(14), 4393–4398.

Hedrick, P. W. (2011). Population genetics of malaria resistance in humans. *Heredity, 107*(4), 283–304.

Hill, G. E., Doucet, S. M., & Buchholz, R. (2005). The effect of coccidial infection on iridescent plumage coloration in wild turkeys. *Animal Behaviour, 69*(2), 387–394.

Innocenti, P., & Morrow, E. H. (2010). The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS B*iology, 8(3), e1000335.

Kalsotra, A., & Cooper, T. A. (2011). Functional consequences of developmentally regulated alternative splicing. *Nature Reviews Genetics, 12*(10), 715–29.

Kasimatis, K. R., Abraham, A., Ralph, P. L., Kern, A. D., Capra, J. A., & Phillips, P. C. (2021). Evaluating human autosomal loci for sexually antagonistic viability selection in two large biobanks. *Genetics*, *217*(1), iyaa015.

Kasimatis, K. R., Nelson, T. C., & Phillips, P. C. (2017). Genomic signatures of sexual conflict. *Journal of Heredity, 108*(7), 780–790.

Kasimatis, K. R., Ralph, P. L., & Phillips, P. C. (2019). Limits to genomic divergence under sexually antagonistic selection. *G3-Genes Genome Genetics, 9*(11), 3813–3824.

Khaitovich, P., Hellmann, I., Enard, W., Nowick, K., Leinweber, M., Franz, H., … Pääbo, S. (2005). Evolution: Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. *Science, 309*(5742), 1850–1854.

Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: A fast spliced aligner with low memory requirements. *Nature Methods, 12*(4), 357–360.

Korneliussen, T. S., Albrechtsen, A., & Nielsen, R. (2014). ANGSD: analysis of next generation sequencing data. *BMC Bioinformatics, 15*, 356.

Korneliussen, T. S., & Moltke, I. (2015). NgsRelate: a software tool for estimating pairwise relatedness from next-generation sequencing data. *Journal of Bioinformatics, 31*(24), 4009–4011.

Kryuchkova-Mostacci, N., & Robinson-Rechavi, M. (2017). A benchmark of gene expression tissue-specificity metrics. *Briefings in Bioinformatics, 18*(2), 205–214.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Journal of Bioinformatics, 25*(16), 2078–2079.

Löytynoja, A., & Goldman, N. (2008). Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science, 320*(5883), 1632–1635.

Mank, J. E. (2017a). The transcriptional architecture of phenotypic dimorphism. *Nature Ecology and Evolution, 1*, 0006.

Mank, J. E. (2017b). Population genetics of sexual conflict in the genomic era. *Nature Reviews Genetics, 18*, 721–730.

Mank, J. E., Hultin-Rosenberg, L., Axelsson, E., & Ellegren, H. (2007). Rapid evolution of female-biased, but not male-biased, genes expressed in the avian brain. *Molecular Biology, 24*(12), 2698–2706.

Mank, J. E., Hultin-Rosenberg, L., Zwahlen, M., & Ellegren, H. (2008). Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. *American Naturalist, 171*(1), 35–43.

Mank, J. E., Nam, K., Brunström, B., & Ellegren, H. (2010). Ontogenetic complexity of sexual dimorphism and sex-specific selection. *Molecular Biology and Evolution, 27*(7), 1570–1578.

Matlin, A. J., Clark, F., & Smith, C. W. J. (2005). Understanding alternative splicing: towards a cellular code. *Nature Reviews Molecular Cell Biology, 6*(5), 386–398.

McIntyre., L. M., Bono, L. M., Genissel, A., Westerman, R., Junk, D., Telonis-Scott, M., … Nuzhdin, S. V. (2006). Sex-specific expression of alternative transcripts in *Drosophila*. *Genome Biology, 7*(8), 1–17.

Meisel, R. P. (2011). Towards a more nuanced understanding of the relationship between sex-biased gene expression and rates of protein-coding sequence evolution. *Molecular Biology and Evolution, 28*(6), 1893–1900.

Melamud, E., & Moult, J. (2009). Stochastic noise in splicing machinery. *Nucleic Acids Research, 37*(14), 4873–4886.

Melé, M., Ferreira, P. G., Reverter, F., DeLuca, D. S., Monlong, J., Sammeth, M., … Guigó, R. (2015). The human transcriptome across tissues and individuals. *Science, 348*(6235), 660–665.

Merkin, J., Russell, C., Chen, P., & Burge, C. B. (2012). Evolutionary dynamics of gene and

isoform regulation in mammalian tissues. *Science, 338(*6114), 1593–1599.

Mi, H., Muruganujan, A., Ebert, D., Huang, X., & Thomas, P. D. (2019). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Research, 47*(D1), D419–D426.

Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S., & Mank, J. E. (2012). W chromosome expression responds to female-specific selection. *Proceedings of the National Academy of Sciences of the United States of America, 109*(21), 8207–8211.

Nielsen, R., Korneliussen, T., Albrechtsen, A., Li, Y., & Wang, J. (2012). SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data. *PLoS ONE, 7*(7), e37558.

Nilsen, T. W., & Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature, 463*, 457–463.

Pál, C., Papp, B., & Hurst, L. D. (2001). Highly expressed genes in yeast evolve slowly. *Genetics, 158*(2), 927–931.

Pan, Q., Bakowski, M. A., Morris, Q., Zhang, W., Frey, B. J., Hughes, T. R., & Blencowe, B. J. (2005). Alternative splicing of conserved exons is frequently species-specific in human and mouse. *Trends in Genetics, 21*(2), 73–77.

Papkostas, S. Vøllestad, L. A., Bruneaux, M., Aykanat, T., Vanoverbeke, J., Ning, M., … Leder, E. H. (2014). Gene pleiotropy constrains gene expression in fish adapted to different thermal conditions. *Nature Communications, 5*, 4071.

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philosophical Transactions of the Royal Society B, 28*(1366), 261–274.

Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T. C., Mendell, J. T., & Salzberg, S. L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature Biotechnology, 33*(3), 290–295.

Pointer, M. A., Harrison, P. W., Wright, A. E., & Mank, J. E. (2013). Masculinization of gene expression is associated with exaggeration of male sexual dimorphism. *PLoS Genetics, 9*(8), e1003697.

Ranz, J. M., Castillo-Davis, C. I., Meiklejohn, C. D., & Hartl, D. L. (2003). Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science, 300*(5626), 1742–1745.

Rice, W. R. (1984). Sex chromosomes and the evolution of sexual dimorphism. *Evolution,*

38(4), 735–742.

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Journal of Bioinformatics, 26*(1), 139–140.

Rockman, M. V, Skrovanek, S. S., Kruglyak, L., Gerke, J. P., & Kruglyak, L. (2010). Selection at linked sites shapes heritable phenotypic variation in *C. elegans*. *Science, 330*(6002), 372–376.

Ruzicka, F., Dutoit, L., Czuppon, P., Jordan, C. Y., Li., X., Olito, C., I., … Connallon, T. (2020). The search for sexually antagonistic genes: Practical insights from studies of local adaptation and genomics. *Evolution Letters, 4*(5), 398–415.

Schütt, C., & Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. *Development, 127*(4), 667–677.

Shen, S., Park, J. W., Lu, Z. X., Lin, L., Henry, M. D., Wu, Y. N., … Xing, Y. (2014). rMATS: Robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proceedings of the National Academy of Sciences of the United States of America, 111*(51), e5593–e5601.

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., & Bergelson, J. (1999). Dynamics of disease resistance polymorphism at the Rpm1 locus of *Arabidopsis*. *Nature, 400*(6745), 667–671.

Su, W. L., Modrek, B., GuhaThakurta, D., Edwards, S., Shah, J. K., Kulkarni, A. V., … Castle, J. C. (2008). Exon and junction microarrays detect widespread mouse strain- and sex-bias expression differences. *BMC Genomics, 9*, 273.

Suzuki, R., & Shimodaira, H. (2006). Pvclust: An R package for assessing the uncertainty in hierarchical clustering. *Journal of Bioinformatics, 22*(12), 1540–1542.

Tress, M. L., Abascal, F., & Valencia, A. (2017a). Alternative splicing may not be the key to proteome complexity. *Trends in Biochemical Sciences, 42*(2), 98–110.

Tress, M. L., Abascal, F., & Valencia, A. (2017b). Most alternative isoforms are not functionally important. *Trends in Biochemical Sciences, 42*(6) 408–410.

Uebbing, S., Künstner, A., Mäkinen, H., Backström, N., Bolivar, P., Burri, R., … Ellegren, H. (2016). Divergence in gene expression within and between two closely related flycatcher species. *Molecular Ecology, 25*(9), 2015–2028.

Wan, Y., & Larson, D. R. (2018). Splicing heterogeneity: separating signal from noise.

Genome Biology, 19, 1–10.

Wang, M., Zhao, Y., & Zhang, B. (2015). Efficient test and visualization of multi-set intersections. *Scientific Reports, 5*, 16923.

Weatheritt, R. J., Sterne-Weiler, T., & Blencowe, B. J. (2016). The ribosome-engaged landscape of alternative splicing. *Natural Structural and Molecular Biology, 23*(12), 1117–1123.

Wright, A. E., Fumagalli, M., Cooney, C. R., Bloch, N. I., Vieira, F. G., Buechel, S. D., … Mank, J. E. (2018). Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evolution Letters, 2*(2), 52–61.

Wright, A. E., Rogers, T. F., Fumagalli, M., Cooney, C. R., & Mank, J. E. (2019). Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Molecular Ecology, 28*(11), 2860–2871.

Yanai, I., Benjamin, H., Shmoish, M., Chalifa-Caspi, V., Shklar, M., Ophir, R., … Shmueli, O. (2005). Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. *Journal of Bioinformatics, 21*(5), 650–659.

Yang, X., Schadt, E. E., Wang, S., Wang, H., Arnold, A. P., Ingram-Drake, L., … Lusis, A. J. (2006). Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Research, 16*(8), 995–1004.

Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution, 24*(8), 1586–1591.

Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., ... Flicek, P. (2018). Ensembl 2018. *Nucleic Acids Research, 46*(D1), D754–D761.

Zhang, Y., Sturgill, D., Parisi, M., Kumar, S., & Oliver, B. (2007). Constraint and turnover in sex-biased gene expression in the genus *Drosophila*. *Nature, 450*(7167), 233–237.

CHAPTER 5. DISCUSSION

In this thesis, I use transcriptomic and genomic data from multiple bird species to investigate the genetic architecture of sexual dimorphism and to understand how sex-specific selection shapes genome evolution. I explore two paths to sexual dimorphism. Firstly, due to their unequal pattern of inheritance, sex chromosomes are hypothesised to facilitate the evolution of sexual dimorphisms by navigating functional constraints that shared portions of the genome are subject to. However, the sex chromosomes have differences in mutation rate, effective population size and recombination rate relative to the autosomes, which may act to reduce the efficacy of selection acting on them. Consistent with this, in Chapter 2 (Wright, Rogers et al., 2019), I show that the avian Z chromosome is not a hotspot of ongoing sexual conflict relative to the autosomes. Additionally, in Chapter 3 (Rogers, Pizzari et al., 2021), I conclude that a combination of adaptive and purifying selection are the dominant modes of evolution of the avian W chromosome. Secondly, I examine the role of differential regulation of the regions of the genome that are shared equally between males and females in the evolution of phenotypic dimorphisms. In Chapter 4 (Rogers, Palmer et al., 2021), I uncover an abundance of autosomal genes with sex differences in expression level and alterative splicing and suggest that differential alternative splicing evolves under sex-specific selection and facilitates sex-specific adaptation when differential expression level is limited by pleiotropic constraints. These findings are key to understanding the role that sex-specific selection has in establishing sexually dimorphic phenotypes, and the relative importance of the sex chromosomes and the autosomes with regards to this. This discussion chapter will examine broad questions that remain in the field and future directions of this work.

5.1 The relative role of the sex chromosomes vs the autosomes in sex-specific adaptation

A well-recognised population genetics model by Rice (1984) predicts that under a certain set of assumptions, sexually antagonistic polymorphisms should accumulate disproportionately on the sex chromosomes relative to the autosomes, and this has been demonstrated by a series of compelling experiments (Chippindale et al., 2001; Connallon & Jakubowski, 2009; Gibson et al., 2002; Pischedda & Chippindale, 2006). However, a review of Rice's (1984) model by Fry (2010) indicates that this may not always be the case. For example, Rice's (1984) model is contingent on the assumption that the dominance of an autosomal allele is the same in

both sexes, which Fry (2010) points out is not necessarily true, particularly in natural populations. Additionally, Fry (2010) states that it cannot be assumed that genetic variation in a sexually dimorphic trait automatically creates a trade-off between the sexes, as in Rice's (1984) model. Empirical studies also dispute Rice's (1984) model. For instance, Fedorka & Mousseau (2004) found that male ground crickets (*Allonemobius socius*) with the greatest mating success transmit alleles associated with reproductive advantage to their sons but sire daughters with significantly lower reproductive success. These male-beneficial alleles must be autosomally inherited because males do not pass on their X chromosomes to their sons. A similar study in lab populations of *Drosophila serrata* (Delcourt et al., 2009) as well as a study of wild populations of side-blotched lizards (*Uta stansburiana*) (Calsbeek & Sinervo, 2004) yielded results consistent with this.

Interestingly, my results are consistent with this growing evidence suggesting that the role of the sex chromosomes in sex-specific adaptation may be overestimated. Firstly, I find that while sexual conflict is significantly associated with patterns of genetic diversity across the autosomes, there was no such signature on the Z chromosome, indicating that the avian Z chromosome is not a hotspot of sexual conflict relative to the autosomes (Wright, Rogers et al., 2019). Secondly, I found no enrichment of sex-biased alternatively spliced genes on the Z chromosome (Rogers, Palmer et al., 2021), which runs counter to the predictions of Rice (1984). This is likely due to the decreased effective population size of the sex chromosomes relative to the autosomes, as well as differences in mutation rate and recombination rate, leading to increased genetic drift (Wright & Mank, 2013; Wright et al., 2015), reducing the efficiency of selection acting on the sex chromosomes. However, my results do suggest that purifying and sex-specific selection act on the avian W. Nevertheless, the W chromosome is lacking in multi-copy gene families in contrast to the Y chromosome, indicating that the W does not play a large role in sex-specific adaptation as Y studies might imply (Rogers, Pizzari et al., 2021).

5.2 Do sex differences in gene regulation have sex-specific functions?

There is some debate over the extent to which sex-biased genes have sex-specific functions, since it is challenging to link gene expression data to particular phenotypes. Sex-biased alternative splicing in the *sex lethal* gene plays a role in regulating sex determination in *Drosophila* (Schütt & Nöthiger, 2000), but studies investigating the broad functional effects

of sex-biased alternatively spliced genes are particularly lacking. A handful of studies have attempted to investigate the functional importance of sexually dimorphic expression using a variety of methods, with mixed results. One notable study by Connallon & Clark (2011) used published data on mutations with well-documented phenotypic fitness effects from *Drosophila melanogaster* along with RNA-seq profiles for the associated genes, and characterised alleles with sex-limited effects. If genes with sex-biased expression have sex-specific functions, then mutations in these genes will have sex-specific phenotypic effects. They found that genes with sexually dimorphic phenotypic effects tended to exhibit sex-biased transcription significantly more than those with similar phenotypic effects between the sexes. However, some genes with highly dimorphic expression had similar mutational effects in males and females, and this was even more common for genes with more moderate sex-biased expression. Therefore, they conclude that although there is some link between sex-biased transcription and sex-specific phenotypes, sex-biased expression does not always equate to sex-specific significance or function.

In addition to this, Innocenti & Morrow (2010) use microarrays to measure expression and found that in *Drosophila melanogaster*, only 7.8% of differentially expressed transcripts were significantly associated with the sex by fitness interaction term, describing putative sexually antagonistic loci. This suggests that sex-biased expression represents the footprint of resolved conflict. Furthermore, the male-biased gene *yellow* in some *Drosophila* species has been found to have a role in sex-specific fitness due to its function in male mating behaviour. Male mating success is mediated by the effect of the gene on pigmentation of male-specific leg structures called sex combs, with loss of *yellow* expression leading to an increase in unsuccessful mating attempts (Massey et al., 2019). Van der Bijl & Mank (2021) also recently studied the effect of gene knockout mutations on hundreds of mouse phenotypes to investigate how changes in gene regulation affect sexual dimorphism. They revealed that both dimorphic and monomorphic traits have a large amount of sex-specific genetic architecture, presumably mediated by differential expression, and that the sexes react to knockout mutations in a variety of ways.

Some studies also use proxies to test for sex-specific effects of differential gene regulation. Population genomic approaches, such as Tajima's *D* and intersexual F_{st} can be used as proxies to detect the signature of ongoing and resolved conflict (Mank et al., 2017). For instance,

Wright et al. (2018) and Wright, Rogers et al. (2019) employ population genomic signatures to show that differential expression can lead to the evolution of separate male and female genomic architectures, thereby resolving sexual conflict. However, it is important to note that recent theoretical work suggests that intersexual divergence in allele frequency may not always be a reliable estimate of sexual conflict over survival, as very large selective pressures and mortality loads are necessary to produce elevated patterns of intersexual F_{ST}, and studies with a small number of samples are particularly problematic (Kasimatis et al., 2017; Kasimatis et al., 2019). Additionally, recently it has been highlighted that elevated F_{st} for male-biased genes could be due to the presence of multi-copy Y-linked genes, rather than representing sexually antagonistic selection over survival (Bissegger et al., 2020).

5.3 What is a sex-biased gene?

RNA-seq approaches are increasingly used to understand the mechanisms underlying complex phenotypes and the selective forces acting on the transcriptome. However, most studies, including ours, use bulk sequencing techniques to assess expression and alternative splicing across whole tissues or body parts which are made up of multiple distinct cell types. Consequently, expression or splicing profiles actually resemble average values across a range of different cell types. Importantly, this means that samples that vary in tissue composition can produce patterns of differential expression that are often mistaken as evidence of regulatory change. Conversely, this approach can also dampen and/or mask known signals of regulatory differences (Hunnicutt et al., 2021; Montgomery & Mank, 2016).

Recent advances in single-cell transcriptomics are providing new insights into tissue composition and how this can vary both within and across species. Within species, dramatic changes in tissue composition are well documented throughout development (Kim et al., 2021; Niu & Spradling, 2020; Witt et al., 2019) and between the sexes (Kim et al., 2021). Most notably, gonad tissue has many sex-specific cell types (Estermann et al., 2020; Lin & Capel, 2015) as well as a mix of diploid and haploid cells at various stages of differentiation (Lei et al., 2007). For example, testes cells in particular have unique expression patterns (Green et al., 2018; Shima et al., 2004) and experience diverse selection pressures (Larson et al., 2018) across ontogeny. However thus far, limited research accounts for the cellular complexity of this tissue, which is predicted to significantly confound the results of studies on regulatory evolution measured from bulk RNA-seq data (Good et al., 2010). Accordingly, differentially

expressed or alternatively spliced genes detected from traditional 'bulk' RNA-seq methods may just be artefacts of disparities in tissue composition as opposed to real regulatory divergence. In this thesis, all RNA samples were taken at the same age to limit variation in tissue composition associated with ontogeny. Furthermore, we used stringent filtering thresholds to partially mitigate the aforementioned problem. Stringent filtering thresholds can largely alleviate the false-positive effects of tissue scaling (Montgomery & Mank, 2016). Montgomery & Mank (2016) suggest that a $\log_2$-fold change of 1 is sufficient, which is the parameter has been used throughout this thesis, however, they note that this will likely also increase the false-negative rate. Future studies would ideally, if possible, utilise single-cell RNA-seq techniques to ensure reliable results without a corresponding increase in false-negative effects.

5.4 How does gene regulation vary over development?

Most research on alternative splicing, including mine, primarily assess transcription in adults, but many adult dimorphisms are established during embryogenesis and it is simply not appropriate to only study them outside of their developmental context. As a result, very little is known about how regulatory variation manifests during development and whether sex-specific selection varies over ontogeny. The few developmental studies which have been performed show that gene expression level (Ingleby et al., 2015; Mank et al., 2010; Perry et al., 2014) and splicing (Kalsotra & Cooper, 2011; Mazin et al., 2021) vary substantially over this period. Yet, it remains unclear whether adult sex differences are the product of gradual increases in the degree of sex-bias over development or temporal shifts in expression across independent sets of genes. Moreover, we know even less about how sex-specific selection varies through development. Therefore, a logical next step in my research would be to conduct a comprehensive analysis of the ontogeny of sex-biased alternative splicing and sex-specific selection.

5.5 What are the relative roles of gene regulatory mechanisms in sex-specific adaptation?

To date, studies of regulatory evolution have primarily focused on differences in gene expression level. However, regulatory variation is highly complex and multi-dimensional. My research has started to provide insight into the relative roles of splicing and expression in sex-specific adaptation (Rogers, Palmer et al. 2021) but the importance of other regulatory

mechanisms is unclear. Recent advances in sequencing methods can be used to study a range of different modes of gene regulation and shed light on this important outstanding question.

Firstly, studies of alternative splicing, including my own, are hampered by the limitations of short-read sequencing technologies in reconstructing isoform sequences and inferring combinations of splice-site usage. This leads to an underestimation in levels of splicing and increased number of false isoforms. Advances in long-read sequencing now make it possible to study splicing with unprecedented precision by directly sequencing full-length isoforms. However, high-throughput Illumina RNA-seq data is still necessary to quantify differences in isoform abundance. Therefore, future studies should, if feasible, use a cutting-edge hybrid sequencing approach to study alternative splicing. Specifically, high-throughput Illumina RNA-seq data should be combined with long read PacBio Iso-seq data to identify a high confidence set of splice events and accurately quantify isoform-specific expression.

Chromatin profiling methods such as Hi-C and ATAC-seq as well as bisulfite sequencing to quantify methylation can now be used to examine how each regulatory process differs between males and females. In eukaryotes, DNA is tightly wound into chromatin. Chromatin remodelling regulates gene expression by presenting transcription apparatus with dynamic access to an otherwise tightly packed genome. DNA methylation is a process by which methyl groups are added to a DNA molecule. When positioned in a gene promotor, it usually acts to repress gene expression, however, when DNA methylation is located in gene bodies, it is known to act to increase transcription (de Mendoza et al., 2020). Recent work suggests that chromatin and methylation profiles vary substantially between the sexes and likely play a role in sexual differentiation in several species (Garcia-Moreno et al., 2019; Mathers et al., 2019; McCarthy et al., 2014; Tsai et al., 2009). However, many outstanding questions remain including, what is the relative role of each of these mechanisms in resolving sexual antagonism, and do they evolve in response to sex-specific selection? Methods employed in this thesis to test for selection on differential gene regulation can be used to answer these questions in the future. For instance, the measurement of isoform specificity (τ_{AS}) adapted from the tissue specificity index (τ) (Yanai et al., 2005) developed by myself and colleagues (Rogers, Palmer et al., 2021), is simply a measurement of variation and therefore can be tailored to test for sex-specific selection on chromatin and methylation variation, in turn allowing us to test how these mechanisms evolve. Furthermore, there is evidence to suggest

that in some cases, various chromatin states can influence patterns of DNA methylation by directing DNA methyltransferases to specific DNA sequences, but mechanistically this is not yet fully understood (Robertson, 2002). Therefore, it would also be worthwhile to investigate the interaction between the two processes, in a similar way to which the interaction between differential gene expression level and alternative splicing was examined in this thesis. Studying a more diverse range of gene regulation methods will provide a more comprehensive understanding of how sex differences evolve.

5.6 How can we infer selection acting on regulatory variation?

More generally, the dominant mode by which gene expression evolves remains controversial. Current evidence supports the notion that global patterns of gene expression evolve predominantly under stabilizing selection but the extent of neutral evolution is heavily debated (Fay & Wittkopp, 2008; Khaitovich et al., 2006; Romero et al., 2012; Signor & Nuzhdin, 2018). Much of this debate is driven by the lack of a consensus neutral model of transcriptome evolution. In contrast to established models of sequence evolution that allow us to predict the phenotypic effects of different types of coding mutations and scan coding sequence data for regions of adaptive evolution, gene regulation can be complex and non-additive in its phenotypic effects. This complexity has resulted in a wide range of approaches to study regulatory evolution (Bedford & Hartl, 2009; Hill et al., 2021; Whitehead & Crawford, 2006). Importantly, these approaches make direct assumptions about how expression evolves across species, many of which have yet to be robustly validated, and these assumptions vary extensively across models. With the exception of a few studies (Catalán et al., 2019; Nourmohammad et al., 2017; Rohlfs & Nielsen, 2015; Rohlfs et al., 2013), multiple models are rarely incorporated into analyses of gene regulatory evolution. Consequently, it is unclear which method is the most reliable and whether discrepancies between studies are biologically meaningful or due to differences in model usage. Future work should focus on developing a consensus model of gene regulatory evolution to infer selection acting on gene expression evolution (Price et al., in revision).

5.7 REFERENCES

Bedford, T., & Hartl, D. L. (2009). Optimization of gene expression by natural selection. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(4), 1133–1138.

Bissegger, M., Laurentino, T. G., Roesti, M., & Berner, D. (2020). Widespread intersex differentiation across the stickleback genome – The signature of sexually antagonistic selection? *Molecular Ecology*, *29*(2), 262–271.

Butler, M. A., & King, A. A. (2004). Phylogenetic comparative analysis: A modeling approach for adaptive evolution. *American Naturalist*, *164*(6), 683–695.

Calsbeek, R., & Sinervo, B. (2004). Within-clutch variation in offspring sex determined by differences in sire body size: Cryptic mate choice in the wild. *Journal of Evolutionary Biology*, *17*(2), 464–470.

Catalán, A., Briscoe, A., & Höhna, S. (2019). Drift and directional selection are the evolutionary forces driving gene expression divergence in eye and brain tissue of Heliconius butterflies. *Genetics*, *213*(2), 581–594.

Charlesworth, B., & Charlesworth, D. (2000). The degeneration of Y chromosomes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *355*(1403), 1563–1572.

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America, 98*(4), 1671–1675.

Connallon, T., & Clark, A. G. (2011). Association between sex-biased gene expression and mutations with sex-specific phenotypic consequences in Drosophila. *Genome Biology and Evolution*, *3*(1), 151–155.

Connallon, T., & Jakubowski, E. (2009). Association between sex ratio distortion and sexually antagonistic fitness consequences of female choice. *Evolution*, *63*(8), 2179–2183.

de Mendoza, A., Lister, R., & Bogdanovic, O. (2020). Evolution of DNA methylome diversity in eukaryotes. *Journal of Molecular Biology*, *432*(6), 1687–1705.

Delcourt, M., Blows, M. W., & Rundle, H. D. (2009). Sexually antagonistic genetic variance for fitness in an ancestral and a novel environment. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1664), 2009–2014.

Engelstädter, J. (2008). Muller's ratchet and the degeneration of Y chromosomes: A simulation study. *Genetics*, *180*(2), 957–967.

Estermann, M. A., Williams, S., Hirst, C. E., Roly, Z. Y., Serralbo, O., Adhikari, D., … Smith, C. A. (2020). Insights into gonadal sex differentiation provided by single-cell transcriptomics in the chicken embryo. *Cell Reports*, *31*(1), 107491.

Fay, J. C., & Wittkopp, P. J. (2008). Evaluating the role of natural selection in the evolution of gene regulation. *Heredity*, *100*, 191–199.

Fedorka, K. M., & Mousseau, T. A. (2004). Female mating bias results in conflicting sex-specific offspring fitness. *Nature*, *429*(6987), 65–67.

Felsenstein, J. (1973). Maximum likelihood estimation of evolutionary trees from continuous characters. *American Journal of Human Genetics*, *25*(5), 471–492.

Felsenstein, J. (1985). Phylogenies and the comparative method. *American Naturalist*, *125*(1), 1–15.

Fry, J. D. (2010). The genomic location of sexually antagonistic variation: Some cautionary comments. *Evolution*, *64*(5), 1510–1516.

Garcia-Moreno, S. A., Futtner, C. R., Salamone, I. M., Gonen, N., Lovell-Badge, R., & Maatouk, D. M. (2019). Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Developmental Biology*, *446*(2), 168–179.

Gibson, J. R., Chippindale, A. K., & Rice, W. R. (2002). The X chromosome is a hot spot for sexually antagonistic fitness variation. *Proceedings of the Royal Society B: Biological Sciences*, *269*(1490), 499–505.

Good, J. M., Giger, T., Dean, M. D., & Nachman, M. W. (2010). Widespread over-expression of the X chromosome in sterile F1 hybrid mice. *PLoS Genetics*, *6*(9), e1001148.

Green, C. D., Ma, Q., Manske, G. L., Shami, A. N., Zheng, X., Marini, S., … Hammoud, S. S. (2018). A comprehensive roadmap of murine spermatogenesis defined by single-cell RNA-Seq. *Developmental Cell*, *46*(5), 651–667.

Gu, X. (2004). Statistical framework for phylogenomic analysis of gene family expression profiles. *Genetics*, *167*(1), 531–542.

Hansen, T. F. (1997). Stabilizing selection and the comparative analysis of adaptation. *Evolution*, *51*(5), 1341–1351.

Hill, M. S., Vande Zande, P., & Wittkopp, P. J. (2021). Molecular and evolutionary processes generating variation in gene expression. *Nature Reviews Genetics*, *22*, 203–215.

Hunnicutt, K. E., Good, J. M., & Larson, E. L. (2021). Unraveling patterns of disrupted gene expression across a complex tissue. *BioRxiv*, 2021.07.08.451646.

Ingleby, F. C., Flis, I., & Morrow, E. H. (2015). Sex-biased gene expression and sexual conflict throughout development. *Cold Spring Harbor Perspectives in Biology*, *7*(1), a017632

Innocenti, P., & Morrow, E. H. (2010). The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS Biology*, *8*(3), e1000335.

Kalsotra, A., & Cooper, T. A. (2011). Functional consequences of developmentally regulated alternative splicing. *Nature Reviews Genetics*, *12*, 715–729.

Kasimatis, K. R., Nelson, T. C., & Phillips, P. C. (2017). Genomic signatures of sexual conflict. *Journal of Heredity*, *108*(7), 780–790.

Kasimatis, K. R., Ralph, P. L., & Phillips, P. C. (2019). Limits to genomic divergence under sexually antagonistic selection. *G3: Genes, Genomes, Genetics*, *9*(11), 3813–3824.

Khaitovich, P., Enard, W., Lachmann, M., & Pääbo, S. (2006). Evolution of primate gene expression. *Nature Reviews Genetics*, *7*, 693–702.

Kim, D. W., Place, E., Chinnaiya, K., Manning, E., Sun, C., Dai, W., … Blackshaw, S. (2021). Single-cell analysis of early hypothalamic development reveals that hypothalamic cells are induced from prethalamic-like progenitors. *BioRxiv*, 2021.04.09.438683.

Larson, E. L., Kopania, E. E. K., & Good, J. M. (2018). Spermatogenesis and the evolution of mammalian sex chromosomes. *Trends in Genetics*, *34*(9), 722–732.

Lei, N., Hornbaker, K. I., Rice, D. A., Karpova, T., Agbor, V. A., & Heckert, L. L. (2007). Sex-specific differences in mouse DMRT1 expression are both cell type- and stage-dependent during gonad development. *Biology of Reproduction*, *77*(3), 466–475.

Lewontin, R. C., & Krakauer, J. (1973). Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics*, *74*(1), 175–195.

Lin, Y. T., & Capel, B. (2015, June 1). Cell fate commitment during mammalian sex determination. *Current Opinion in Genetics and Development*, *32*, 144–152.

Mank, J. E. (2017). Population genetics of sexual conflict in the genomic era. *Nature Reviews Genetics*, *18*(12), 721–730.

Mank, J. E., Nam, K., Brunström, B., & Ellegren, H. (2010). Ontogenetic complexity of sexual dimorphism and sex-specific selection. *Molecular Biology and Evolution*, *27*(7), 1570–1578.

Massey, J. H., Chung, D., Siwanowicz, I., Stern, D. L., & Wittkopp, P. J. (2019). The *yellow* gene influences *Drosophila* male mating success through sex comb melanization. *ELife*, *8*, e49388.

Mathers, T. C., Mugford, S. T., Percival-Alwyn, L., Chen, Y., Kaithakottil, G., Swarbreck, D., … van Oosterhout, C. (2019). Sex-specific changes in the aphid DNA methylation

landscape. *Molecular Ecology*, *28*(18), 4228–4241.

Mazin, P. V., Khaitovich, P., Cardoso-Moreira, M., & Kaessmann, H. (2021). Alternative splicing during mammalian organ development. *Nature Genetics*, *53*(6), 925–934.

McCarthy, N. S., Melton, P. E., Cadby, G., Yazar, S., Franchina, M., Moses, E. K., … Hewitt, A. W. (2014). Meta-analysis of human methylation data for evidence of sex-specific autosomal patterns. *BMC Genomics*, *15*, 981.

Montgomery, S. H., & Mank, J. E. (2016). Inferring regulatory change from gene expression: the confounding effects of tissue scaling. *Molecular Ecology*, *25*(20), 5114–5128.

Niu, W., & Spradling, A. C. (2020). Two distinct pathways of pregranulosa cell differentiation support follicle formation in the mouse ovary. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(33), 20015–20026.

Nourmohammad, A., Rambeau, J., Held, T., Kovacova, V., Berg, J., & Lässig, M. (2017). Adaptive evolution of gene expression in *Drosophila*. *Cell Reports*, *20*(6), 1385–1395.

Pennell, M. W., & Harmon, L. J. (2013). An integrative view of phylogenetic comparative methods: Connections to population genetics, community ecology, and paleobiology. *Annals of the New York Academy of Sciences*, *1289*(1), 90–105.

Perry, J. C., Harrison, P. W., & Mank, J. E. (2014). The ontogeny and evolution of sex-biased gene expression in *Drosophila melanogaster*. *Molecular Biology and Evolution*, *31*(5), 1206–1219.

Pischedda, A., & Chippindale, A. K. (2006). Intralocus sexual conflict diminishes the benefits of sexual selection. *PLoS Biology*, *4*(11), 2099–2103.

Price, P. A., Palmer, D. H., Taylor, D. J., Kim, D. W., Place, E. S., Rogers, T. F., … Wright, A. E. (in revision). Detecting signatures of selection in regulatory variation.

Rice, W. R. (1984). Sex chromosomes and the evolution of sexual dimorphism. *Evolution*, *38*(4), 735–742.

Robertson, K. D. (2002). DNA methylation and chromatin - Unraveling the tangled web. *Oncogene*, *21*(35), 5361–5379.

Rogers, T. F., Palmer, D. H., & Wright, A. E. (2021). Sex-specific selection drives the evolution of alternative splicing in birds. *Molecular Biology and Evolution*, *38*(2), 519–530.

Rogers, T. F., Pizzari, T., & Wright, A. E. (2021). Multi-copy gene family evolution on the avian W chromosome. *Journal of Heredity*, *112*(3), 250–259.

Rohlfs, R. V., & Nielsen, R. (2015). Phylogenetic ANOVA: The expression variance and

evolution model for quantitative trait evolution. *Systematic Biology*, *64*(5), 695–708.

Rohlfs, R. V, Harrigan, P., & Nielsen, R. (2013). Modeling gene expression evolution with an extended Ornstein–Uhlenbeck process accounting for within-species variation. *Molecular Biology and Evolution*, *31*(1), 201–211.

Romero, I. G., Ruvinsky, I., & Gilad, Y. (2012). Comparative studies of gene expression and the evolution of gene regulation. *Nature Reviews Genetics*, *13*, 505–516.

Schütt, C., & Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. *Development*, *127*(4), 667–677.

Shima, J. E., McLean, D. J., McCarrey, J. R., & Griswold, M. D. (2004). The murine testicular transcriptome: Characterizing gene expression in the testis during the progression of spermatogenesis. *Biology of Reproduction*, *71*(1), 319–330.

Signor, S. A., & Nuzhdin, S. V. (2018). The evolution of gene expression in *cis* and *trans*. *Trends in Genetics*, *34*(7), 532–544.

Tsai, H. W., Grant, P. A., & Rissman, E. F. (2009). Sex differences in histone modifications in the neonatal mouse brain. *Epigenetics*, *4*(1), 47–53.

van der Bijl, W., & Mank, J. E. (2021). Widespread cryptic variation in genetic architecture between the sexes. *Evolution Letters*, *5*(4), 359–369.

Whitehead, A., & Crawford, D. L. (2006). Neutral and adaptive variation in gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(14), 5425–5430.

Witt, E., Benjamin, S., Svetec, N., & Zhao, L. (2019). Testis single-cell RNA-seq reveals the dynamics of de novo gene transcription and germline mutational bias in drosophila. *ELife*, *8*, e47138.

Wright, A. E., Fumagalli, M., Cooney, C. R., Bloch, N. I., Vieira, F. G., Buechel, S. D., … Mank, J. E. (2018). Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evolution Letters*, *2*(2), 52–61.

Wright, A. E., Harrison, P. W., Zimmer, F., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Variation in promiscuity and sexual selection drives avian rate of Faster-Z evolution. *Molecular Ecology*, *24*(6), 1218–1235.

Wright, A. E., & Mank, J. E. (2013). The scope and strength of sex-specific selection in genome evolution. *Journal of Evolutionary Biology*, *26*(9), 1841–1853.

Wright, A. E., Rogers, T. F., Fumagalli, M., Cooney, C. R., & Mank, J. E. (2019). Phenotypic

sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Molecular Ecology*, *28*(11), 2860–2871.

Yanai, I., Benjamin, H., Shmoish, M., Chalifa-Caspi, V., Shklar, M., Ophir, R., … Shmueli, O. (2005). Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. *Bioinformatics*, *21*(5), 650–659.

CHAPTER 6. SUPPLEMENTARY INFORMATION

S6.1. CONTRASTING PATTERNS OF SEXUAL CONFLICT ON THE AVIAN Z CHROMOSOME RELATIVE TO THE AUTOSOMES

Wright, A. E., **Rogers, T. F.**, Fumagalli, M., Cooney, C. R., & Mank, J. E. (2019). Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Molecular Ecology*, *28*(11), 2860-2781.

Supplementary Material available online:

https://onlinelibrary.wiley.com/doi/10.1111/mec.15115

S6.1.1 Calculating relatedness and inbreeding coefficients

We identified and removed related individuals from our analyses using ngsRelate (Korneliussen & Moltke, 2015) to avoid violating Hardy Weinberg assumptions. First, we estimated allele frequencies with ANGSD using the SAMtools genotype likelihood model and inferred the major and minor allele from the genotype likelihood. Sites with SNP pvalue > 1e-6 or with MAF < 0.05 were removed from the analysis. We extracted allele frequencies and calculated relatedness estimates for all pairs of individuals in each species using ngsRelate. We excluded four peacock, two wild turkey and two swan goose individuals from subsequent analyses.

We used ANGSD to estimate allele frequencies using the SAMtools genotype likelihood model and assuming the reference base being one of the two possible alleles. Triallelic sites and sites with SNP pvalue > 1e-6 were removed. We estimated pairwise linkage disequilibrium from unphased expected genotypes calculated from genotype likelihoods using ngsLD (Fox et al., 2019). We then pruned linked sites using a threshold on r^2 of 0.3 and retrieved unlinked sites for further analysis.

Inbreeding coefficients were calculated using an EM algorithm with the ngsF package in ngsTools (Fumagalli et al., 2014). The maximum root-mean-square deviation between iterations to assume convergence was 0.001. For all species, inbreeding coefficients were <0.03 with the exception of the peacock where we identified two inbred individuals.

Table S6.1.1. Statistics of transcriptome assembly.

Species	No. transcripts	No. 'best isoforms'	No. 2FPKM filtered 'best isoforms'	% Complete BUSCOs	No. pairwise reciprocal orthologs	No. autosomal/Z-linked genes
Mallard duck	1,158,494	691,200	64,017	75.6%	11,608	8,305/342
Swan goose	1,096,179	709,410	50,187	70.0%	11,479	8,180/345
Wild turkey	1,066,855	676,369	56,876	69.9%	11,454	10,285/532
Common pheasant	1,160,278	699,055	74,515	68.4%	11,558	10,328/544
Guineafowl	1,110,494	686,549	55,050	77.6%	11,419	10,233/531
Indian peafowl	1,057,022	655,810	57,425	68.4%	11,085	9,886/528

Table S6.1.2. Tissue-biased genes identified in males.

	Autosomes		Z chromosome	
Species	No. spleen-biased genes	No. gonad-biased genes	No. spleen-biased genes	No. gonad-biased genes
Mallard duck	971	1647	43	66
Swan goose	893	1545	29	62
Wild turkey	1367	2052	44	133
Common pheasant	1236	2267	49	139
Guineafowl	1379	1964	108	61
Indian peafowl	1278	2113	53	130

Table S6.1.3. Tissue-biased genes identified in females.

	Autosomes		Z chromosome	
Species	**No. spleen-biased genes**	**No. gonad-biased genes**	**No. spleen-biased genes**	**No. gonad-biased genes**
Mallard duck	689	1283	25	48
Swan goose	651	1262	29	44
Wild turkey	890	1624	39	86
Common pheasant	774	1533	33	89
Guineafowl	1004	1446	52	70
Indian peafowl	891	1506	45	77

Table S6.1.4. The effect of tissue-bias on Tajima's *D* and F_{st} after controlling for multiple factors using multiple regression.

	Tajima's D		F_{ST}
Species	**All genes p-value (F value)**	**Unbiased genes p-value (F value)**	**Unbiased genes p-value (F value)**
Mallard duck	**< 2.2e-16** (38.093)	**< 0.001** (8.525)	0.687 (0.375)
Swan goose	**< 2.2e-16** (85.478)	**3.184e-05** (10.383)	0.421 (0.866)
Wild turkey	**< 2.2e-16** (157.101)	**< 2.2e-16** (51.923)	0.498 (0.698)
Common pheasant	**< 2.2e-16** (42.168)	**0.025** (3.697)	0.509 (0.676)
Guineafowl	**< 2.2e-16** (90.883)	**9.695e-07** (13.889)	0.480 (0.734)
Indian peafowl	**0.024** (3.718)	0.114 (2.174)	0.767 (0.265)

Tissue-biased genes were identified from male expression data. Only autosomal genes are included in the analyses. Multi-predictor model: (TD/F_{ST} ~ Tissue bias + log(tW) + log(Gene length) + log(GC) + log (Gene expression level)).

Table S6.1.5. Intersexual F_{ST} across autosomal tissue-biased genes.

Species	Gonad-biased		Spleen-biased		Non-tissue-biased
	Median F_{ST}	p-value	Median F_{ST}	p-value	Median F_{ST}
Mallard duck	-0.006	0.660	-0.007	0.891	-0.007
Swan goose	-0.061	0.068	-0.064	**0.041**	-0.078
Wild turkey	0.001	0.359	0.014	**0.015**	0.001
Common pheasant	-0.014	0.311	-0.009	0.754	-0.008
Guinea fowl	-0.010	0.452	-0.020	0.181	-0.017
Indian peafowl	0.012	0.771	0.003	0.253	0.010

Only unbiased genes were used in this analysis. Tissue-biased genes were identified from male expression data. P- values are relative to non-tissue-biased genes and were calculated using Wilcoxon rank tests.

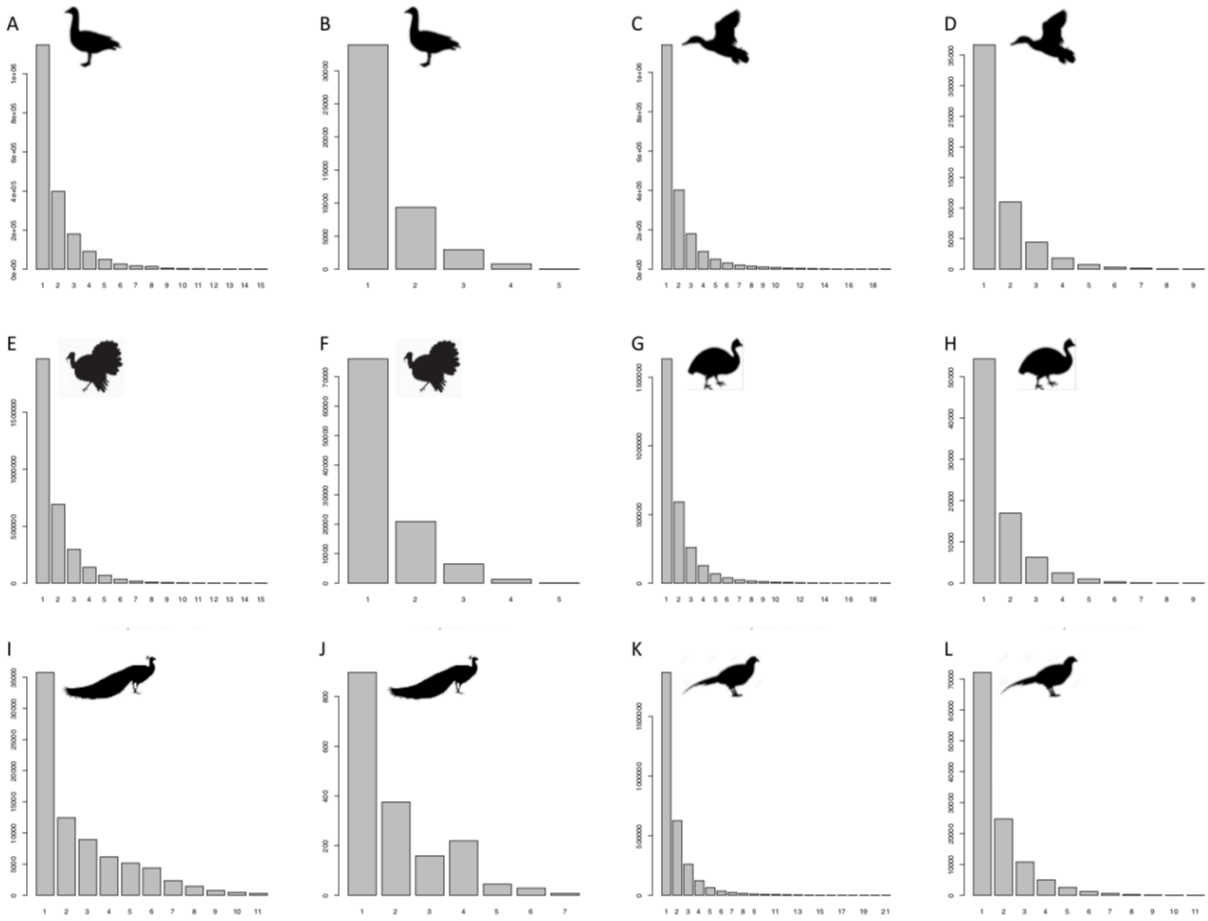

Figure S6.1.1. Site frequency spectrum for the Z chromosome and autosomes. Panels A, C, E, G, I, K show the autosomal SFS for the swan goose, mallard duck, wild turkey, helmeted guineafowl, peacock, common pheasant. Panels B, D, F, H, J, L show the Z-linked SFS for the swan goose, mallard duck, wild turkey, helmeted guineafowl, peacock, common pheasant.

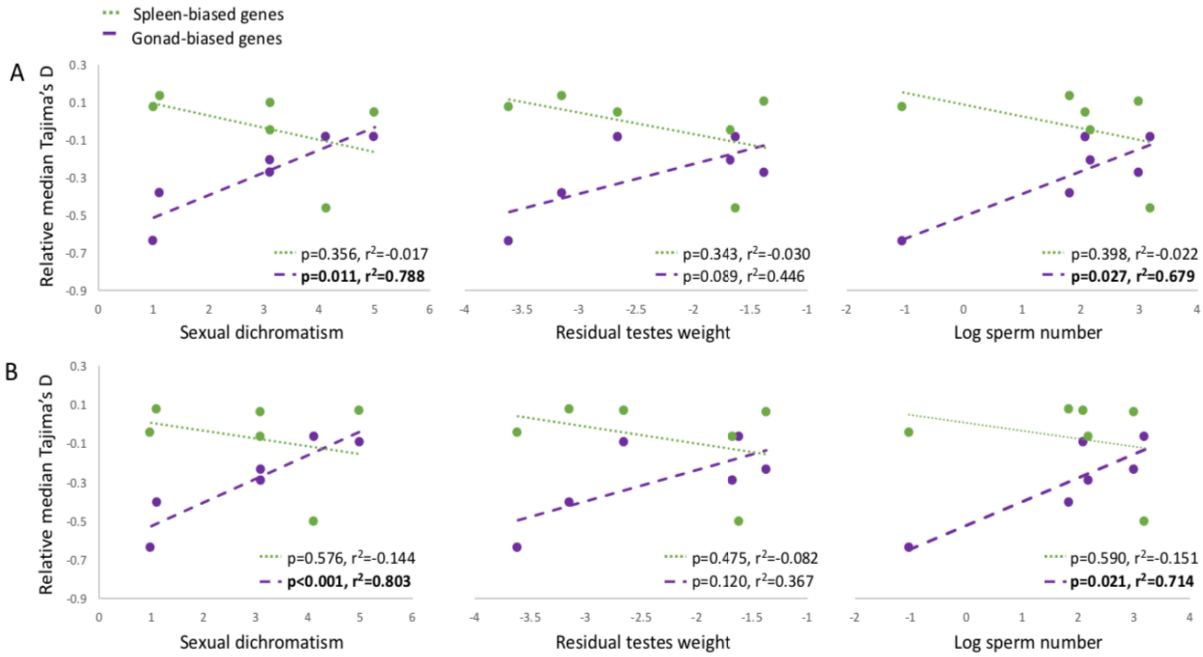

Figure S6.1.2. Phylogenetically controlled regression between proxies of sperm competition and relative Tajima's *D* for autosomal genes with unbiased expression between males and females. Relative *D* is calculated as the difference between median *D* for tissue-biased genes compared to non-tissue-biased genes. Tissue-biased genes were identified from male expression data (panel A) and female expression (panel B).

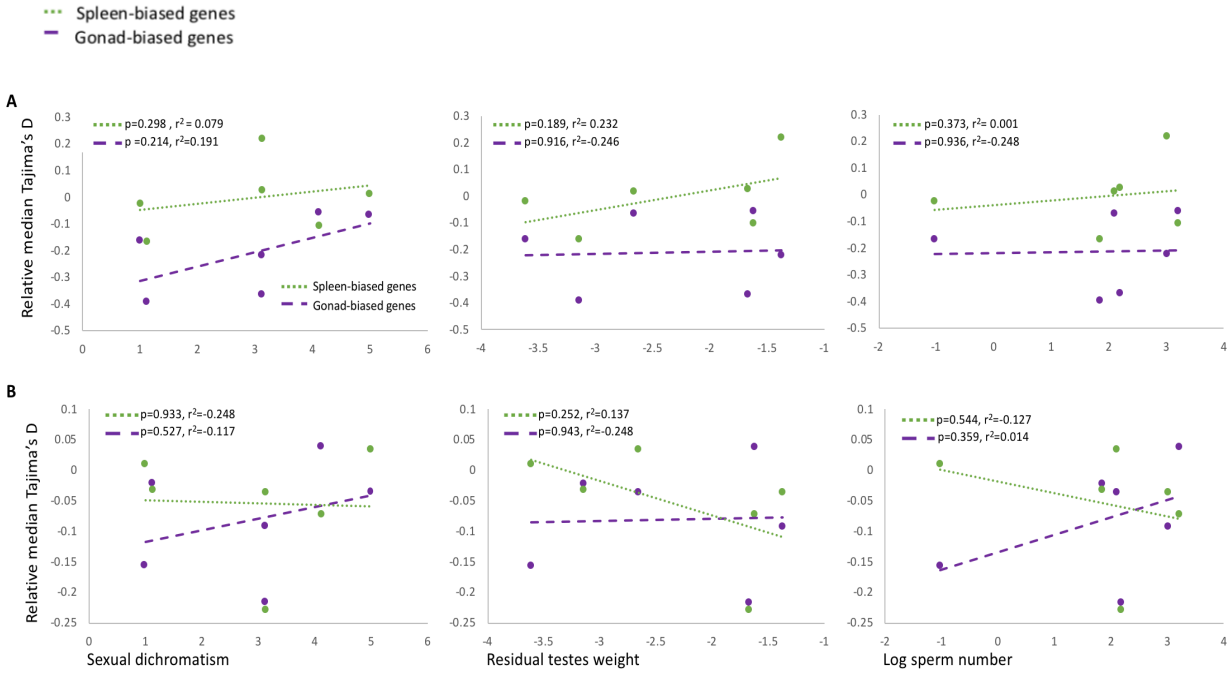

Figure S6.1.3. Phylogenetically controlled regression between proxies of sperm competition and relative Tajima's *D* for Z-linked genes. Relative *D* is calculated as the difference between median *D* for tissue-biased genes compared to non-tissue-biased genes. Tissue-biased genes were identified from female expression data (panels A) or male data (panels B).

S6.1.2 REFERENCES

Fox, E. A., Wright, A. E., Fumagalli, M., & Vieira, F. G. (2019). NgsLD: Evaluating linkage disequilibrium using genotype likelihoods. *Bioinformatics*, *35*(19), 3855–3856.

Fumagalli, M., Vieira, F. G., Linderoth, T., & Nielsen, R. (2014). NgsTools: Methods for population genetics analyses from next-generation sequencing data. *Bioinformatics*, *30*(10), 1486–1487.

Korneliussen, T. S., & Moltke, I. (2015). NgsRelate: A software tool for estimating pairwise relatedness from next-generation sequencing data. *Bioinformatics*, *31*(24), 4009–4011.

S6.2 MULTI-COPY GENE FAMILY EVOLUTION ON THE AVIAN W CHROMOSOME

Rogers, T. F., Pizzari, T. & Wright, A. E. (2021). Multi-copy gene family evolution on the avian W chromosome. *Journal of Heredity, 112*(3), 250-259.

Supplementary Material available online:

https://academic.oup.com/jhered/article/112/3/250/6184574#supplementary-data

Table S6.2.1. Primers used to sequence HINTW.

Species	Primer Sequence (5'-3')	Fragment size (bp)
Mallard	F: GGGTTATCCGAAGCAGAAGATTC R: GCCCAGGTTAGCAGCACACTT	702

Table S6.2.2. Probes for Nanostring nCounter.

Identifier	Type	Species	Ensembl ID	Accession	Position	Target Sequence	Status
Chicken_INV_1	Invariant	Chicken	ENSGALG00000009722	GA09722_43955.1	1-100	AGCTACAAACCAGCAAGCACATTTTCATTTTCTAGCCATATTTCAAAGTAAATCAGCATACTCATTTTTGTACATTTCAAGTACAACCAGCATGAGCAGT	
Chicken_INV_2	Invariant	Chicken	ENSGALG00000016947	ENSGALG016947_42721.1	92-191	TACTCTCCGATCAAACCGTGTGCCTATTTTTATCTTTTTATGAGTAATTATGTATAACAGATGAACCCTCTTACTCTGTGTCCTCTCTGCTGTACGTGT	
Chicken_INV_3	Invariant	Chicken	ENSGALG00000032974	ENSGA74_40681.1	213-312	CCTAAAACACAGATTCCTTACTGCAGTCTAATAAGTAACATTCAGAGTAACTTTCATTAGGAACTCAACAATAAATAATGTACTGTTTCTTCAGTCAGCT	
Chicken_INV_4	Invariant	Chicken	ENSGALG00000040666	ENSGALG040666_11933.1	110-209	TCTATACCTGAGAAAATCCATGCTTGCGCACGAGACAGTACCAATTTTTGCCAGTGAAAAATACTGTTCACATTTCTCATTGCATTTTAGCCTGGTGACA	
Chicken_INV_5	Invariant	Chicken	ENSGALG00000009722	ENSGALG09722_19343.1	42-141	ATTCCAGCAGTAGGTATTGCTTTTCTCATATTTTGGGGATTTGAGTGTATTTTGGGACTGACTTGAGTGTGTGAGATCTCTAAATTTATTTTGTTCAGAT	
Chicken_INV_6	Invariant	Chicken	ENSGALG00000016947	ENSGALG16947_26228.1	3-102	CTAGGGAGAGAAACGGCAAAATGTGATTGTGAAACTACGGTAACAACTTGTGATCCCTAGGGCTGGCCACTGTCAGGCAATTAATGTTTAACTTGCTTTT	
Chicken_INV_7	Invariant	Chicken	ENSGALG00000032974	ENSGALG32974_128322.1	11-110	CACTTCTCTGCGTTTTCTTTATATTGCTATCCTTAGGTTATGGGATGCTGCAAATCAGTAAACAGGGCACATGCTGTGCATTTTTCTTCAAAAGGGAAAA	
Chicken_INV_8	Invariant	Chicken	ENSGALG00000040666	ENSGALG40666_19677.1	47-146	ACAGTCTGTTCTCTCTGCTGACATTCAGGTAAACCAAAGATAAAGCAGATGTGACAAAGATTACTGGTGCGGTAGGAAACAATGTGTTATCATGAGACTT	
Chicken_INV_9	Invariant	Chicken	ENSGALG00000003010	ENSGALG03010_25416.1	1-100	AGCTGAATGGAGAGCAGTTCTCCTGTTCATCCCAATAACTGGCACTGCTCATTGGGATACAGGGCATGGATGGCAGAACGGTGCTGCGGGTTATGTGGCA	
Chicken_INV_10	Invariant	Chicken	ENSGALG00000042458	ENSGALG42458_33255.1	55-154	TTGTTATGTGGTCATCATGCCAAACTTTATAATAACGCAAAGTTTAGAAGGTCTGTGGTTTGGTTTTAATGTGTTTCTTGGCGACATAGACCCTGTCACC	
HINTW_chicken_1	W-linked	Chicken	ENSGALG00000035489	ENSGALG035489_17968.1	4-103	TTTCTGTACACGAAACGGTGCCAGTAAGTGGTATGTAATTTAGTAGGCTCTGTCCCGTCGCCTCGTTTCCTCTCGATGTTGCAAAGAGGCACAGTAGGCT	
HINTW_chicken_2	W-linked	Chicken	ENSGALG00000035489	ENSGALG035489_8807.1	89-188	CACCTTTCGGGTAGTTGTAAAGAGGCGATAAGGTCTCTCTCCCCTGAGCCTCCCCTCCTCCAGACGAAACAATCCCAGTTCCCTCAACTGCTACCCATAG	Removed from analysis as binds to Z-linked ortholog
HINTW_chicken_3	W-linked	Chicken	ENSGALG00000035489	ENSGALG035489_4864.1	135-234	ATTCTCCTTGGCCCTCCTCTTGCTCTTATTAATATGTTCGTAAAAACATAGTTTTAATCATCTCAAACAATGGCGGCCATATTAAGGTCGTGCTGGGCTT	Removed from analysis as binds to Z-linked ortholog
Duck_INV_1	Invariant	Duck	ENSAPLG00000002841	LG002841_61305.1	10-109	TCAGGGATGCTCTTTTTCCTCCCCAATGTTTTGCTTTTTTTCTCTTCTCTGTCTACAATTTAGTGCTAGTTTAACGCCAACTTAGTAACTCTACCTGCCG	
Duck_INV_2	Invariant	Duck	ENSAPLG00000012467	ENSAPLG012467_23246.1	1-100	AGCTAACCAGTCCTTAAATTCGGGAAGAAACATTTGTTAATATAAAAACATGAACTGTACAGAAACATAAATTAACCGCTGCTTAGTCTGCAGGGAAGTG	
Duck_INV_3	Invariant	Duck	ENSAPLG00000013057	ENSAPLG013057_1135.1	57-156	GATGAAGGGATGTGGAGAAGCAGAGGGAAGATAACTTTCAAAGAGTGAAACCCTTCTGTTGTTGCATAGTGTTACTTAATTTGGCTCCACCATGTAAAAC	
Duck_INV_4	Invariant	Duck	ENSAPLG00000013367	ENSAPLG013367_22283.1	151-250	ACAGACAAGAGTCCTGAAAACCCTACTGCTTAAACCTTCAAATTATTTTAACAAACAAGATAGGGATAAAAAGATTCATTTATTTCAAGTCCTCTGTTTT	
Duck_INV_5	Invariant	Duck	ENSAPLG00000003053	ENSAPLG003053_15739.1	37-136	GAAATTGCATAGGGGAAATATCAGCGTGGCTTGGCTTTGAAAGAAGATTATCTCAGTATGTAAGCAAGGGGAAAAAAATGGATTATGACTTTGAGTGTGC	
Duck_INV_6	Invariant	Duck	ENSAPLG00000007178	ENSAPLG007178_5323.1	1-100	AGCTTCATATCTGAAGGTGTTGCACATAACTGTTGAAGGAATGAGTTGGACATGTGACTGACCAACTTCTCTTTGATTCACATTTGCGGTATTTTTGAC	
Duck_INV_7	Invariant	Duck	ENSAPLG00000008931	ENSAPLG008931_1712.1	92-191	CAGTTCCTGAATATCCTTGTGTTGTAAGGGCCTCTGTGGATTGTTAAAATTTCTGTTTGTTGTTCTGCTGCTTATTTTAGCAGAAAAGGATGAAAGCT	
Duck_INV_8	Invariant	Duck	ENSAPLG00000009853	ENSAPLG009853_3346.1	45-144	CGCTCTGTCTCTAAAGTTCCGATACAACATTTTTCAATAATAACTTGTATTTACTGAAACTACTGTATGCTTGTTTTCCTTAAAACGCAGCGGCTTTTTA	
Duck_INV_9	Invariant	Duck	ENSAPLG00000012716	ENSAPLG012716_40807.1	4-103	TCATGCCTGTGACTTGAATAAATCACGTAAGTTGCATGTTAAACAGAGGAATGGTCTGTCAGGAGCCTCATCAAATTCAACAAGGAAAAATGTCAAGTCT	
Duck_INV_10	Invariant	Duck	ENSAPLG00000015973	ENSAPLG015973_75975.1	21-120	AAAAAATATTCTTTCTAATCCTTTGACATAAGAACGCTCTTCAGGACAGCCAGGAGCAGAGAACAGTTTGTAGAATTTCCATCTTTGGAGATGCTCAGAG	
ATP5A1_1	W-linked	Duck	ENSAPLG00000009007	ENSAPLG009007_1018.1	46-145	TGCTCCTCAATAGCCATGGGAACTGCATGAAAGAAAACATTCATACTATTAGCAACTCTTGTGTCAGGATACATCAGTATCAGTCTCTCTGAAGATCAGC	Removed from analysis as binds to Z-linked ortholog
ATP5A1_2	W-linked	Duck	ENSAPLG00000009007	ENSAPLG009007_3183.1	232-331	ATGTTTTTAGAAGCAATTCAACAGTTTCAGGGCATCATACTACTTTTCTAATCCAATGAAGTAAAAGTGTCCCATCCCTGAAAATGGCTGTAAAAACTTG	
ATP5A1_3	W-linked	Duck	ENSAPLG00000009007	ENSAPLG009007_5689.1	56-155	TTAAGGACACAAGGTCAAACTCAAGCAGGCAAGAGAAAGCCATCCTTTCCCTAATGTTGCCAACAAGCAGACAGGAAATTGGATTTGAGTAATTTTTCAG	
BTF3_1	W-linked	Duck	ENSAPLG00000004652	ENSAPLG004652_492.1	106-205	ATCTGAAAAATAAATAAAGACTTTGAACATATGTATGCTAGCAAATGTTTAAGTTACGGTTTGAGTTTTTTAGCAAGGACTCATTAATAATAGTTTATAT	
BTF3_2	W-linked	Duck	ENSAPLG00000004652	ENSAPLG004652_3648.1	123-222	TATGTGGCTCTGAGGATTAGAGTTTGTGAAGAACTACTTAAATACTAATTTTATTTTACTTTATGCAAAGGGTACTGCCCGCAGAAAGAAGAAGGTTGTC	
BTF3_3	W-linked	Duck	ENSAPLG00000004652	ENSAPLG004652_6747.1	174-273	TTACAGAATTTGAACGGGTTACGTATCTGTTTTCAGACACTGTGAGACTGGGTGAACCATTGGTTTGAGGTAGCATTTGTCCTGTGGAAGAGTAATAACT	
CHD_1	W-linked	Duck	ENSAPLG00000005191	ENSAPLG005191_982.1	45-144	TATAAAAGAAGAAAAATATTCTCATCTGTATATTCAGACGTGTTGCATGGAGCGTGTGCATTTCACGTATTAAATTACTTTCAAGACAATGATTCATGAA	
CHD_2	W-linked	Duck	ENSAPLG00000005191	ENSAPLG005191_5301.1	19-118	TTTCCAGGTATACGAAGGACAAGAAAACCATCAGGAGGAGCCAGCATGGATTCACCAAGAGGAAGTCATGCTTGACCAATCTGATAAGTTTCAATAATGA	
CHD_3	W-linked	Duck	ENSAPLG00000005191	ENSAPLG005191_10058.1	152-251	CAATCATCATAAATACTCCAATGTTTCTGACTAATCCGTTATTACTACTGATAATGATTCTTCAGGTAGCAACTTTTTCCAGGCTCAGGAAAAAAAATCA	
HINTW_duck_1	W-linked	Duck	HINTW_HB1	HINTW_HB1_27.1	27-126	ACCTGTAACTGGTTTGTAACTTCCTAGACTCTTTCCCGTCCCCTCTTTTCCTCTGGATGTTGCAGAGGGGCGCACAGTAGGCTATTTGGGGTTGTCCGAT	
HINTW_duck_2	W-linked	Duck	HINTW_HB1	HINTW_HB1_233.1	11-110	TTGCAGTGCAAAAAGCAGCGTTGTTCCGACGTGCGGTCTGATGCCCGTTTTCACGCTTTCCGGACAGCGGGGGTGGTGCCTTGACTATGCAGAAGAGGGC	
HNRPK_1	W-linked	Duck	ENSAPLG00000010986	ENSAPLG010986_533.1	9-108	GATCTAGAATGGGTCTTCAGTAATTAATTCATATGTAGGCTGATGAAGTAAAAACCTGTTTCTTTGAGTGTGTAGATGTTTGAAGTTAGAGTTGGGCTAA	
HNRPK_2	W-linked	Duck	ENSAPLG00000010986	ENSAPLG010986_6668.1	39-138	TGTTGTAAGAACTTGGAAGCAAGCATGCACTTCATTACTGGGAGTTTAATATTCTGGTTTGAGTCTTTCTTTAAAACTGTTTTAGTGTATTACCCTTTTT	
HNRPK_3	W-linked	Duck	ENSAPLG00000010986	ENSAPLG010986_13686.1	16-115	TAGTTTTTGGAAGGGCAGGATTTAAATAACTATCGGACTTTTCTAGTGGTAGTTCTGCCTTTCTTTTCTGTTCTTCTAACTAAAAAGATTAAAAATTCCT	
KCMF1_1	W-linked	Duck	ENSAPLG00000003026	ENSAPLG003026_195.1	126-225	ATTGTTGATGATATTACTAAGCCTAACAGTTGGACTGACGCATGCTGAATAACATCTGAAAATCATTCCGTTTGAAAACAGCCTAGGTACTACTGCACTT	
KCMF1_2	W-linked	Duck	ENSAPLG00000003026	ENSAPLG003026_3522.1	132-231	AATAAATAAAATATGGCATGAAGGCATGAATTGCTTTCTTCCAAAGTTCAAGACCGACAAATTCTGTCCATGCTAAGCATTTCCTGAAATAAATGTTAAT	
KCMF1_3	W-linked	Duck	ENSAPLG00000003026	ENSAPLG003026_7669.1	125-224	AAACCACATCACTCAAAGTTGATCACTTAACAAGGATTCTTATACTTTAAATATCATACTCTAATCCTGTATCTAGATGGCCACGAGTTTAAAGCAGTAA	
MIER3_1	W-linked	Duck	ENSAPLG00000010850	ENSAPLG010850_611.1	70-169	AGGAGAAATTACTGAAGTTGCTTTTAGAGTTGTATTATCTAATATCTCTTCTAATTTTTGTAAGTCAGAAATAAGTTGAAATTGCACATTTTGAGTACTA	
MIER3_2	W-linked	Duck	ENSAPLG00000010850	ENSAPLG010850_9002.1	114-213	GGAATTCAGAAATCATCCACTTCCTTCTACGTAATGTGAATCTGTGAAAGCCTCTTCTATACTCCCTGAAGAGGTGGCATTGGCAAACGTGTTTATATTT	
MIER3_3	W-linked	Duck	ENSAPLG00000010850	ENSAPLG010850_17762.1	371-470	TGTCTGCTGTCAAAGCCTTGGCAGTTTCTGACTTTCCCAAAAGGAATGAGTACCCCGAGTCTTTTCTGCTTCACTTCTTTTAGGTCATTAATCTAATGAG	Remvoed from analysis due to elevated COV
NIPBL_1	W-linked	Duck	ENSAPLG00000002953	ENSAPLG002953_2.1	72-171	AGTCTTTGCCTCTTTTGAACATTTAATTTTGTTAAATATACAAAAAGCACACATATCTAGTGTAATAACATTCAGTGTCAGCAGTGACTGAGAGCACAAT	Removed from analysis as binds to Z-linked ortholog
NIPBL_2	W-linked	Duck	ENSAPLG00000002953	ENSAPLG002953_1985.1	94-193	TCCAACTCCTAACTCCACACAGGACTACCTAAAAAATAAGTTTTCACTATACAGTTCTGAATGACTTCAGCAATATGAAAAATAAGTGAAAAGGTGACGG	
NIPBL_3	W-linked	Duck	ENSAPLG00000002953	ENSAPLG002953_5691.1	7-106	AGTTATGTGCCATCAAATTAGTGACTGCAGTAAGTAATTTTTAATTTGTGATTTCATGGTCCTGTATATCCGAAAGTTATTAGGACATTTACGTAGGCTG	
RASA1_1	W-linked	Duck	ENSAPLG00000005611	ENSAPLG005611_30.1	117-216	AATTTGTCTTTGAGTAAGTCTTTCCTATCAAATTCCTGAATTTCACTCAAATTCAATGCAACTTTGAAGATTCTTTACACTCTACTGTTTTTCATAAGCT	
RASA1_2	W-linked	Duck	ENSAPLG00000005611	ENSAPLG005611_2679.1	38-137	CTTGTACAGGTTATTGCTTGGTAACTTTAGGTACCAACACCACCAAAAAAAAAAGTGAATTAATGATTTTGTCATTCTTGGGTTGATTTGGGCAATAAGC	
RASA1_3	W-linked	Duck	ENSAPLG00000005611	ENSAPLG005611_3869.1	78-177	TTTACTGGAACTGGGGAGGGAAGGACTCCCATGTCTTCGTTTCTGTTTCCTCCCTTAACTAAGAGAGGCATATCAAACTGAGGACAACATATTTTTCCTC	
SMAD2_1	W-linked	Duck	ENSAPLG00000004964	ENSAPLG004964_439.1	61-160	ACCAGACATTGACACACTTCTATTACCATTCTATAACTTAAAAACACTCTGGCAACATTAAAGAGATCAAACTATCAAACACTGAACCAAACAAAGGAAA	
SMAD2_2	W-linked	Duck	ENSAPLG00000004964	ENSAPLG004964_4288.1	1-100	AGCTACACTGTAGGTGAAATAGATCTCTTTGTAGACATACTGAGACAGTATTGTGGTACAATACTACCACAGTTAGTCTGGTTTACTGCTAAAGAAAAGT	Removed from analysis as binds to Z-linked ortholog
SMAD2_3	W-linked	Duck	ENSAPLG00000004964	ENSAPLG004964_5485.1	63-162	TTCAGGCCTTCCATTGGAGTTAATGCAGAAAAATCAACAAGATTTAATTGGTATGTTTTTCAATTCATTTAATCACTTCGTGAAATCAACAACAATGCAG	
SPIN_1	W-linked	Duck	ENSAPLG00000002923	ENSAPLG002923_4640.1	192-291	CAGGACAGTGACCATCTTATTCCTAAAGAAAGATTTTTCCATTTGTGGCTCACTTTTTTCAAGCCACAGATATATTATTTTTGTGTTGCAAATCTGCTCA	
SPIN_2	W-linked	Duck	ENSAPLG00000002923	ENSAPLG002923_6138.1	316-415	CTGAAGATGCCCACTCAAACTAAATTAATCTGCCTATTTTAGTTATCCCCAAAACATTATTCTTAGAAGAACTGCTTTAGAAATCCAAATCCAGAGCCTG	
SPIN_3	W-linked	Duck	ENSAPLG00000002923	ENSAPLG002923_7162.1	240-339	TAAACAAACTGTTATAATGCAAGGTAAAGGGAGACCTTTACTTGCTATAGGCCATAGCAATAAATAATGTCTGTATTTTCAATATTTGTATTGTGGGGG	
UBAP2_1	W-linked	Duck	ENSAPLG00000016004	ENSAPLG016004_1862.1	94-193	TAAGCAGCCAATTTGATAGCAGTTGTAATCTTTTACATGCAGTAATTCTGAAAAAAATGTTTGTATGCATTTTGAGTTGCTTTCTAATGCACTTTGCCAA	Remvoed from analysis due to elevated COV
UBAP2_2	W-linked	Duck	ENSAPLG00000016004	ENSAPLG016004_11367.1	72-171	ATATATCTGGTCTGTGGTTGCTTACCACATTGATTTTGTTGTGGGGCATTGCAGTTTTAATGTTTTAAATTCCGCAAAGTGCATCGAGGAAATGACAATA	
UBAP2_3	W-linked	Duck	ENSAPLG00000016004	ENSAPLG016004_32095.1	175-274	TTTTTCTGCATCTTGCAGACTAATTATTAATTTCCCATCTGCACCAAAGGTCAACAAACAGCACAAAAAATATTCAGGATTTGGTCAATTAAACAACACC	Removed from analysis due to low binding affinity
UBE2R2_1	W-linked	Duck	ENSAPLG00000016000	ENSAPLG016000_1026.1	32-131	CATGCACCTCTGGGGCTGCAGCAGCACGTGTGGCAATGGAAGGACAACATAGATGTTTCCCAATATCCAGGCAAACTGCCAAGCACCAGCAGGATTCAGC	
UBE2R2_2	W-linked	Duck	ENSAPLG00000016000	ENSAPLG016000_4244.1	120-219	TTTTCTACTAGTTGGTATCTTAGAAGAGTTTGAATAACATGCCTTGGGAAAGGTTAAAATGTTTGAGATCGCTTTAAGAACATGTAAACCTGTGAATATG	
UBE2R2_3	W-linked	Duck	ENSAPLG00000016000	ENSAPLG016000_14516.1	35-134	CCCTGTTATATCTTGTTGTATCACACAAAACAGTGGGATAAGCCAAAAACAAAGATGTACTTGAACAGCATACAGATAACCACTAGTTGTGTAAGTATCA	
VCP_1	W-linked	Duck	ENSAPLG00000005806	ENSAPLG005806_193.1	183-282	AAACTAAATCTGTCTCTCAGAATTCGCATACTCTTATGCTGAGGACACAAAGGTGGAAGTAAACAAAAATGCCTCATGGTATGTTGGTTAGGGGAAGTAT	
VCP_2	W-linked	Duck	ENSAPLG00000005806	ENSAPLG005806_1465.1	113-212	ATGCAAGAGTGCCTCTCTTTTAGGTGCAATGTCTAATTGTTTATCTGAAGCATGTGAGCACGACATGATAGCATTGTTTAATGACGTTTCTTCTAACGAT	
VCP_3	W-linked	Duck	ENSAPLG00000005806	ENSAPLG005806_2180.1	93-192	GTCTGGAAACTTCTGGTGTCTGCATCGAGATGGTTTCTTTCTGATACATCCCTGCTCTCAGTTGTCACTATAGAGGCAATGTGTCTGTGAGTTAAAAATA	
ZFR_1	W-linked	Duck	ENSAPLG00000015519	ENSAPLG015519_129.1	107-206	AAGACTGATAAAGTTTTGGGCAGAACATTGATACTTGCAAATAAACTCTTCAGTCTGTACATCACGTGTAATCATGTTTGCATTTTATGGAAGTTAAAAA	
ZFR_2	W-linked	Duck	ENSAPLG00000015519	ENSAPLG015519_2647.1	182-281	ACTTTCAAAAAGCCATTCCTTTTTACTTACCCTCTGGAATTCCCTGGTATTTGCACAATTACTAGAAATGAGTTAGTGAGGACATCCAAGAATTTATTTA	Removed from analysis as binds to Z-linked ortholog
ZFR_3	W-linked	Duck	ENSAPLG00000015519	ENSAPLG015519_4067.1	147-246	CTGGAGAAGAGAGAGCGTCAGTAGGTCCTAAGAGCCTTCTAATGTCTGTGCCTTAGAAAGACGATGGAGCCGGGTTCTTTGTAGTGGTGAGATCAAGATA	
ZSWIM6_1	W-linked	Duck	ENSAPLG00000013555	ENSAPLG013555_1276.1	11-110	AGCAGCAGTTCAAACTGTGTCTGACCCTACGCTGGAGTGGATGAGGAGTACTTGCACAATTTATGTAGCCACTTCTGCTACAAAGAATAGAATAGAATAG	
ZSWIM6_2	W-linked	Duck	ENSAPLG00000013555	ENSAPLG013555_6473.1	126-225	CTGGGTTGGGGCAATCCCAGAGGTGAGTACAGACTAGGAGAAGAACTCATTGAGAGCAGCCCTGTGGAGAAGGACTTGATGGATGAAAAGTTCGACATGA	Removed from analysis as binds to Z-linked ortholog
ZSWIM6_3	W-linked	Duck	ENSAPLG00000013555	ENSAPLG013555_9296.1	266-365	AACAAAAAAGATTATTCACGCTGACTTCTTACTGGTTCACTTATACGTTACATTACCACTATGATCATTTGCAATGGAGAGTTAAAGACACCTCATTGAC	
CHD1_ENSAPLG00000002506_1	W-linked	Duck	ENSAPLG00000002506	ENSAPLG02506_1703.1	55-154	CCCTCCAGCCATGCAACTTTTTTTTTCTCATGCTGCTCTTGAGAAATATAATAATGGAATTTCTGTAACCTTCTACCTGGGTTTCCATGCAGAAAAGTAA	
CHD1_ENSAPLG00000002506_2	W-linked	Duck	ENSAPLG00000002506	ENSAPLG02506_2806.1	32-131	GAGATCAATACTAAATAAAGCATCTCTGTGCAAATGCATGACCGGCAAAGCACCAACAGTCCATGTGATAGCAGAAAGTGATCTGCATAGTCTCTTCAAG	
CHD1_ENSAPLG00000002506_3	W-linked	Duck	ENSAPLG00000003022	ENSAPLG03022_7188.1	45-144	GGTATTGTCTTTAGGAGACATTCAGACAATTGCTGTCTCTAGGGTCCAGTGTATGTGGAAAGAGGAAAGGAACTGGTAAACAACTGGCTGACTTATTTAA	
KCMF1_ENSAPLG00000003106_1	W-linked	Duck	ENSAPLG00000003106	ENSAPLG03106_174.1	22-121	TTTGGTTGTTGTTGTTGGGAGATGATAGCGACAAATCTGTATCATTATAACAATGTAAAATTATTTTTCCTGTATTTTTTTTATTCACAGGTGTCAGC	
KCMF1_ENSAPLG00000003106_2	W-linked	Duck	ENSAPLG00000003106	ENSAPLG03106_31.1	1-100	AGCTTAACCATCTATCAGTTTAGGTGACTGACTGTGTTTTTTAATTTATTTTACTTTAAGTTTAACTTTCAATTTGGGTGACTGTTGCCTTTTTAAAAAT	
NIPBL_ENSAPLG00000003022_1	W-linked	Duck	ENSAPLG00000003022	ENSAPLG03022_10659.1	201-300	TGCATGAGTAAAACTCTTTAGATTTGGTTATTTATAATCTTCAGCACCTAAGGAAACATGTAGCAGTCTTGTTCCAGGGAACATTGTGATAGATACAAAA	
NIPBL_ENSAPLG00000003022_2	W-linked	Duck	ENSAPLG00000003022	ENSAPLG03022_15827.1	168-267	TGTTTAAAAGGACAAGTGCCCTTTAAACTCATGTTTTAAGTGTTTGCTGTTGTCTTTGGCAATTTCAGGCATGGTTTTCATGTAATTCAAATTGAATTGT	
NIPBL_ENSAPLG00000005315_1	W-linked	Duck	ENSAPLG00000005315	ENSAPLG05315_1343.1	104-203	TAATGTAATAGGATGTAATAATTTGAAGCATCTCTTCCAGTCTAAAATTGTCTTTAATTTATAGTTCACAATGTTTTGCACAGTGAATAGTACACAAACA	
NIPBL_ENSAPLG00000005315_2	W-linked	Duck	ENSAPLG00000005315	ENSAPLG05315_1805.1	1-100	AGCTGACATAAGCATACACATTTGGTAAAATATTCTGTTCATGTATGAACATTTCAGTATTCTTGAAAGTAGTTTAAGTTGGAATACCTTGCTGCAGTTG	
NIPBL_ENSAPLG00000005315_3	W-linked	Duck	ENSAPLG00000005315	ENSAPLG05315_3429.1	56-155	GCAAGAAAAGTGTGCTGTATTTCATTTCATATGGTGGTATATAAGAACCCCAGTCATGGTATAATATCTGTTGTTACGGCAATACACAACCCTGTAAGAA	
NIPBL_ENSAPLG00000010290_1	W-linked	Duck	ENSAPLG00000010290	ENSAPLG10290_1832.1	47-146	CTGGGACCATATTATATTCAAGTTGTATAAAACACTTGTTCAGTTAACAGAACAAATTTCTATTACGTATAACATTTTCCAGCAAATACTAGTAGCAGTG	
NIPBL_ENSAPLG00000010290_2	W-linked	Duck	ENSAPLG00000010290	ENSAPLG10290_5371.1	19-118	AGAGATACTTTTGTAATGCAAAATCCTTGTGTACTTGTAGTAATTATTATAAAAACAGTATGTATCCAGTGTAAATTAATGCACACTTACTACTAAATTG	
NIPBL_ENSAPLG00000010290_3	W-linked	Duck	ENSAPLG00000010290	ENSAPLG10290_10294.1	271-370	TATATTTTAATGATTATGACTTTGGGCAGGTTTTCAGAGCAGTCTGGGAGATAGACTGCTAGAGCGTTTGTTTTAGTTCTACATATCTCCACAAAAATCT	
NIPBL_ENSAPLG00000010560_1	W-linked	Duck	ENSAPLG00000010560	ENSAPLG10560_169.1	100-199	TCTCTTGTCTATATAACCCTGCCAATCATATGGTCAAGACTTCTTATTTTATATACTTCCTTCTTATGCATGTAGACCTAAGAATGCCCTCTGTAAGTAT	
NIPBL_ENSAPLG00000010560_2	W-linked	Duck	ENSAPLG00000010560	ENSAPLG10560_1533.1	51-150	TGAGAGGGATGTTTGTGAGAATCTGAAAATCTGAATGTGCAGGTTGTTTGTTAGGCTTGTAGCAGATTATCAGTGTATAAGAGCCAATTATTTCAGTCAA	
NIPBL_ENSAPLG00000010560_3	W-linked	Duck	ENSAPLG00000010560	ENSAPLG10560_1734.1	91-190	AGACAAACTATATATCCAGCATTATACGAAAATAAACTTTTTGGTGAGACCCATGTAAGACCCATGTCAATTCTGATTAGGTATTGAACTTTAATATTAA	
RASA1_ENSAPLG00000010611_1	W-linked	Duck	ENSAPLG00000010611	ENSAPLG10611_169.1	59-158	CTTTCCTTACATTGGCTTGGGGGAGCAAGTGGCTACAGGTTCAATTACTGGTAGACAGTGGAACACTTTTGGGAGGTAGGAGACCAAGTTTTCCTCCTTC	
RASA1_ENSAPLG00000010611_2	W-linked	Duck	ENSAPLG00000010611	ENSAPLG10611_5446.1	63-162	CACACCCTCTCCACATGCACACTTTTGCACTACATATTCCTTAGAGTGGCATTTTTCTATTGGTACAGCATTTTGAAACACCACTTTTATTTGTACTCAT	
RASA1_ENSAPLG00000010611_3	W-linked	Duck	ENSAPLG00000010611	ENSAPLG10611_10046.1	35-134	ATATTTTAAACTATCTAATTACAGGTCTGGTAGGCTAATGTTGATGGTTCTCAAGCAAGGTGTTTGTTTTTCTCTAGTTGCTAATGTAGGGTTGTACTCT	
RASA1_ENSAPLG00000011371_1	W-linked	Duck	ENSAPLG00000011371	ENSAPLG11371_361.1	56-155	GATGGTTTTGAGTCTATTCCTCAGTCACTGCAGTTTACAGGGAATACTTGAATGTTGAAGTTGTAGTAAATCTGTGTGCTTCCTTTTTAAGAAAAATAAC	
RASA1_ENSAPLG00000011371_2	W-linked	Duck	ENSAPLG00000011371	ENSAPLG11371_1646.1	73-172	GAGTCCATGAAAACTAAAATTCTCTGTGGATGTAGTAGTATGCTTTTAGTGAGTTGCAGAGTGATATAAAATATTAGGAAGGAAAACTTTTCATCTTGTC	
RASA1_ENSAPLG00000011371_3	W-linked	Duck	ENSAPLG00000011371	ENSAPLG11371_2437.1	22-121	TGATCAAGTTGTTATTTTTAGTATGTCTACTAGACTAACTGTAGAAAAGTGACTAATTCTTCGAGGTATTTCTGTATAATTAAATGCAGTTATTTGTTGT	
UBAP2_ENSAPLG00000016155_1	W-linked	Duck	ENSAPLG00000016155	ENSAPLG16155_3889.1	1-100	AGCTGTACCAGAACCATATGTTATACTTTATTGGCTTGATGACTTTCTGAAACTGAACTTTTTGCAGAAGAAAACATGAAACGGTCAGAGATGGGAGTGT	
UBAP2_ENSAPLG00000016155_2	W-linked	Duck	ENSAPLG00000016155	ENSAPLG16155_5536.1	62-161	TGAAGATGTCAAACAAGACCAGAACAGCACCAACCCCTGGGGAACACCGCCAGTCATGGGTCTCCCGACAGACTTTGTGCCGCCGATCACCACCCTCTGA	Removed from analysis as binds to Z-linked ortholog
UBAP2_ENSAPLG00000016155_3	W-linked	Duck	ENSAPLG00000016155	ENSAPLG16155_14404.1	61-160	AATGTTCCAAAAAGATGGGATTAAAATACTTTGTCTTGGCAGTGATGGGAGGGAAATGGAGGAACTGTGGTGAGTTTCAGTTTTTCATGACCCACTGTGG	
ZSWIM6_ENSAPLG00000014338_1	W-linked	Duck	ENSAPLG00000014338	ENSAPLG14338_795.1	86-185	ACAAGAATTTGACCCTGTGTTGATGGGTACAGTTCTTTAAGTCACTGAGGAACATACCATCTTGGGCTCAGTTTTTAGATTCTGTTTGCTGATGTAAAGC	
ZSWIM6_ENSAPLG00000014338_2	W-linked	Duck	ENSAPLG00000014338	ENSAPLG14338_2691.1	75-174	ATGTATTGAGCAGAAGTGAAATACTTCTTTGGCTACATGGGTTATGGTATAAATTAACTTCAGTTCCTCCTTAATCAAGACATGGATACGTTTTATTTGA	
ZSWIM6_ENSAPLG00000014338_3	W-linked	Duck	ENSAPLG00000014338	ENSAPLG14338_4397.1	33-132	AAGTCATCACTATAGGCATTTAAATAATATCTGGATATATCTGGAATTGTATATATATCACTGTAGAATAAACAGACAGTCTGTATTGTTGAAGTCAATT	

Table S6.2.3. Estimated copy number of W-linked genes in duck.

Ensembl ID	Gene name	Mallard																Caguya																Aylesbury												Indian Runner												Khaki Campbell											
		1				2			1				2				3				1				2				3				1				2				3				1				2				3				1				2				3			
		CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower
ENSAPLG09007	ATP5A1W	0.84	0.97	0.74	0.80	0.99	0.68	0.86	1.04	0.75	0.78	0.96	0.67	0.73	0.89	0.63	0.87	1.22	0.72	0.86	1.21	0.70	0.78	0.90	0.69	0.87	1.02	0.78	0.79	0.97	0.68	0.88	1.04	0.76	0.83	1.01	0.72	0.83	0.98	0.72	0.84	1.02	0.73
ENSAPLG04652	BTF3W	0.68	0.79	0.60	0.62	0.77	0.53	0.65	0.78	0.56	0.58	0.71	0.50	0.59	0.72	0.51	0.66	0.92	0.54	0.65	0.92	0.53	0.60	0.70	0.53	0.70	0.82	0.62	0.58	0.71	0.50	0.67	0.79	0.58	0.65	0.79	0.56	0.64	0.76	0.56	0.65	0.79	0.56
ENSAPLG05191, ENSAPLG02506	CHD1W	0.98	1.14	0.87	0.90	1.10	0.76	1.04	1.25	0.90	0.95	1.16	0.81	0.92	1.12	0.79	1.01	1.41	0.83	1.02	1.44	0.83	0.92	1.06	0.81	0.94	1.10	0.83	0.94	1.15	0.81	1.09	1.29	0.94	1.02	1.25	0.89	0.93	1.11	0.81	1.00	1.21	0.86
ENSAPLG10986	HNRPKW	0.96	1.11	0.85	0.97	1.20	0.83	1.04	1.25	0.90	0.97	1.19	0.83	0.98	1.19	0.84	1.14	1.59	0.94	1.11	1.57	0.91	1.03	1.19	0.91	0.99	1.17	0.88	1.01	1.24	0.87	1.04	1.23	0.90	1.05	1.29	0.92	1.05	1.24	0.91	1.05	1.28	0.91
ENSAPLG03026, ENSAPLG03106	KCMF1W	2.57	2.99	2.28	2.29	2.82	1.94	2.79	3.37	2.42	2.52	3.09	2.16	2.47	3.02	2.13	2.69	3.74	2.20	2.81	3.96	2.29	2.44	2.83	2.16	2.46	2.90	2.19	2.55	3.12	2.18	2.89	3.42	2.51	2.75	3.36	2.39	2.51	2.97	2.18	2.63	3.19	2.27
ENSAPLG10850	MIER3W	0.62	0.72	0.55	0.61	0.76	0.52	0.67	0.80	0.58	0.58	0.71	0.50	0.60	0.73	0.51	0.66	0.92	0.54	0.67	0.94	0.54	0.61	0.71	0.54	0.64	0.76	0.57	0.58	0.71	0.50	0.65	0.76	0.56	0.65	0.79	0.56	0.63	0.74	0.55	0.62	0.75	0.54
ENSAPLG02953, ENSAPLG03022, ENSAPLG05315, ENSAPLG10290, ENSAPLG10560	NIPBLW	0.70	0.81	0.62	0.65	0.80	0.55	0.73	0.88	0.64	0.68	0.83	0.58	0.67	0.82	0.58	0.72	1.01	0.59	0.75	1.05	0.61	0.67	0.78	0.59	0.67	0.79	0.60	0.68	0.83	0.58	0.73	0.87	0.63	0.71	0.86	0.61	0.68	0.81	0.59	0.70	0.85	0.61
ENSAPLG05611, ENSAPLG10611, ENSAPLG11371	RASA1W	0.67	0.78	0.60	0.61	0.75	0.52	0.74	0.89	0.64	0.66	0.81	0.57	0.66	0.81	0.57	0.72	1.00	0.59	0.73	1.03	0.60	0.64	0.74	0.57	0.65	0.76	0.58	0.67	0.82	0.57	0.75	0.89	0.65	0.72	0.88	0.62	0.67	0.79	0.58	0.70	0.85	0.61
ENSAPLG00000004964	SMAD2W	0.69	0.81	0.62	0.69	0.85	0.59	0.75	0.91	0.65	0.71	0.86	0.60	0.70	0.85	0.60	0.75	1.05	0.62	0.76	1.07	0.62	0.71	0.82	0.63	0.71	0.84	0.63	0.69	0.84	0.59	0.72	0.85	0.63	0.74	0.91	0.65	0.68	0.81	0.59	0.72	0.87	0.62
ENSAPLG00000002923	SPIN1W	0.67	0.78	0.59	0.59	0.73	0.50	0.65	0.78	0.56	0.60	0.73	0.51	0.58	0.70	0.50	0.66	0.92	0.54	0.63	0.89	0.52	0.63	0.73	0.56	0.68	0.80	0.60	0.58	0.72	0.50	0.71	0.84	0.62	0.66	0.80	0.57	0.62	0.74	0.54	0.65	0.79	0.56
ENSAPLG16004, ENSAPLG16155	UBAP2W	0.60	0.70	0.54	0.62	0.76	0.52	0.60	0.73	0.53	0.58	0.70	0.49	0.56	0.69	0.49	0.60	0.83	0.49	0.61	0.85	0.49	0.57	0.66	0.50	0.62	0.72	0.55	0.56	0.69	0.48	0.61	0.73	0.53	0.60	0.74	0.52	0.59	0.69	0.51	0.62	0.75	0.54
ENSAPLG16000	UBE2R2W	0.76	0.89	0.68	0.75	0.93	0.64	0.77	0.92	0.67	0.73	0.90	0.63	0.71	0.86	0.61	0.78	1.08	0.64	0.78	1.10	0.64	0.72	0.83	0.63	0.79	0.93	0.70	0.75	0.92	0.64	0.79	0.94	0.69	0.76	0.93	0.66	0.76	0.90	0.66	0.77	0.93	0.66
ENSAPLG05806	VCPW	0.90	1.05	0.80	0.91	1.13	0.78	0.88	1.06	0.76	0.82	1.00	0.70	0.84	1.02	0.72	0.94	1.31	0.77	0.88	1.25	0.72	0.87	1.00	0.77	0.93	1.10	0.83	0.87	1.06	0.74	0.94	1.12	0.82	0.90	1.10	0.78	0.89	1.06	0.78	0.89	1.08	0.77
ENSAPLG15519	ZFRW	0.71	0.82	0.63	0.64	0.78	0.54	0.72	0.88	0.63	0.67	0.82	0.57	0.66	0.80	0.57	0.70	0.98	0.58	0.72	1.01	0.58	0.65	0.75	0.58	0.69	0.81	0.61	0.66	0.81	0.56	0.75	0.89	0.65	0.70	0.86	0.61	0.65	0.77	0.57	0.68	0.82	0.59
ENSAPLG13555, ENSAPLG14338	ZSWIM6W	0.82	0.95	0.72	0.73	0.90	0.62	0.86	1.04	0.74	0.77	0.94	0.66	0.77	0.93	0.66	0.81	1.13	0.67	0.83	1.17	0.68	0.75	0.87	0.67	0.77	0.91	0.69	0.75	0.92	0.65	0.96	1.14	0.83	0.82	1.01	0.71	0.76	0.89	0.66	0.81	0.98	0.70
-	HINTW	18.49	21.53	16.40	17.57	21.64	14.90	17.96	21.70	15.60	15.61	19.13	13.39	15.49	18.88	13.33	17.21	23.98	14.12	16.79	23.71	13.71	15.69	18.16	13.91	17.29	20.36	15.41	16.49	20.19	14.11	17.89	21.17	15.50	16.97	20.75	14.74	16.98	20.07	14.76	16.54	20.09	14.32

Table shows copy number as well as upper and lower estimates in brackets.

Copy number was estimated relative to invariant gene count data.

Upper and lower estimates were calculated using bootstrapping of count data across invariant genes.

Raw count data for all genes was higher than background noise in every individual suggesting minimum single copy.

Table S6.2.4. Estimated copy number of W-linked genes in duck where each annotated gene is analysed separately.

		Mallard						Caguya									Aylesbury									Indian Runner									Khaki Campbell								
Ensembl ID	Gene name	1			2			1			2			3			1			2			3			1			2			3			1			2			3		
		CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower	CN	Upper	Lower
ENSAPLG00000009007	ATP5A1W	0.84	0.97	0.74	0.80	0.99	0.68	0.86	1.04	0.75	0.78	0.96	0.67	0.73	0.89	0.63	0.87	1.22	0.72	0.86	1.21	0.70	0.78	0.90	0.69	0.87	1.02	0.78	0.79	0.97	0.68	0.88	1.04	0.76	0.83	1.01	0.72	0.83	0.98	0.72	0.84	1.02	0.73
ENSAPLG00000004652	BTF3W	0.68	0.79	0.60	0.62	0.77	0.53	0.65	0.78	0.56	0.58	0.71	0.50	0.59	0.72	0.51	0.66	0.92	0.54	0.65	0.92	0.53	0.60	0.70	0.53	0.70	0.82	0.62	0.58	0.71	0.50	0.67	0.79	0.58	0.65	0.79	0.56	0.64	0.76	0.56	0.65	0.79	0.56
ENSAPLG00000005191	CHD1W	1.17	1.37	1.04	1.06	1.31	0.90	1.23	1.48	1.07	1.10	1.35	0.95	1.08	1.32	0.93	1.18	1.64	0.97	1.19	1.67	0.97	1.06	1.23	0.94	1.11	1.31	0.99	1.08	1.33	0.93	1.29	1.53	1.12	1.20	1.46	1.04	1.09	1.29	0.95	1.17	1.42	1.01
ENSAPLG00000002506	CHD1W	0.78	0.91	0.69	0.73	0.90	0.62	0.85	1.03	0.74	0.80	0.98	0.68	0.76	0.93	0.66	0.84	1.17	0.69	0.86	1.21	0.70	0.77	0.89	0.68	0.76	0.89	0.68	0.80	0.98	0.68	0.88	1.04	0.76	0.84	1.03	0.73	0.78	0.92	0.68	0.83	1.00	0.72
ENSAPLG00000010986	HNRPKW	0.96	1.11	0.85	0.97	1.20	0.83	1.04	1.25	0.90	0.97	1.19	0.83	0.98	1.19	0.84	1.14	1.59	0.94	1.11	1.57	0.91	1.03	1.19	0.91	0.99	1.17	0.88	1.01	1.24	0.87	1.04	1.23	0.90	1.05	1.29	0.92	1.05	1.24	0.91	1.05	1.28	0.91
ENSAPLG00000003026	KCMF1W	0.68	0.79	0.60	0.57	0.70	0.48	0.71	0.85	0.61	0.65	0.80	0.56	0.60	0.74	0.52	0.67	0.94	0.55	0.69	0.97	0.56	0.63	0.73	0.56	0.69	0.81	0.61	0.63	0.77	0.54	0.79	0.93	0.68	0.71	0.87	0.62	0.64	0.76	0.56	0.68	0.82	0.58
ENSAPLG00000003106	KCMF1W	1.89	2.20	1.68	1.72	2.11	1.46	2.08	2.52	1.81	1.87	2.29	1.60	1.87	2.28	1.61	2.01	2.81	1.65	2.12	3.00	1.73	1.81	2.09	1.60	1.77	2.09	1.58	1.92	2.34	1.64	2.10	2.49	1.82	2.04	2.49	1.77	1.87	2.21	1.62	1.95	2.37	1.69
ENSAPLG00000010850	MIER3W	0.62	0.72	0.55	0.61	0.76	0.52	0.67	0.80	0.58	0.58	0.71	0.50	0.60	0.73	0.51	0.66	0.92	0.54	0.67	0.94	0.54	0.61	0.71	0.54	0.64	0.76	0.57	0.58	0.71	0.50	0.65	0.76	0.56	0.65	0.79	0.56	0.63	0.74	0.55	0.62	0.75	0.54
ENSAPLG00000002953	NIPBLW	0.61	0.71	0.54	0.60	0.74	0.51	0.66	0.80	0.58	0.61	0.75	0.52	0.61	0.75	0.53	0.66	0.92	0.54	0.68	0.96	0.56	0.61	0.70	0.54	0.58	0.68	0.52	0.62	0.76	0.53	0.61	0.73	0.53	0.63	0.77	0.54	0.60	0.71	0.52	0.62	0.75	0.54
ENSAPLG00000003022	NIPBLW	0.64	0.75	0.57	0.57	0.70	0.48	0.63	0.76	0.55	0.61	0.74	0.52	0.59	0.72	0.51	0.65	0.91	0.53	0.65	0.92	0.53	0.61	0.71	0.54	0.62	0.73	0.56	0.57	0.70	0.49	0.68	0.80	0.59	0.62	0.76	0.54	0.61	0.72	0.53	0.61	0.74	0.52
ENSAPLG00000005315	NIPBLW	0.80	0.93	0.71	0.76	0.93	0.64	0.85	1.03	0.74	0.79	0.97	0.68	0.79	0.97	0.68	0.85	1.18	0.70	0.87	1.22	0.71	0.77	0.89	0.69	0.76	0.89	0.67	0.80	0.98	0.68	0.85	1.00	0.73	0.82	1.00	0.71	0.80	0.95	0.70	0.83	1.01	0.72
ENSAPLG00000010290	NIPBLW	0.59	0.69	0.52	0.53	0.66	0.45	0.60	0.72	0.52	0.55	0.67	0.47	0.55	0.67	0.47	0.57	0.80	0.47	0.60	0.85	0.49	0.54	0.63	0.48	0.54	0.63	0.48	0.55	0.67	0.47	0.61	0.72	0.52	0.58	0.71	0.51	0.55	0.65	0.48	0.58	0.70	0.50
ENSAPLG00000010560	NIPBLW	0.85	0.99	0.75	0.78	0.96	0.66	0.91	1.10	0.79	0.85	1.04	0.73	0.81	0.99	0.70	0.89	1.24	0.73	0.92	1.30	0.75	0.82	0.95	0.73	0.84	0.99	0.75	0.85	1.04	0.73	0.92	1.09	0.80	0.88	1.07	0.76	0.85	1.00	0.74	0.87	1.05	0.75
ENSAPLG00000005611	RASA1W	0.64	0.75	0.57	0.57	0.70	0.48	0.70	0.84	0.60	0.62	0.76	0.53	0.63	0.77	0.54	0.68	0.95	0.56	0.70	0.99	0.57	0.60	0.70	0.53	0.59	0.69	0.52	0.63	0.77	0.54	0.69	0.81	0.60	0.64	0.78	0.56	0.60	0.71	0.52	0.64	0.78	0.55
ENSAPLG00000010611	RASA1W	0.63	0.73	0.56	0.56	0.68	0.47	0.68	0.82	0.59	0.62	0.76	0.53	0.62	0.75	0.53	0.68	0.94	0.55	0.69	0.97	0.56	0.60	0.69	0.53	0.61	0.72	0.55	0.63	0.77	0.54	0.73	0.87	0.64	0.68	0.83	0.59	0.62	0.73	0.54	0.66	0.80	0.57
ENSAPLG00000011371	RASA1W	0.75	0.87	0.66	0.70	0.86	0.59	0.84	1.01	0.73	0.74	0.91	0.64	0.74	0.90	0.63	0.80	1.12	0.66	0.80	1.13	0.65	0.72	0.83	0.64	0.74	0.88	0.66	0.75	0.92	0.64	0.83	0.98	0.72	0.83	1.02	0.72	0.79	0.93	0.69	0.80	0.97	0.69
ENSAPLG00000004964	SMAD2W	0.69	0.81	0.62	0.69	0.85	0.59	0.75	0.91	0.65	0.71	0.86	0.60	0.70	0.85	0.60	0.75	1.05	0.62	0.76	1.07	0.62	0.71	0.82	0.63	0.71	0.84	0.63	0.69	0.84	0.59	0.72	0.85	0.63	0.74	0.91	0.65	0.68	0.81	0.59	0.72	0.87	0.62
ENSAPLG00000002923	SPIN1W	0.67	0.78	0.59	0.59	0.73	0.50	0.65	0.78	0.56	0.60	0.73	0.51	0.58	0.70	0.50	0.66	0.92	0.54	0.63	0.89	0.52	0.63	0.73	0.56	0.68	0.80	0.60	0.58	0.72	0.50	0.71	0.84	0.62	0.66	0.80	0.57	0.62	0.74	0.54	0.65	0.79	0.56
ENSAPLG00000016004	UBAP2W	0.89	1.04	0.79	0.88	1.09	0.75	0.88	1.06	0.76	0.84	1.03	0.72	0.81	0.99	0.70	0.86	1.20	0.71	0.87	1.23	0.71	0.84	0.97	0.74	0.92	1.08	0.82	0.81	0.99	0.69	0.90	1.07	0.78	0.88	1.08	0.77	0.86	1.02	0.75	0.90	1.09	0.78
ENSAPLG00000016155	UBAP2W	0.31	0.37	0.28	0.35	0.43	0.30	0.33	0.40	0.29	0.31	0.38	0.27	0.32	0.39	0.27	0.33	0.46	0.27	0.34	0.48	0.28	0.30	0.34	0.26	0.31	0.37	0.28	0.31	0.38	0.27	0.33	0.39	0.28	0.32	0.39	0.28	0.31	0.36	0.27	0.34	0.41	0.29
ENSAPLG00000016000	UBE2R2W	0.76	0.89	0.68	0.75	0.93	0.64	0.77	0.92	0.67	0.73	0.90	0.63	0.71	0.86	0.61	0.78	1.08	0.64	0.78	1.10	0.64	0.72	0.83	0.63	0.79	0.93	0.70	0.75	0.92	0.64	0.79	0.94	0.69	0.76	0.93	0.66	0.76	0.90	0.66	0.77	0.93	0.66
ENSAPLG00000005806	VCPW	0.90	1.05	0.80	0.91	1.13	0.78	0.88	1.06	0.76	0.82	1.00	0.70	0.84	1.02	0.72	0.94	1.31	0.77	0.88	1.25	0.72	0.87	1.00	0.77	0.93	1.10	0.83	0.87	1.06	0.74	0.94	1.12	0.82	0.90	1.10	0.78	0.89	1.06	0.78	0.89	1.08	0.77
ENSAPLG00000015519	ZFRW	0.71	0.82	0.63	0.64	0.78	0.54	0.72	0.88	0.63	0.67	0.82	0.57	0.66	0.80	0.57	0.70	0.98	0.58	0.72	1.01	0.58	0.65	0.75	0.58	0.69	0.81	0.61	0.66	0.81	0.56	0.75	0.89	0.65	0.70	0.86	0.61	0.65	0.77	0.57	0.68	0.82	0.59
ENSAPLG00000013555	ZSWIM6W	0.95	1.11	0.84	0.82	1.01	0.69	0.97	1.18	0.85	0.86	1.06	0.74	0.87	1.06	0.75	0.92	1.28	0.75	0.95	1.34	0.77	0.84	0.97	0.74	0.88	1.04	0.79	0.84	1.03	0.72	1.17	1.39	1.02	0.92	1.13	0.80	0.85	1.00	0.74	0.90	1.10	0.78
ENSAPLG00000014338	ZSWIM6W	0.68	0.79	0.60	0.64	0.79	0.54	0.74	0.90	0.64	0.67	0.83	0.58	0.67	0.81	0.57	0.71	0.99	0.58	0.72	1.01	0.58	0.67	0.77	0.59	0.67	0.78	0.59	0.67	0.82	0.57	0.75	0.89	0.65	0.72	0.88	0.63	0.66	0.78	0.58	0.72	0.87	0.62
-	HINTW	18.49	21.53	16.40	17.57	21.64	14.90	17.96	21.70	15.60	15.61	19.13	13.39	15.49	18.88	13.33	17.21	23.98	14.12	16.79	23.71	13.71	15.69	18.16	13.91	17.29	20.36	15.41	16.49	20.19	14.11	17.89	21.17	15.50	16.97	20.75	14.74	16.98	20.07	14.76	16.54	20.09	14.32

Table shows copy number as well as upper and lower estimates in brackets.

Copy number was estimated relative to invariant gene count data.

Upper and lower estimates were calculated using bootstrapping of count data across invariant genes.

Raw count data for all genes was higher than background noise in every individual suggesting minimum single copy.

Table S6.2.5. Coefficient of variation of count data across duck breeds.

Ensembl ID	Gene name	CV across individuals	CV across breeds
ENSAPLG09007	ATP5A1W	0.098	0.073
ENSAPLG04652	BTF3W	0.096	0.060
ENSAPLG05191, ENSAPLG02506	CHD1W	0.103	0.091
ENSAPLG10986	HNRPKW	0.105	0.106
ENSAPLG03026, ENSAPLG03106	KCMF1W	0.112	0.104
ENSAPLG10850	MIER3W	0.102	0.083
ENSAPLG02953, ENSAPLG03022, ENSAPLG05315, ENSAPLG10290, ENSAPLG10560	NIPBLW	0.097	0.090
ENSAPLG05611, ENSAPLG10611, ENSAPLG11371	RASA1W	0.112	0.105
ENSAPLG04964	SMAD2W	0.100	0.093
ENSAPLG02923	SPIN1W	0.096	0.075
ENSAPLG16004, ENSAPLG16155	UBAP2W	0.084	0.064
ENSAPLG16000	UBE2R2W	0.085	0.071
ENSAPLG05806	VCPW	0.078	0.060
ENSAPLG15519	ZFRW	0.096	0.083
ENSAPLG13555, ENSAPLG14338	ZSWIM6W	0.105	0.088
-	HINTW	0.080	0.043

Table S6.2.6. Coefficient of variation of count data across duck breeds where each annotated gene is analysed separately.

Ensembl ID	Gene name	CV across individuals	CV across breeds
ENSAPLG00000009007	ATP5A1W	0.098	0.073
ENSAPLG00000004652	BTF3W	0.096	0.060
ENSAPLG00000005191	CHD1W	0.102	0.084
ENSAPLG00000002506	CHD1W	0.106	0.102
ENSAPLG00000010986	HNRPKW	0.105	0.106
ENSAPLG00000003026	KCMF1W	0.115	0.099
ENSAPLG00000003106	KCMF1W	0.115	0.107
ENSAPLG00000010850	MIER3W	0.102	0.083
ENSAPLG00000002953	NIPBLW	0.102	0.094
ENSAPLG00000003022	NIPBLW	0.093	0.082
ENSAPLG00000005315	NIPBLW	0.101	0.097
ENSAPLG00000010290	NIPBLW	0.093	0.082
ENSAPLG00000010560	NIPBLW	0.103	0.097
ENSAPLG00000005611	RASA1W	0.114	0.103
ENSAPLG00000010611	RASA1W	0.114	0.108
ENSAPLG00000011371	RASA1W	0.113	0.107
ENSAPLG00000004964	SMAD2W	0.100	0.093
ENSAPLG00000002923	SPIN1W	0.096	0.075
ENSAPLG00000016004	UBAP2W	0.084	0.064
ENSAPLG00000016155	UBAP2W	0.091	0.067
ENSAPLG00000016000	UBE2R2W	0.085	0.071
ENSAPLG00000005806	VCPW	0.078	0.060
ENSAPLG00000015519	ZFRW	0.096	0.083
ENSAPLG00000013555	ZSWIM6W	0.113	0.084
ENSAPLG00000014338	ZSWIM6W	0.103	0.096
-	HINTW	0.080	0.043

Table S6.2.7. Tukey multiple comparisons of average copy number of HINTW across duck breeds.

Breed	diff	lwr	upr	p adj
Mallard - Aylesbury	1.46	-1.17	4.10	0.40
Cayuga - Aylesbury	-0.21	-2.57	2.15	1.00
Indian Runner - Aylesbury	0.66	-1.70	3.02	0.87
Khaki Campbell - Aylesbury	0.26	-2.09	2.62	0.99
Cayuga - Mallard	-1.68	-4.32	0.96	0.28
Indian Runner - Mallard	-0.81	-3.44	1.83	0.84
Khaki Campbell - Mallard	-1.20	-3.84	1.44	0.57
Indian Runner - Cayuga	0.87	-1.49	3.23	0.73
Khaki Campbell - Cayuga	0.48	-1.88	2.84	0.96
Khaki Campbell - Indian Runner	-0.39	-2.75	1.97	0.98

Table S6.2.8. Tukey multiple comparisons of average copy number of HINTW across chicken breeds.

Breed	diff	lwr	upr	p adj
Black Sumatra -Black Minorca	-5.08	-9.37	-0.78	**0.02**
Red Jungle Fowl -Black Minorca	-9.82	-14.62	-5.02	**0.00**
Oxford Old English -Black Minorca	-4.50	-8.80	-0.20	**0.04**
White Leghorn -Black Minorca	-3.26	-7.56	1.03	0.16
Red Jungle Fowl -Black Sumatra	-4.74	-9.55	0.06	0.05
Oxford Old English -Black Sumatra	0.58	-3.72	4.87	0.99
White Leghorn -Black Sumatra	1.81	-2.48	6.11	0.63
Oxford Old English -Red Jungle Fowl	5.32	0.52	10.12	**0.03**
White Leghorn -Red Jungle Fowl	6.56	1.75	11.36	**0.01**
White Leghorn - Oxford Old English	1.24	-3.06	5.53	0.86

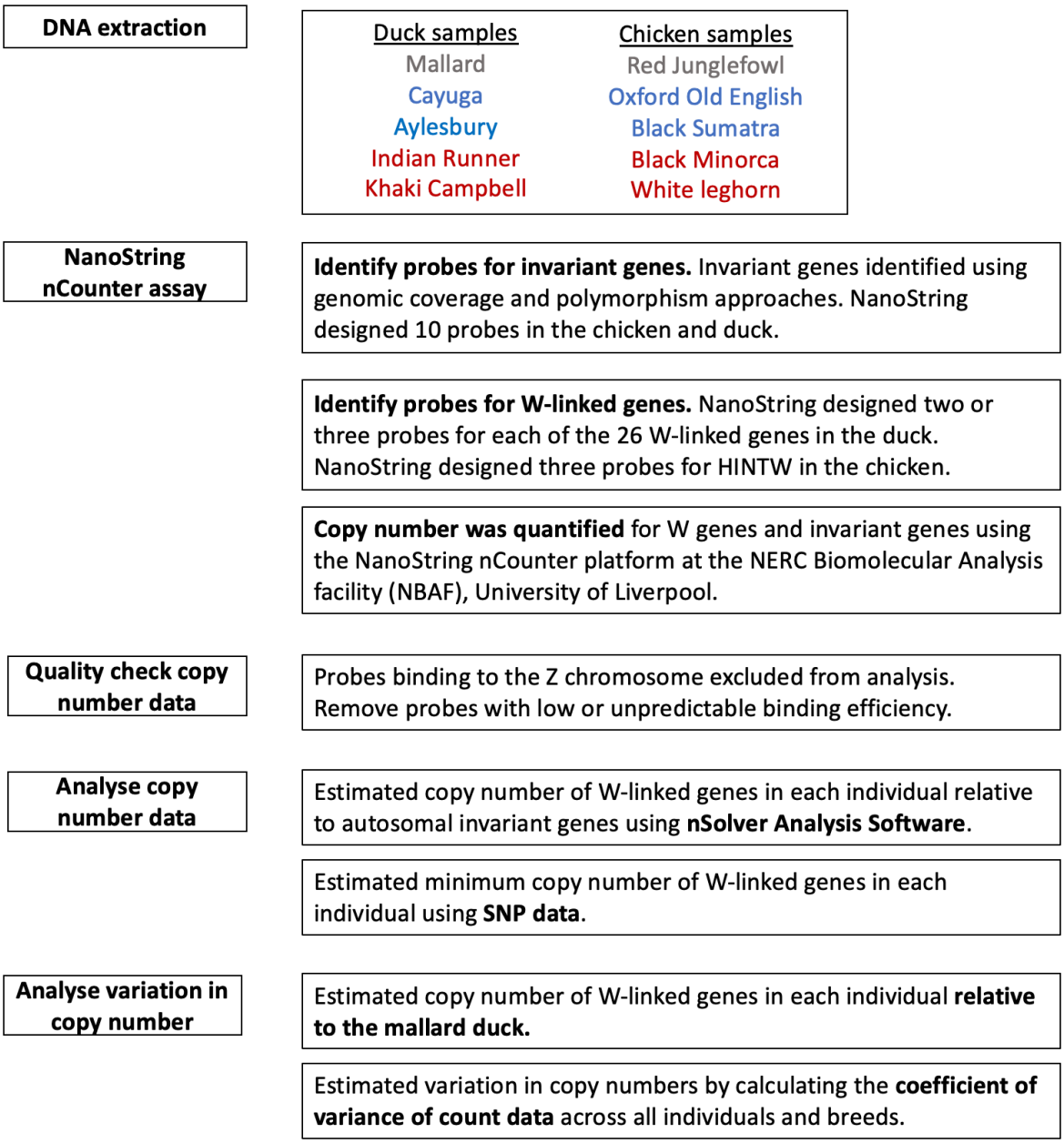

Figure S6.2.1. Workflow used in this study to quantify copy number and variability of 26 W-linked genes across duck breeds and their modern ancestor, and HINTW across chicken breeds and their modern ancestor. In the top box, modern ancestor birds are written in grey, male-selected breeds in blue, and female-selected breeds in red.

S6.3 SEX-SPECIFIC SELECTION DRIVES THE EVOLUTION OF ALTERNATIVE SPLICING IN BIRDS

Rogers, T. F., Palmer, D. H. & Wright, A. E. (2020). Sex-specific selection drives the evolution of alternative splicing in birds. *Molecular Biology and Evolution*, *38*(2), 519-530.

Supplementary Material available online:

https://academic.oup.com/mbe/article/38/2/519/5909999#supplementary-data

Table S6.3.1. Number of autosomal alternative splicing events and alternatively spliced genes in each species and tissue. Alternative splicing events are defined as those with 0 < PSI < 1 and a minimum of 20 reads spanning the splice junction in more than half the individuals in each sample. Genes were classified as alternatively spliced if they had at least one alternative splicing event. M and F denote males and females respectively. We categorized alternative splicing events as one of five splice types; skipped exons (SE), mutually exclusive exons (MXE), alternative 5' and 3' splice sites (A5'SS and A3'SS), and retained intron (RI) events.

			Alternative splicing events												Alternatively spliced genes												Total proportion of alternatively spliced genes	
Species	Tissue	Total expressed mutiple exon genes	M A3'SS	F A3'SS	M A5'SS	F A5'SS	M MXE	F MXE	M R	F RI	M SE	F SE	M total	F total	M A3'SS	F A3'SS	M A5'SS	F A5'SS	M MXE	F MXE	M RI	F RI	M SE	F SE	M total	F total	Males	Females
Duck	Gonad	8413	47	52	38	36	989	960	90	78	2421	2038	3585	3164	45	49	34	32	713	709	84	73	1572	1450	1939	1877	23%	22%
Duck	Spleen	8290	35	36	28	30	880	923	77	80	1716	2047	2736	3116	35	36	25	26	640	662	70	73	1192	1415	1572	1756	19%	21%
Turkey	Gonad	9114	33	41	10	13	893	909	4	5	1921	1953	2861	2921	33	40	10	12	691	715	4	5	1419	1495	1779	1895	20%	21%
Turkey	Spleen	8704	29	30	18	13	355	330	5	5	1411	1068	1818	1446	28	29	16	12	309	296	5	5	1142	894	1295	1079	15%	12%
Guineafowl	Gonad	9514	63	82	44	50	1386	1397	71	63	3045	2597	4609	4189	58	75	39	44	1002	1032	70	62	1984	1835	2453	2415	26%	25%
Guineafowl	Spleen	9728	71	77	56	56	1199	1255	69	68	2419	2668	3814	4124	63	71	53	53	887	918	66	65	1689	1835	2175	2319	22%	24%

Table S6.3.2. The total proportion of alternatively spliced autosomal genes in each species and tissue under different filtering parameters. The read count filter column refers to the total reads required to support the splice junction in more than half the individuals in each sample.

				Proportion of autosomal genes that are alternatively spliced	
Species	**Tissue**	**Read count filter**	**PSI filter**	**Males**	**Females**
Duck	Gonad	5	0 < PSI < 1	27%	25%
Duck	Gonad	10	0 < PSI < 1	25%	24%
Duck	Gonad	15	0 < PSI < 1	24%	23%
Duck	Gonad	20	0 < PSI < 1	23%	22%
Duck	Spleen	5	0 < PSI < 1	23%	24%
Duck	Spleen	10	0 < PSI < 1	22%	23%
Duck	Spleen	15	0 < PSI < 1	20%	22%
Duck	Spleen	20	0 < PSI < 1	19%	21%
Turkey	Gonad	5	0 < PSI < 1	22%	23%
Turkey	Gonad	10	0 < PSI < 1	21%	22%
Turkey	Gonad	15	0 < PSI < 1	21%	21%
Turkey	Gonad	20	0 < PSI < 1	20%	21%
Turkey	Spleen	5	0 < PSI < 1	18%	14%
Turkey	Spleen	10	0 < PSI < 1	17%	14%
Turkey	Spleen	15	0 < PSI < 1	16%	13%
Turkey	Spleen	20	0 < PSI < 1	15%	12%
Guineafowl	Gonad	5	0 < PSI < 1	30%	29%
Guineafowl	Gonad	10	0 < PSI < 1	29%	28%
Guineafowl	Gonad	15	0 < PSI < 1	27%	27%
Guineafowl	Gonad	20	0 < PSI < 1	26%	25%
Guineafowl	Spleen	5	0 < PSI < 1	26%	28%
Guineafowl	Spleen	10	0 < PSI < 1	25%	26%
Guineafowl	Spleen	15	0 < PSI < 1	24%	25%
Guineafowl	Spleen	20	0 < PSI < 1	22%	24%

Table S6.3.3. List of zebra finch, chicken, mallard duck, wild turkey and helmeted guineafowl orthologs expressed in the gonad of the three species studied, and whether they are sex-biased (SB) or unbiased (UB) in splicing. This table can be found online under 'Table S3' at:
https://academic.oup.com/mbe/article/38/2/519/5909999#supplementary-data

Table S6.3.4. Number of autosomal alternative splicing events and alternatively spliced genes in each species and tissue under different filtering parameters. The read count filter column refers to the total reads required to support the splice junction in more than half the individuals in both males and females. We defined sex-biased splicing from significant differences in percent spliced-in (PSI) between males and females (FDR p-value <0.05). We then imposed two different filtering thresholds based on the magnitude of sex differences in PSI. We used a Δ PSI (average PSI of male samples – average PSI of female samples) threshold of 0.1 following previous approaches (Grantham & Brisson MBE 2018). We also used a male: female $\log_2$ fold change PSI value of 1 for analyses comparing splicing and expression to ensure equivalent thresholds were implemented. We categorized alternative splicing events as one of five splice types; skipped exons (SE), mutually exclusive exons (MXE), alternative 5' and 3' splice sites (A5'SS and A3'SS), and retained intron (RI) events.

						Significant differentially spliced events						Significant differentially spliced genes						
Species	Tissue	Read count filter	Threshold to define sex-bias	FDR	Total expressed mutiple exon genes	A3'SS	A5'SS	MXE	RI	SE	Total	A3'SS	A5'SS	MXE	RI	SE	Total	Total proportion of genes with sex differences in splicing
Duck	Gonad	20	m:f logFC PSI	< 0.05	8413	0	3	46	9	278	336	0	3	42	9	238	277	3.3%
Duck	Gonad	20	m:f Δ 0.1 PSI	< 0.05	8413	7	11	181	10	677	886	7	10	148	10	551	640	7.6%
Duck	Spleen	20	m:f logFC PSI	< 0.05	8290	1	0	1	0	1	3	1	0	1	0	1	3	0.0%
Duck	Spleen	20	m:f Δ 0.1 PSI	< 0.05	8290	0	0	7	0	27	31	0	0	6	0	26	34	0.4%
Turkey	Gonad	20	m:f logFC PSI	< 0.05	9114	3	0	14	1	92	110	3	0	14	1	87	102	1.1%
Turkey	Gonad	20	m:f Δ 0.1 PSI	< 0.05	9114	5	0	91	2	481	579	5	0	78	2	421	475	5.2%
Turkey	Spleen	20	m:f logFC PSI	< 0.05	8704	0	0	0	0	3	3	0	0	0	0	3	3	0.0%
Turkey	Spleen	20	m:f Δ 0.1 PSI	< 0.05	8704	0	0	2	0	39	41	0	0	2	0	38	40	0.5%
Guineafowl	Gonad	20	m:f logFC PSI	< 0.05	9514	3	3	54	3	260	323	3	3	46	3	229	263	2.8%
Guineafowl	Gonad	20	m:f Δ 0.1 PSI	< 0.05	9514	15	16	219	7	720	977	13	16	174	7	596	701	7.4%
Guineafowl	Spleen	20	m:f logFC PSI	< 0.05	9728	0	0	3	0	3	6	0	0	3	0	3	6	0.1%
Guineafowl	Spleen	20	m:f Δ 0.1 PSI	< 0.05	9728	0	0	1	0	13	14	0	0	1	0	13	13	0.1%

Table S6.3.5. Distribution of F_{ST} and Tajima's *D* (TD) for each bird species. Only autosomal genes expressed in the gonad were included in the analyses. We split the data into three quantiles to define high F_{ST} and low Tajima's *D*.

		Number of genes with sex differences in splicing			
Species	Measurement	Observed	Expected	Chi squared value	p-value
Duck	High FST, Low TD	55	37.14	8.591	0.003
Turkey	High FST, Low TD	29	35.64	1.238	0.266
Guineafowl	High FST, Low TD	73	58.55	3.565	0.059

Table S6.3.6. Results of multi-predictor model to test for the relationship between isoform diversity, expression level and sex (isoform diversity ~ expression level + sex).

Species	Gene category	Parameter	Estimate	Standard Error	p-value
Duck	Male-biased	Intercept	0.371	0.037	0.000
		Expression	0.064	0.011	0.000
		Sex [M]	0	0.025	0.000
	Female-biased	Intercept	0.288	0.088	0.001
		Expression	0.057	0.02	0.004
		Sex [M]	0.094	0.038	0.014
	Unbiased	Intercept	0.34	0.035	0.000
		Expression	0.058	0.009	0.000
		Sex [M]	-0.002	0.016	0.892
Turkey	Male-biased	Intercept	0.39	0.045	0.000
		Expression	0.063	0.013	0.000
		Sex [M]	-0.126	0.031	0.000
	Female-biased	Intercept	0.426	0.089	0.000
		Expression	0.028	0.021	0.171
		Sex [M]	0.044	0.04	0.267
	Unbiased	Intercept	0.277	0.036	0.000
		Expression	0.072	0.009	0.000
		Sex [M]	-0.003	0.017	0.843
Guineafowl	Male-biased	Intercept	0.386	0.034	0.000
		Expression	0.064	0.011	0.000
		Sex [M]	-0.116	0.025	0.000
	Female-biased	Intercept	0.412	0.072	0.000
		Expression	0.027	0.017	0.105
		Sex [M]	0.047	0.035	0.178
	Unbiased	Intercept	0.308	0.03	0.000
		Expression	0.061	0.008	0.000
		Sex [M]	-0.012	0.014	0.38

Table S6.3.7. List of differentially expressed (DE) genes and differentially alternatively spliced (DS) genes in each species. This table can be found online under 'Table S7' at:
https://academic.oup.com/mbe/article/38/2/519/5909999#supplementary-data

Table S6.3.8. Hypergeometric tests for whether the overlap between differentially spliced and expressed genes was significantly less than expected across different splicing events and filtering thresholds. Sex-biased splicing is defined using significant differences in percent spliced-in (PSI) between males and females (FDR p-value <0.05). We also used a male: female $\log_2$ fold change PSI value of 1 or 0 for analyses comparing splicing and expression to ensure equivalent thresholds were implemented

SE only												
Species	Threshold to define sex-bias	Total expressed multiple exon genes	Total DEGs (observed)	Total DSGs (observed)	DEGs only (observed)	DSGs only (observed)	DEGs and DSGs (observed)	DEGs and DSGs (expected)	Representation Factor	Total genes minus DSGs	Hypergeometric dist p-value (phyper)	Benjamini Hochberg correction
Duck	m:f 1 logFC PSI & P<0.05	8441	3820	238	3735	153	85	107.71	0.79	8203	0.00	0.03
Duck	m:f 0 logFC PSI & P<0.05	8441	6039	868	5425	254	614	621.00	0.99	7573	0.30	0.54
Turkey	m:f 1 logFC PSI & P<0.05	9130	4172	87	4141	56	31	39.76	0.78	9043	0.04	0.22
Turkey	m:f 0 logFC PSI & P<0.05	9130	6831	707	6312	188	519	528.97	0.98	8423	0.20	0.44
Guineafowl	m:f 1 logFC PSI & P<0.05	9548	4228	229	4133	134	95	101.40	0.94	9319	0.21	0.43
Guineafowl	m:f 0 logFC PSI & P<0.05	9548	7281	974	6539	232	742	742.74	1.00	8574	0.49	0.68

MXE only												
Species	Threshold to define sex-bias	Total expressed multiple exon genes	Total DEGs (observed)	Total DSGs (observed)	DEGs only (observed)	DSGs only (observed)	DEGs and DSGs (observed)	DEGs and DSGs (expected)	Representation Factor	Total genes minus DSGs	Hypergeometric dist p-value (phyper)	Benjamini Hochberg correction
Duck	m:f 1 logFC PSI & P<0.05	8441	3820	42	3800	22	20	19.01	1.05	8399	0.68	0.82
Duck	m:f 0 logFC PSI & P<0.05	8441	6039	185	5909	55	130	132.36	0.98	8256	0.38	0.62
Turkey	m:f 1 logFC PSI & P<0.05	9130	4172	14	4163	5	9	6.40	1.41	9116	0.95	0.95
Turkey	m:f 0 logFC PSI & P<0.05	9130	6831	108	6751	28	80	80.80	0.99	9022	0.47	0.70
Guineafowl	m:f 1 logFC PSI & P<0.05	9548	4228	46	4206	24	22	20.37	1.08	9502	0.74	0.83
Guineafowl	m:f 0 logFC PSI & P<0.05	9548	7281	218	7111	48	170	166.24	1.02	9330	0.75	0.80

SE & MXE combined												
Species	Threshold to define sex-bias	Total expressed multiple exon genes	Total DEGs (observed)	Total DSGs (observed)	DEGs only (observed)	DSGs only (observed)	DEGs and DSGs (observed)	DEGs and DSGs (expected)	Representation Factor	Total genes minus DSGs	Hypergeometric dist p-value (phyper)	Benjamini Hochberg correction
Duck	m:f 1 logFC PSI & P<0.05	8441	3820	266	3720	166	100	120.38	0.83	8175	0.01	0.06
Duck	m:f 0 logFC PSI & P<0.05	8441	6039	940	5379	280	660	672.51	0.98	7501	0.18	0.54
Turkey	m:f 1 logFC PSI & P<0.05	9130	4172	99	4133	60	39	45.24	0.86	9031	0.12	0.55
Turkey	m:f 0 logFC PSI & P<0.05	9130	6831	757	6278	204	553	566.38	0.98	8373	0.13	0.47
Guineafowl	m:f 1 logFC PSI & P<0.05	9548	4228	256	4122	150	106	113.36	0.94	9292	0.19	0.49
Guineafowl	m:f 0 logFC PSI & P<0.05	9548	7281	1064	6469	252	812	811.37	1.00	8484	0.50	0.65

Table S6.3.9. The effect of sex-bias on the ratio of nonsynonymous (dN) to synonymous (dS) substitutions (dN/dS) for unbiased (UB), differentially expressed (DE) and differentially spliced (DS) genes after controlling for gene length (bp) and gene expression level (logCPM) using multiple regression. Estimates and p-values for DE and DS genes relative to UB genes are from a multiple linear regression (dN/dS ~ sex bias + gene length + gene expression level). The F values are calculated using analysis of variance (ANOVA) along with p-values for the categorical variable.

	Estimate				
Species	**UB**	**DE (p-value)**	**DS (p-value)**	**p-value**	**F value**
Duck	0.155	0.159 (0.355)	0.146 (0.341)	0.126	2.073
Turkey	0.172	0.188 (0.076)	0.145 (0.239)	0.040	3.228
Guineafowl	0.147	0.155 (0.021)	0.142 (0.590)	0.035	3.346

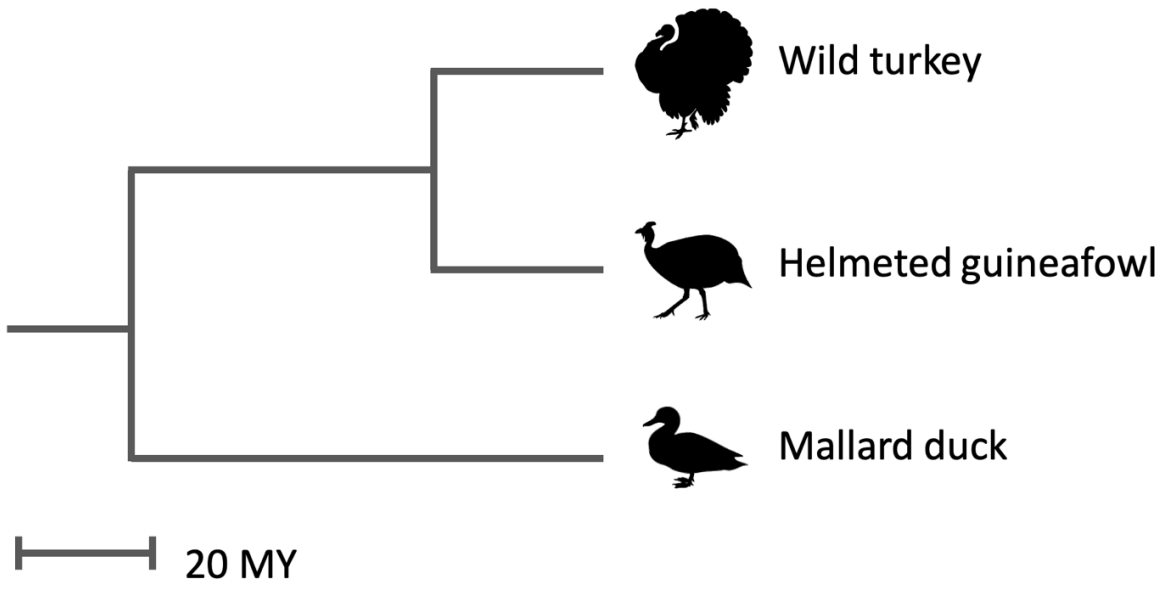

Figure S6.3.1. Phylogenetic relationships across the three avian species used in this study. The mallard duck, wild turkey and helmeted guineafowl shared a common ancestor approximately 90 million years ago.

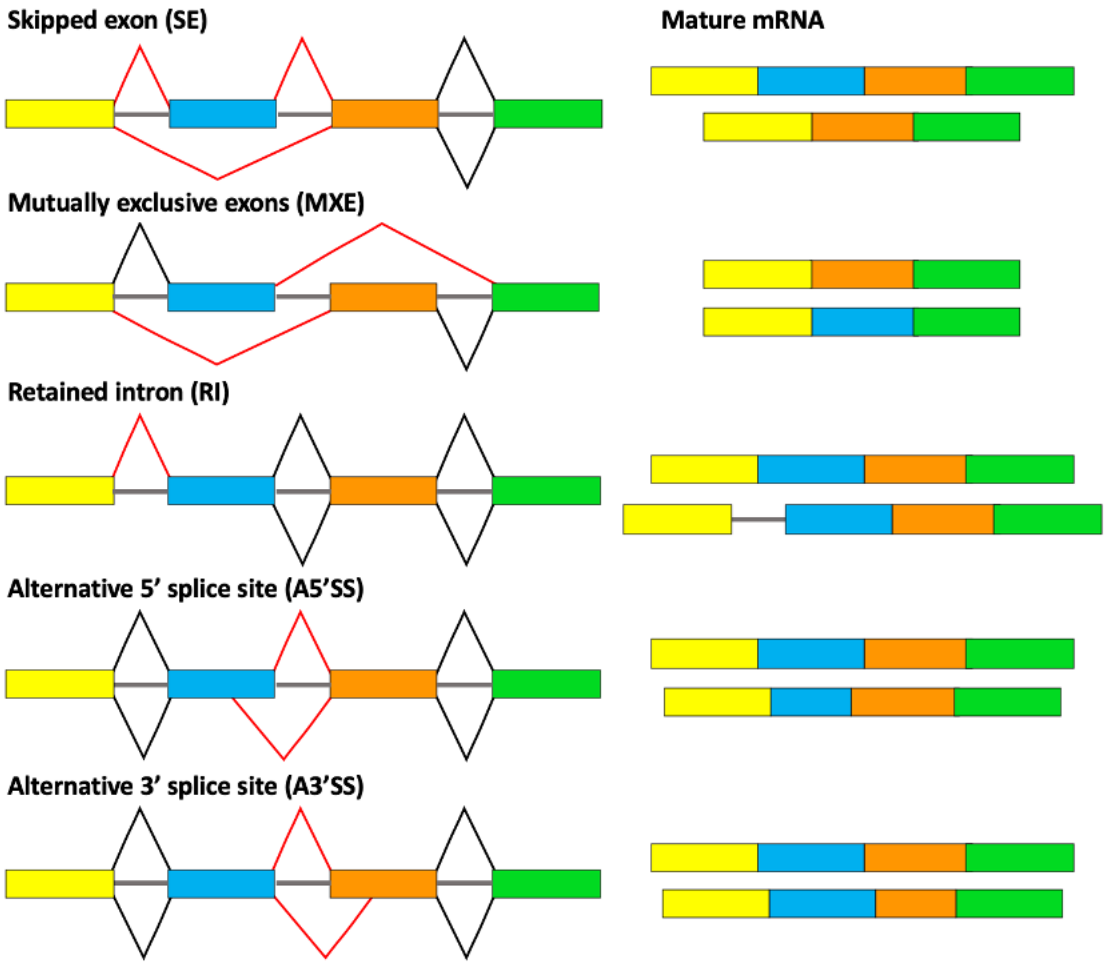

Figure S6.3.2. Schematic diagram of the different types of alternative splicing events. Exons are represented by coloured boxes and introns by grey lines. Lines connecting exons represent regions that are removed from the final transcript. Red lines indicate the two alternative splice events.

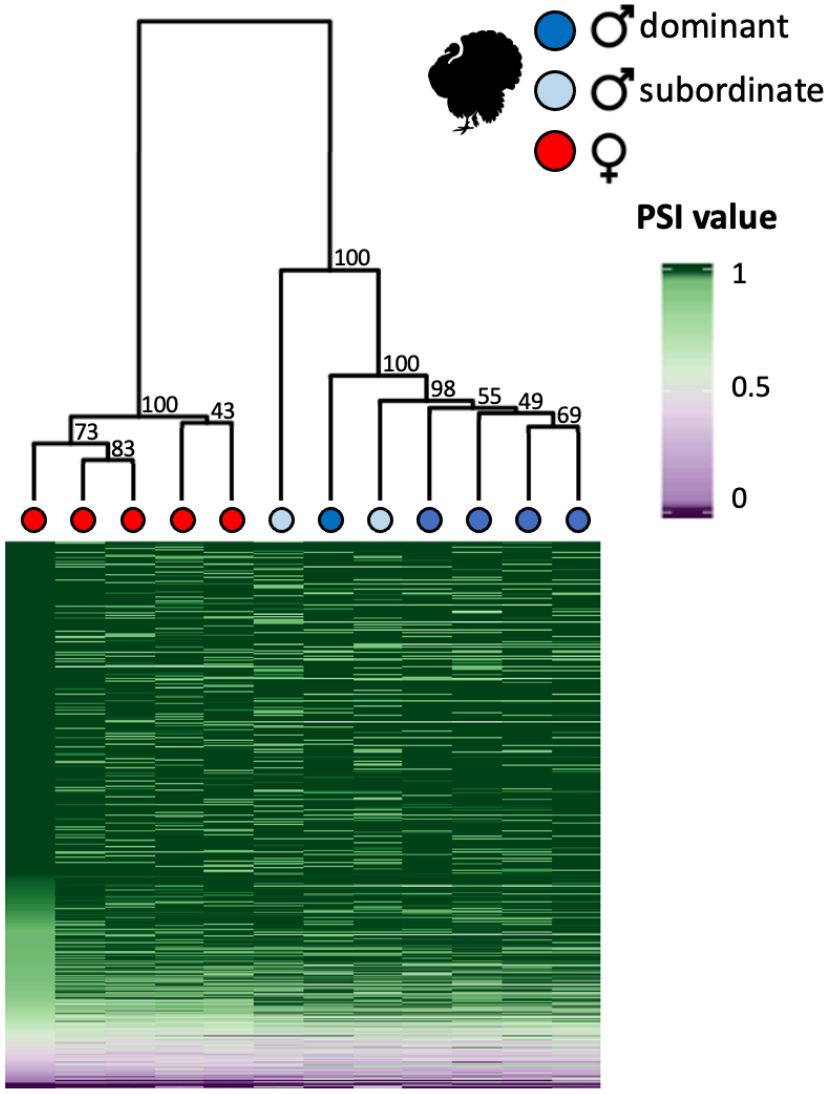

Figure S6.3.3. Heatmap and hierarchical clustering of alternative splicing in the gonad across dominant male, subordinate male and female turkey individuals. Percent spliced-in values (PSI) refer to the proportion of long to short isoforms of each splice site expressed per sample. Green depicts greater inclusion of the long isoform. If a gene undergoes multiple splice events, the average PSI is shown. Numbers on each branch represent the bootstrap probability values. Only autosomal genes are included in the analysis.

Figure S6.3.4. Workflow to identify alternative splicing events and differences in gene expression level in this study.

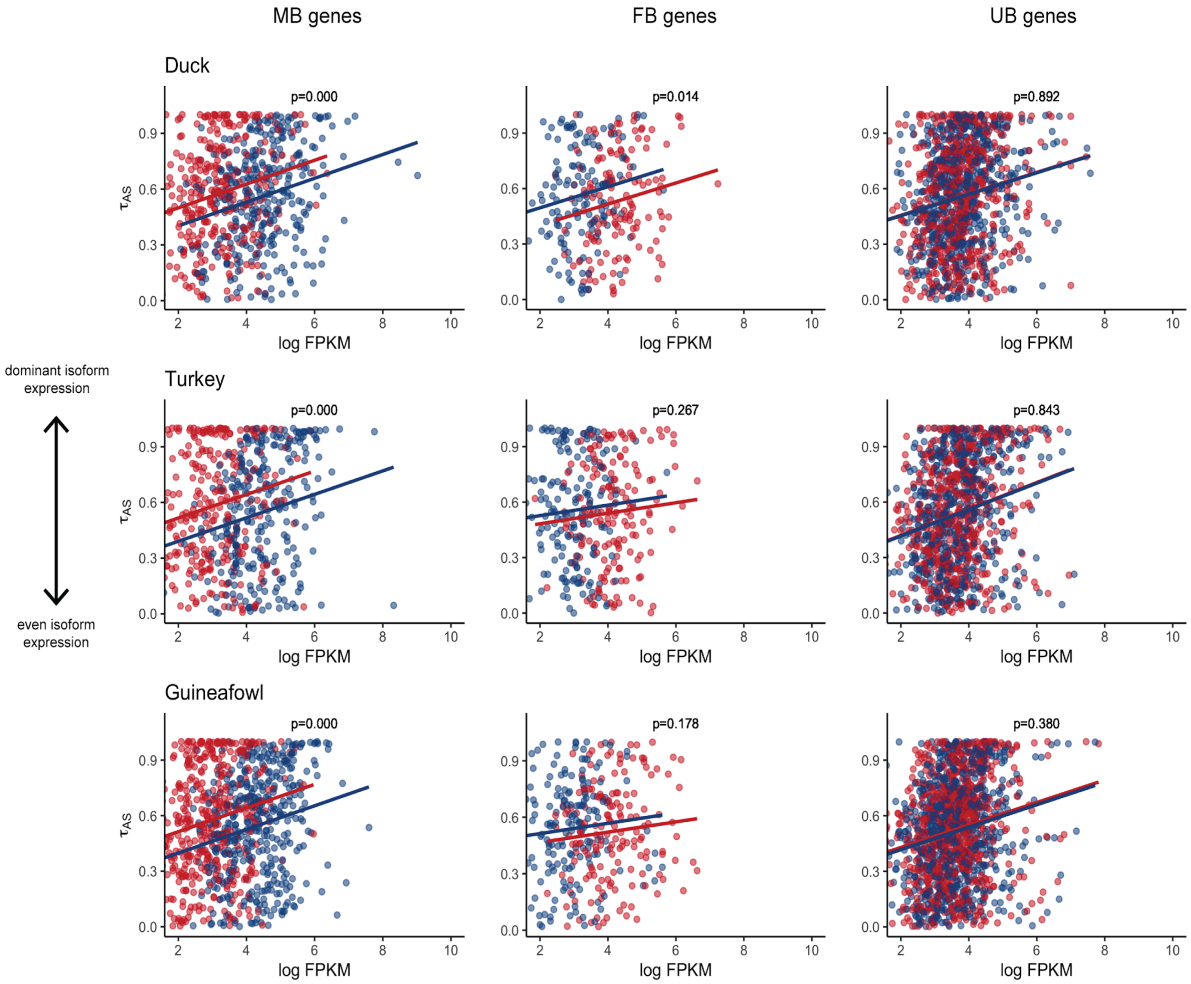

Figure S6.3.5. Linear regressions of τ_{AS} over expression level and sex for male-biased (MB), female-biased (FB), and unbiased (UB) genes for duck, turkey, and guineafowl. Blue lines represent male values and red lines represent female values. Each p value denotes the statistical significance of male versus female intercepts.

CHAPTER 7. KEY PUBLICATIONS

Wright, A. E., **Rogers, T. F.**, Fumagalli, M., Cooney, C. R. & Mank, J. E. Phenotypic sexual dimorphism associated with genomic signatures of resolved sexual conflict (2019). *Molecular Ecology, 28*(11), 2860–2871.

Palmer, D. H**.**, **Rogers, T. F.**, Dean, R. & Wright, A. E. (2019). How to identify sex chromosomes and their turnover. *Molecular Ecology, 28*(21), 4709–4724.

Rogers, T. F., Pizzari, T. & Wright, A. E. Multi-copy gene family evolution on the avian W chromosome. *Journal of Heredity, 112*(3), 250–259.

Rogers, T. F., Palmer, D. H. & Wright, A. E. (2021). Sex-specific selection drives the evolution of atlernative splicing in birds. *Molecular Biology and Evolution, 38*(2), 519–530*.*

Price, P. D., Palmer, D. H., Taylor, J. A., Kim, D. W., Place, E. S., **Rogers, T. F.**, Placzek, M., Mank, J. E., Cooney, C. R. & Wright, A. E. (2021). Detecting signatures of selection in regulatory variation. ***EcoEvoRxiv.*** In revision for *Nature Ecology and Evolution*.

Received: 21 November 2018 | Revised: 8 April 2019 | Accepted: 22 April 2019

DOI: 10.1111/mec.15115

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict

Alison E. Wright[1] | **Thea F. Rogers**[1] | **Matteo Fumagalli**[2] | **Christopher R. Cooney**[1] | **Judith E. Mank**[3,4,5]

[1]Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK

[2]Department of Life Sciences, Imperial College London, London, UK

[3]Department of Genetics, Evolution and Environment, University College London, London, UK

[4]Department of Organismal Biology, Uppsala University, Uppsala, Sweden

[5]Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada

Correspondence
Alison E. Wright, Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK.
Email: a.e.wright@sheffield.ac.uk

Funding information
Natural Environment Research Council, Grant/Award Number: NE/N013948/1; H2020 European Research Council, Grant/Award Number: 260233 and 680951

Abstract

Intralocus sexual conflict, where an allele benefits one sex at the expense of the other, has an important role in shaping genetic diversity of populations through balancing selection. However, the potential for mating systems to exert balancing selection through sexual conflict on the genome remains unclear. Furthermore, the nature and potential for resolution of sexual conflict across the genome has been hotly debated. To address this, we analysed de novo transcriptomes from six avian species, chosen to reflect the full range of sexual dimorphism and mating systems. Our analyses combine expression and population genomic statistics across reproductive and somatic tissue, with measures of sperm competition and promiscuity. Our results reveal that balancing selection is weakest in the gonad, consistent with the resolution of sexual conflict and evolutionary theory that phenotypic sex differences are associated with lower levels of ongoing conflict. We also demonstrate a clear link between variation in sexual conflict and levels of genetic variation across phylogenetic space in a comparative framework. Our observations suggest that this conflict is short-lived, and is resolved via the decoupling of male and female gene expression patterns, with important implications for the role of sexual selection in adaptive potential and role of dimorphism in facilitating sex-specific fitness optima.

KEYWORDS
molecular evolution, population genetics, sexual conflict, transcriptomics

1 | INTRODUCTION

Males and females in many species often have divergent evolutionary interests and are subject to conflicting selection pressures (Andersson, 1994). However, with the exception of the sex chromosomes, the sexes share an identical genome, and this can give rise to intralocus sexual conflict, where an allele benefits one sex at the expense of the other (Parker & Partridge, 1998). This shared genomic architecture is thought to hamper males and females simultaneously evolving towards their respective fitness peaks, and in turn acts as a constraint in the evolution of sexual dimorphism (Mank, 2017; Rowe, Chenoweth, & Agrawal, 2018; Stewart & Rice, 2018).

Recently, studies have used population genomic statistics to detect the signature of sexual conflict across the genome (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Lucotte, Laurent, Heyer, Ségurel, & Toupance, 2016; Mank, 2017; Mostafavi et al., 2017; Rowe et al., 2018; Wright et al., 2018). Ongoing sexual conflict can arise from several different factors and these leave distinct population genomic signatures in sequence data (Mank, 2017; Wright et al., 2018). Sexual conflict can result in over-reproduction, where

an allele increases the reproductive fitness of one sex at a cost to the other (Barson, et al. 2015; Lonn et al., 2017). Alternatively, sexual conflict can result when an allele has differential effects on survival between males and females (Czorlich, Aykanat, Erkinaro, Orell, & Primmer, 2018). Both of these scenarios are predicted to produce in elevated genetic diversity and higher Tajima's *D*, a population genomic statistic that estimates the proportion of polymorphic nucleotide sites in a given sequence within a population.

To distinguish between sexual conflict arising over reproduction or survival, it is necessary to employ contrasts with intersexual F_{ST} (Lewontin & Krakauer, 1973), which measures divergence in allele frequency between males and females within a generation. As allele frequencies are identical between the sexes at conception, different allele frequencies in male and female adults are assumed to be the result of sexual conflict over survival. Elevated F_{ST} can therefore be used to identify alleles that have differential effects on survival parameters, including viability, mortality or predation. By contrasting these two population genomic statistics, it is possible to determine the relative importance of conflict over reproduction, which only leads to increased Tajima's *D*, versus conflict over survival, which leads to elevated Tajima's *D* and intersexual F_{ST} (Mank, 2017; Wright et al., 2018).

Population genomic approaches such as these have made it possible to investigate the manifestation of different types of intralocus sexual conflict at the genomic level and the mechanisms by which they can be resolved. In theory, sexual conflict should be most prevalent in genes with similar expression patterns in males and females, where mutational inputs will be manifest in both sexes. Ultimately, sexual conflict is thought to be resolved via the evolution of sex-biased gene expression (Connallon & Knowles, 2005; Ellegren & Parsch, 2007), which, because of primary expression in one sex or the other, in principle allows for the emergence of male- and female-specific fitness optima (Mank, 2017). However, the exact nature of the relationship between sex-biased gene expression and resolved sexual conflict has been hotly debated, with some recent studies suggesting that sex-biased genes are subject to ongoing sexual antagonism (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018). If true, this suggests that sexual conflict can persist even after gene expression diverges between males and females, and is potentially an unrelenting constraint on sex-specific optima. It would also suggest that, although expressed primarily in one sex, sex-biased genes function similarly in both males and females, and are therefore not appropriate for studying molecular signatures of sex-specific selection, as is often done (Ellegren & Parsch, 2007).

Moreover, the signature of balancing selection for sex-biased genes detected by recent studies is discordant with the rapid molecular evolutionary rates of directional selection (Meiklejohn, Parsch, Ranz, & Hartl, 2003; Pröschel, Zhang, & Parsch, 2006; Zhang, Sturgill, Parisi, Kumar, & Oliver, 2007) and relaxed constraint (Dapper & Wade, 2016; Gershoni & Pietrokovski, 2014; Harrison et al., 2015) observed in this class of genes across a wide variety of species. At the same time, and consistent with the molecular signatures observed, other work has suggested that sex-biased genes represent resolved conflict, and therefore exhibit lower average levels of balancing selection than unbiased genes (Connallon & Knowles, 2005; Innocenti & Morrow, 2010; Mank, 2009; Wright et al., 2018). If broadly true, this suggests that conflict is prevalent in genes with similar expression patterns between the sexes, and is primarily resolved through regulatory decoupling of males and females into separate male and female genetic architectures. This conclusion is intuitively concordant with the fact that sex-biased genes are primarily expressed in either males or females, and also suggests that sexual conflict is a short-lived constraint, given the rapid turnover in sex-biased gene expression across related species (Harrison et al., 2015; Zhang et al., 2007).

Importantly, recent theoretical work indicates that implausibly large selective pressures and mortality loads are required to generate the patterns of intersexual F_{ST} observed in the literature attributed to ongoing sexual antagonism (Kasimatis, Nelson, & Phillips, 2017; Kasimatis, Ralph, & Phillips, 2019). This calls into question the application of F_{ST}-based approaches for detecting sexual conflict arising from survival differences between the sexes. Consistent with this, a recent study found evidence that elevated intersexual F_{ST} for sex-biased genes is actually the product not of sexual conflict, but of sex-specific genetic architecture (Wright et al., 2018), where an allele only affects one sex or the other. Sex-specific genetic architecture invokes relatively lower genetic loads, and there is increasing evidence that many loci exhibit profound sex differences in their phenotypic effects (Dapper & Wade, 2016; Gilks, Abbott, & Morrow, 2014; Karp et al., 2017). Similarly, recent analyses of large genomic data sets identified only a very small number of loci subject to antagonistic selection on survival (Czorlich et al., 2018; Mostafavi et al., 2017).

Furthermore, a major challenge in evolutionary biology is to explain the maintenance and variation in genetic diversity across many species. The existence of elevated genetic diversity relative to neutral expectations across species is puzzling, as directional selection and drift are both expected to erode variation. However, there is increasing evidence that intralocus sexual conflict, through balancing selection, can significantly increase genome-wide patterns of variability (Chippindale, Gibson, & Rice, 2001; Delcourt, Blows, & Rundle, 2009; Foerster et al., 2007; Hawkes et al., 2016; Lonn et al., 2017; Mokkonen et al., 2011). Therefore, variation in sexual conflict across lineages, probably mediated by mating systems, could drive variation in genetic diversity across species and resolve this apparent paradox. However, the exact nature of the relationship between sexual conflict, mating system and genetic diversity remains unclear. Sexual conflict also has important implications for sexual selection, adaptation and evolvability. For instance, on the one hand, balancing selection would be expected to slow rates of sequence evolution arising from directional selection. However, balancing selection can also facilitate rapid adaptation from standing variation by maintaining multiple alleles within the population at high allele frequencies (Charlesworth, 2006; Hartl & Clark, 2006).

To assess the degree to which sex-biased genes exhibit signatures of unresolved conflict and the potential for mating systems

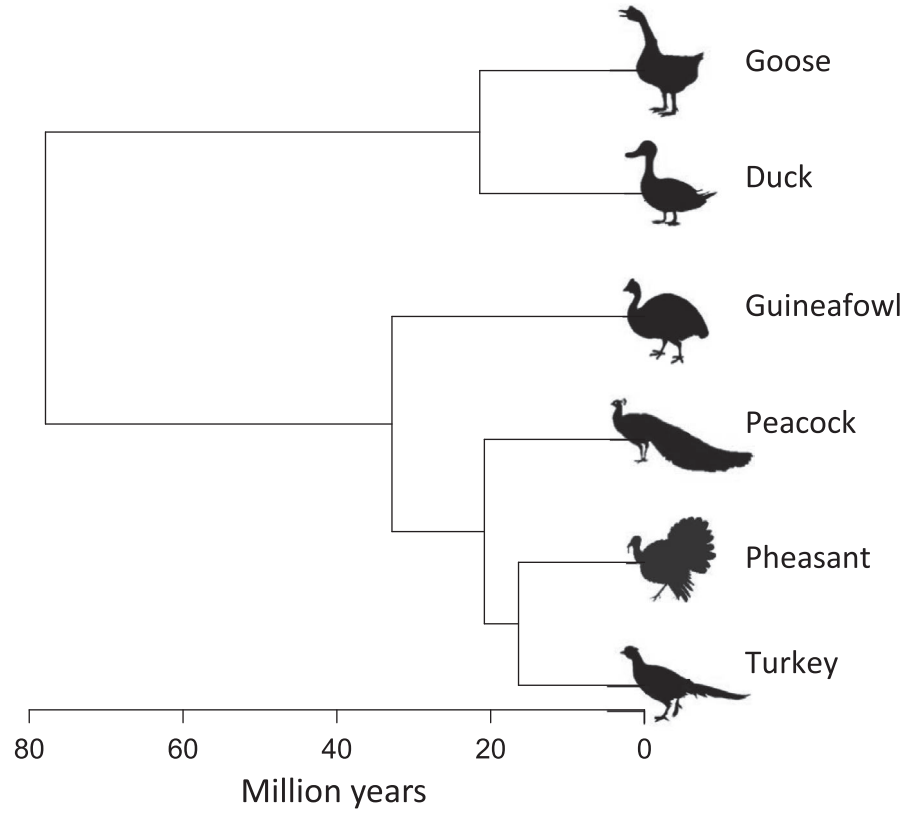

FIGURE 1 Phylogenetic relationships across the six avian species in this study. These species were chosen to reflect the full range of mating system and sexual dimorphism. The intensity of sexual conflict in each species was estimated using three proxies: sexual dichromatism score, sperm number and relative testes size

to exert balancing selection through sexual conflict on the genome, it is necessary to compare population genomic patterns of species and tissues with different levels of sexual dimorphism. We therefore estimated population genomic statistics for genes expressed in reproductive and somatic tissue across six avian species spanning the full range of mating systems and sexual selection in birds. Reproductive tissue has multiple sex-specific functions and is phenotypically more sexually dimorphic, whereas the function of many somatic tissues is largely similar in males and females. By exploiting natural variation in the magnitude of sexual conflict across the body plan within individuals, as well as across mating systems between species, we were able to study the manifestation and resolution of sexual conflict, and subsequent genomic and phenotypic consequences. Our results reveal that the resolution of genomic sexual conflict is associated with the evolution of phenotypic sex differences. We demonstrate a clear link between variation in sexual conflict over reproduction and levels of genetic variation across phylogenetic space in a comparative framework.

2 | MATERIALS AND METHODS

2.1 | Tissue collection

We previously extracted RNA from the left gonad and spleen of individuals with the RNeasy Kit (Qiagen), following the manufacturer's instructions, from the following captive avian populations: mallard (*Anas platyrynchos*), wild turkey (*Meleagris gallopavo*), common pheasant (*Phasianus colchicus*), helmeted guinea fowl (*Numida meleagris*), Indian peafowl (*Pavo cristatus*) and swan goose (*Anser cynoides*) (Harrison et al., 2015) (Figure 1). These captive populations are not maintained under sterile or biosafety conditions. Samples were collected during the first breeding season from five males and five females of each species, with the exception of the pheasant, where six male gonad and spleen samples were collected, and turkey where four male and two female spleens were collected.

These six species were deliberately chosen to reflect a full range of sexual dimorphism, ranging from monogamous and sexually monomorphic species such as the swan goose and guinea fowl, to polygynous and sexually dimorphic species such as the peafowl and wild turkey. We estimated the intensity of sexual conflict in each species using three proxies of sperm competition and male promiscuity: sexual dichromatism score, sperm number and relative testes size, obtained from Harrison et al., 2015.

2.2 | Transcriptome assembly

Samples were sequenced on an Illumina HiSeq 2000 device with 100-bp paired-end reads and are available in the NCBI SRA (BioProject ID PRJNA271731). We assembled and filtered transcriptomes for each species using previously implemented approaches

(Harrison et al., 2015). Briefly, we quality filtered RNA data using TRIMMOMATIC version 0.36 (Bolger, Lohse, & Usadel, 2014) to filter reads containing adaptor sequences and trim reads if the sliding window average Phred score over four bases was < 15 or if the leading/trailing bases had a Phred score < 3. Reads were removed after filtering if either read pair was < 36 bases in length. We assembled a de novo transcriptome for each species using TRINITY version 2.4.0 (Grabherr et al., 2011) with default parameters. We then filtered each transcriptome to remove spurious and low-confidence genes. First, we selected the "best isoform" per gene to avoid redundancy. We used the TRINITY script align_and_estimate_abundance.pl to map RNA-seq reads to transcriptomes using BOWTIE 2 and to quantify expression for each sample using RSEM. We suppressed unpaired and discordant alignments for paired reads. We then picked the most highly expressed isoform per gene to obtain a set of "best isoforms" for each species. RNA-seq reads were remapped to the set of "best isoforms" in each species using the same approach as above to ensure consistency between expression and sequence data. Second, we filtered the transcriptome to remove lowly expressed genes. Specifically, we removed genes with expression < 2 FPKM (fragments per kilobase of transcript per million mapped reads) in half or more of the individuals in either tissue. We assessed the completeness of our transcriptome assembly using eukaryota_odb9 BUSCO version 3.0.2 (Waterhouse et al., 2018) (Table S1).

2.3 | Identification of orthologues

We used BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) to identify orthologous genes across the six species. First, we identified pairwise reciprocal orthologues between the chicken reference genome (Gallus_gallus-5.0) and the wild turkey, common pheasant, helmeted guinea fowl and Indian peafowl, and between the duck reference genome (BGI_duck_1.0) and mallard and swan goose (Zerbino et al., 2018). We downloaded cDNA sequences from Ensembl (Zerbino et al., 2018) and selected the longest transcript per gene. We ran reciprocal BLASTN with an e-value cut-off of 1×10^{-10} and selected the best hit reciprocal orthologue using a minimum percentage identity of 30% and the highest bitscore following previous approaches (Harrison et al., 2015; Wright et al., 2018). If two hits shared the same highest bitscore, then the hit with the highest percentage identity was chosen. If both hits had the same highest bitscore and percentage identity, the gene was discarded.

For the wild turkey, common pheasant, helmeted guinea fowl and Indian peafowl, we assigned chromosomal location and gene position from the pairwise reciprocal orthologue in the chicken reference genome. Chromosomal positional information is not available in the duck reference genome and so we used a synteny-based approach to obtain chromosomal location using MSCANX (Wang et al., 2012). Briefly, we downloaded chicken and duck protein sequences from Ensembl, selected the longest protein per gene in each species, and then conducted a reciprocal BLASTP with an e-value cut-off of 1×10^{-10}. We restricted the number of BLASTP hits for each gene to the top five, generated gff files, and concatenated the duck and chicken results as recommended by MSCANX. We then identified syntenic regions between the duck and chicken reference genome using MSCANX run with default parameters. For the mallard and swan goose, we assigned chromosomal location and gene position from the syntenic information available for the pairwise reciprocal orthologue in the duck reference genome. For all species, we split genes into autosomal or Z-linked based on location in the chicken reference genome (Table S1) as evolutionary forces including sexual conflict act differently across these genomic regions (Rice, 1984; Wright & Mank, 2013).

Second, we identified reciprocal orthologues using the same approach across all species using the chicken and duck reference genomes to assign chromosomal location. This resulted in 1,457 autosomal reciprocal orthologues, which we used to contrast population genetic statistics across species. Finally, potential immune loci were identified from Gene Ontology terms in Biomart in the chicken and duck reference genomes (Zerbino et al., 2018). Specifically, we removed all loci with the terms "immune" or "MHC" in their Gene Ontology annotations from subsequent analyses. This was to reduce any potential confounding effects as heterozygote advantage in immunity can produce patterns of balancing selection independent of sexual conflict (Ghosh, Andersen, Shapiro, Gerke, & Kruglyak, 2012; Hedrick, 2011; Stahl, Dwyer, Mauricio, Kreitman, & Bergelson, 1999).

2.4 | Gene expression analyses

Read counts for autosomal and Z-linked genes were extracted for all gonad and spleen samples and normalized using TMM in EDGER (Robinson, McCarthy, & Smyth, 2010). We identified gonad-biased, spleen-biased and non-tissue-biased genes using a standard $\log_2$ fold change value of 2 (Wright et al., 2018) in each species (Tables S2 and S3). The gonad is transcriptionally more sexually dimorphic than the spleen and so we identified tissue-biased genes in each sex separately instead of combining all samples to avoid biasing our analyses against highly sex-biased or sex-limited genes. We report results from tissue-biased genes identified in males in the main text but results based on tissue-biased genes identified from female expression data are fully detailed in the Supporting Information. The results are qualitatively identical unless otherwise indicated. Sex-biased genes were identified in each set of tissue-biased genes using a $\log_2$ fold change value of 1. We identified tissue-biased genes on the Z chromosome separately due to the unique expression profile of the avian Z chromosome arising from incomplete dosage compensation (Itoh et al., 2007; Mank & Ellegren, 2008; Wright, Moghadam, & Mank, 2012).

2.5 | Filtering data for population genomic analyses

Population genomic analyses were conducted on BAM files generated by mapping RNA-seq data to the set of "best isoforms" in each species with RSEM. For each individual, we merged the spleen and gonad BAM files using SAMTOOLS (Li et al., 2009). The exception was

the turkey, where the spleen and gonad were not sequenced for all individuals so we used only gonad data for subsequent analyses.

We used ANGSD (Korneliussen, Albrechtsen, & Nielsen, 2014) to estimate population genetic summary statistics, following our previous approach (Wright et al., 2018) as ANGSD implements methods to account for sequencing uncertainty and is appropriate for uneven sequencing depth associated with transcriptome data. We filtered BAM files to discard reads if they did not uniquely map, had a flag ≥ 256, had a mate that was not mapped or had a mapping quality below 20. Bases were filtered if base quality fell below 13 or there was data in fewer than half the individuals. Mapping quality scores were adjusted for excessive mismatches and quality scores were adjusted around indels to rule out false single nucleotide polymorphisms (SNPs).

We identified and removed related individuals (four peacock, two wild turkey and two swan goose individuals) from our analyses using NGSRELATE (Korneliussen & Moltke, 2015) to avoid violating Hardy–Weinberg assumptions, and calculated inbreeding coefficients using an EM algorithm with the NGSF package in NGSTOOLS (Fumagalli, Vieira, Linderoth, & Nielsen, 2014) (full details in Methods S1). For all species, inbreeding coefficients were < 0.03 with the exception of the peacock where we identified two inbred individuals. We incorporated inbreeding coefficients for the peacock in subsequent analyses.

2.6 | Calculating Tajima's *D*

ANGSD was used for each species to calculate sample allele frequency likelihoods at each site from genotype likelihoods calculated with the SAMTOOLS model. We calculated allele frequency likelihoods separately for the Z chromosome and the autosomes as they are subject to different evolutionary pressures and differ in ploidy. The Z chromosome is diploid in males yet haploid in females, and therefore we used only male samples to estimate allele frequency to avoid violating Hardy–Weinberg assumptions. Next, we estimated the overall unfolded site frequency spectrum (SFS) for each species (Nielsen, Korneliussen, Albrechtsen, Li, & Wang, 2012) (Figure S1). Specifically, at each site we randomly sampled an allele frequency according to its likelihood, as calculated by ANSGD. Finally, we computed genetic diversity indices, including allele frequency posterior probability and Tajima's *D* using the SFS as prior information with ANGSD thetaStat (Korneliussen et al., 2014).

For each species, we calculated a relative measure of Tajima's *D* for spleen-biased and gonad-biased genes. Specifically, we quantified median *D* relative to non-tissue-biased genes, our neutral estimate of *D* for each species. Calculating a relative measure of Tajima's *D* makes it possible to circumvent problems arising from demographic changes in population size that would otherwise bias comparative analyses of population genetic statistics across species.

2.7 | Calculating intersexual F_{ST}

Intersexual F_{ST} was calculated using the same procedure and filtering criteria as Tajima's *D*, except that RNA-seq data were instead filtered to remove bases where we had data in fewer than half the individuals in males and females separately. This ensures we do not exclude sex-limited genes from the analysis. Hudson's F_{ST}, which is less sensitive to small sample sizes (Bhatia, Patterson, Sankararaman, & Price, 2013), was estimated as implemented in ANGSD (Korneliussen et al., 2014). Estimates across loci were obtained using weighted averages (see Fumagalli et al 2014, Equations 4 and 12), where per-gene F_{ST} is the ratio between the sum of the between-populations variance across loci and the sum of the total variance across loci. Given the Z chromosome is haploid in females, we do not have the power to analyse patterns of F_{ST} across the Z chromosome in this study.

3 | RESULTS

3.1 | Lower levels of ongoing sexual conflict in reproductive versus somatic tissue

Reproductive tissue, such as the gonad, has many sex-specific functions whereas the function of somatic tissue, such as the spleen, is more aligned between male and female fitness. To test whether phenotypic sexual dimorphism is associated with resolved sexual conflict at the genomic level, we contrasted population genomic statistics between genes expressed in the gonad versus the spleen.

As heterozygote advantage in immunity can produce patterns of balancing selection independent of sexual conflict (Ghosh et al., 2012; Hedrick, 2011; Stahl et al., 1999), we removed all loci with potential immune function from downstream analyses. We found that median Tajima's *D* is significantly lower for gonad-biased genes relative to genes expressed in both tissues in all species across the autosomes (Figure 2a and Figure S2A). This result is consistent with lower levels of ongoing sexual antagonism in the gonad. In contrast, we found no significant difference in Tajima's *D* between spleen-biased genes and loci expressed in both tissues in the majority of species. We observed consistent patterns on the Z chromosome (Figure S5), although our power to detect statistically significant differences is reduced due to limited numbers of tissue-biased Z-linked genes (Table S1).

The proportion of sex-biased genes varies across the spleen and gonad (Harrison et al., 2015) and sex-biased genes are subject to different selective pressures (Ellegren & Parsch, 2007; Harrison et al., 2015) as well as distinct patterns of balancing selection relative to unbiased genes (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Wright et al., 2018). To ensure that differences in the number of sex-biased genes between the two tissues are not responsible for the lower Tajima's *D* we observe in gonad-biased genes, we repeated the analyses using Tajima's *D* calculated only from unbiased genes in each tissue. We found a consistent pattern across the majority of species, where Tajima's *D* is significantly lower in gonad-biased but not spleen-biased genes relative to loci expressed similarly in both tissues (Figure S3). However, these species differ in mating system, which could explain the variation in the strength of balancing selection we observe across species, addressed in more detail below.

It is important to note that multiple factors can influence population genetic statistics for any particular locus. Therefore, we

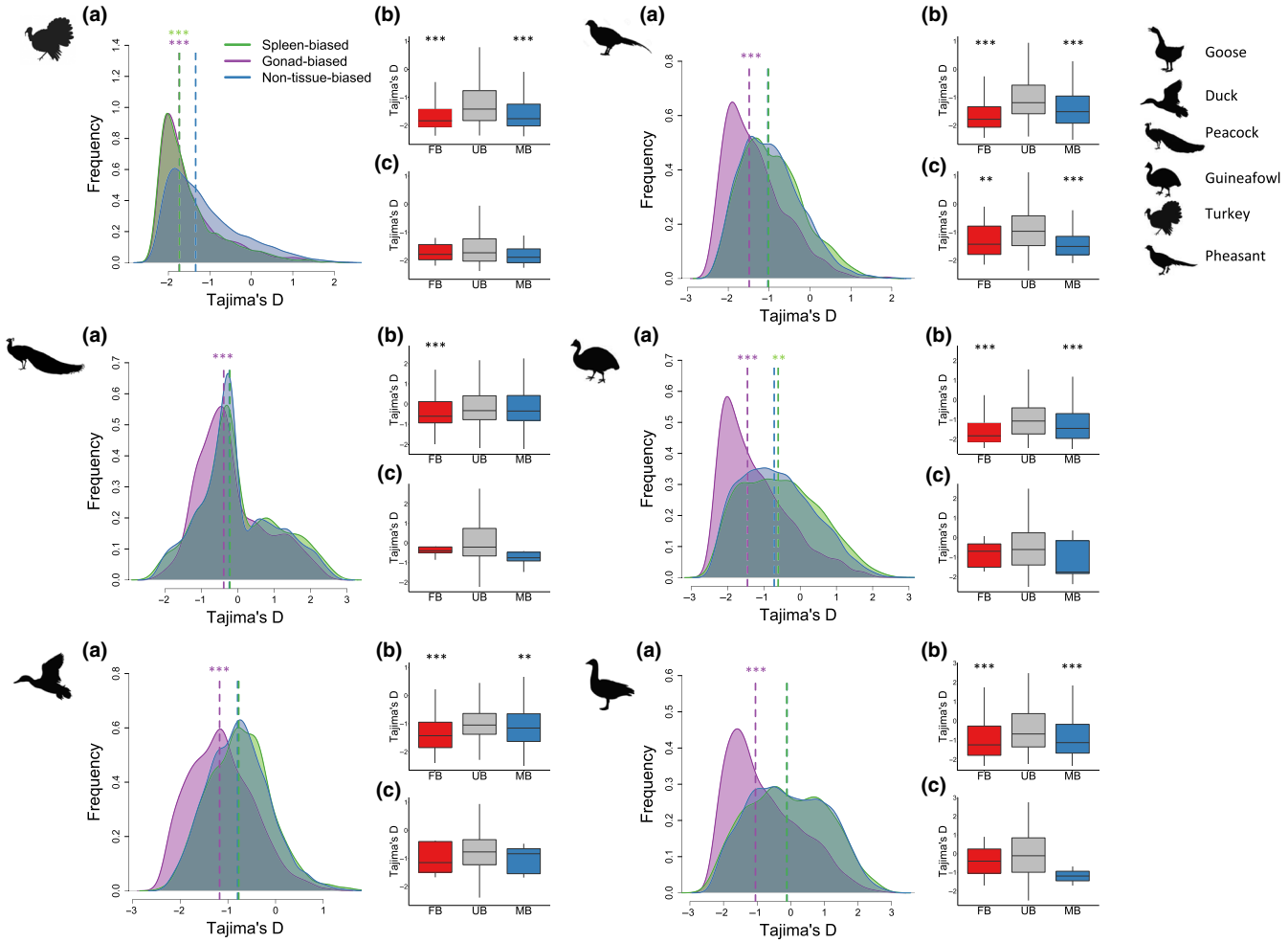

FIGURE 2 Patterns of Tajima's *D* for tissue-biased and sex-biased genes across species. (a) The distribution of *D* for autosomal genes for spleen-biased, gonad-biased and non-tissue-biased genes. Dotted lines show median *D* for each set of genes and asterisks denote a significant difference relative to non-tissue-biased genes (Wilcoxon test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$). Tissue-biased genes were identified from male expression data. (b, c) The relationship between *D* and expression for genes with gonad-biased expression (b) or spleen-biased expression (c). Asterisks denote a significant difference relative to unbiased genes (Wilcoxon test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$). FB, UB, MB refer to female-biased, unbiased and male-biased genes, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

tested whether our results could also be attributed to the effect of covariates that might vary across tissue-biased genes. We incorporated measures of gene length, average expression level, GC content and Watterson's theta into a multiple regression (TD ~ Tissue bias + log(tW) + log(Gene length) + log(GC) + log(Gene expression level)). Tissue-bias remains a significant factor in explaining variation in Tajima's *D* once accounting for these covariates (Table S11). However, the effect size in some species is relatively small, indicating that the pattern we detect is subtle and influenced by multiple factors.

3.2 | Limited power of intersexual F_{ST} to detect sexual conflict arising over survival

We tested the power of intersexual F_{ST} to detect sexual conflict arising over survival through contrasts between the spleen and gonad. Given its role in the lymphatic system and in filtering blood components, we might expect the spleen to be subject to viability selection more so than the gonad, whose role is primarily reproductive. We removed sex-biased genes from this analysis to avoid biasing the results, as the abundance of sex-biased expression differs between reproductive and somatic tissue and previously we have shown that intersexual F_{ST} is often elevated for sex-biased genes (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Wright et al., 2018).

We contrasted intersexual F_{ST} for gonad- and spleen-biased genes using three approaches. First, we found no significant difference in median F_{ST} for unbiased genes expressed primarily in the gonad relative to those expressed broadly across both the gonad and the spleen (Table S4). We observed the same pattern in the spleen, with the exception of the goose and turkey where F_{ST} was elevated marginally. Second, there was no significant difference in the number of unbiased genes with elevated intersexual F_{ST} that were expressed primarily in the gonad compared to those expressed in both tissues (Table 1). We observe the same result in the spleen, with the exception of the turkey. However,

TABLE 1 Observed and expected number of genes with intersexual $F_{ST} > 0$ across tissue-biased genes

	Gonad-biased			Spleen-biased		
Species	E	O	*p*-value	E	O	*p*-value
Mallard	116	118	0.875	112	111	0.956
Swan goose	56	65	0.248	56	70	0.056
Wild turkey	166	160	0.644	204	236	**0.026**[a]
Common pheasant	165	163	0.520	187	174	0.532
Guinea fowl	112	124	0.269	151	142	0.461
Indian peafowl	200	209	0.520	217	208	0.532

Note: Only unbiased genes were used in this analysis. Tissue-biased genes were identified from male expression data. Only autosomal genes are included in the analyses. The expected number of genes with intersexual $F_{ST} > 0$ was calculated from observations of F_{ST} in non-tissue-specific genes. *p*-values were calculated using chi-squared tests.

[a]*p*-values in bold are significant ($p < 0.05$)

all of these differences become nonsignificant when we analyse tissue-biased genes identified from female expression data (Table S5 and Table S6). Last, we found no significant effect of tissue bias on F_{ST} after accounting for gene length, average expression level, GC content and Watterson's theta in a multiple regression (TD ~ Tissue bias + log(tW) + log(Gene length) + log(GC) + log (Gene expression level)) (Table S11).

Intriguingly, despite the limited potential role of the gonad in survival, elevated intersexual F_{ST} has been previously detected in gonad-expressed genes in flycatchers (Dutoit et al., 2018). Consistent with this, we find a weak relationship between intersexual F_{ST} and sex-biased gene expression in the gonad, where F_{ST} is significantly elevated in sex-biased genes in some species (Figure S7, Table S12). However, note that our power to quantify intersexual F_{ST} is limited by our sample size. Whilst our results are consistent with flycatchers, the associated effect sizes are weak (sex-bias and F_{ST} for gonad-biased genes r^2 = 0.000–0.042, spleen-biased genes r^2 = 0.000–0.008). Most importantly, our results are consistent with theoretical work suggesting that intersexual divergence in allele frequency may not always be a reliable indicator of ongoing sexual conflict over viability (Kasimatis et al., 2017, 2019), particularly in studies with low numbers of samples.

3.3 | Regulatory evolution is associated with resolved conflict over long evolutionary time frames

We contrasted population genomic statistics across sex-biased and unbiased genes to test the role of regulatory variation in sexual conflict resolution. We found that autosomal sex-biased genes expressed in the gonad have significantly lower Tajima's *D* than unbiased genes across all six species, consistent with largely resolved sexual conflict (Figure 2 and Figure S2). However, male- and female-biased genes also have significantly elevated intersexual F_{ST} in many species (Figure S7), even after accounting for potential covariates (Table S12). These results are consistent with a potential role of regulatory evolution in conflict resolution via the evolution of sex-specific architecture (Wright et al., 2018). We observed a similar pattern across spleen-biased genes (Figure 2 and Figure S2), although the differences are nonsignificant, probably because of reduced power due to limited numbers of sex-biased genes in somatic tissue.

Employing discrete thresholds to identify sex-biased genes has been shown to have a major effect on the number of genes identified (Ingleby, Flis, & Morrow, 2015). We therefore next investigated the relationship between Tajima's *D* and sex-bias using a polynomial approach (Cheng & Kirkpatrick, 2016). These results confirmed our finding that sex-biased genes have lower Tajima's *D* (Tables S7, S8, S9 and S10). It is important to note that the variance in Tajima's *D* that is accounted for by these associations is extremely low (sex-bias and *D* for gonad-biased genes r^2 = 0.007–0.147, spleen-biased genes r^2 = 0.000–0.018), similar to findings of previous somatic studies in fish (Wright et al., 2018), probably resulting, at least in part, from the inherent noise in Tajima's *D* estimates.

To quantify the pervasiveness of sexual conflict and extent to which balancing selection shapes patterns of genetic diversity across related species, we identified reciprocal orthologues across the six species, which last shared a common ancestor 90 million years ago. Across reciprocal orthologues on the autosomes, we identified genes with elevated Tajima's *D* in all species: specifically, where Tajima's *D* was in the top 10% quantile in each species separately. The average range of Tajima's *D* values for this highest 10% class across species was 1.41–3.26. Using ancestral reconstructions of gene expression levels (Harrison et al., 2015) (Methods S1), we identified gonadal genes that were ancestrally and universally either sex-biased or unbiased across all six species. We found that gonadal genes that were ancestrally sex-biased across the clade were significantly less likely to show elevated Tajima's *D* across all six species than expected from random permutations (245 genes, χ^2 $p < 0.001$, 1,000 permutations). In contrast, universally unbiased genes were significantly enriched in genes with elevated Tajima's *D* across all species (141 genes, χ^2 $p < 0.001$, 1,000 permutations). Our results are robust across multiple quantile thresholds used to define elevated Tajima's *D* (Results S1). This indicates that sexual conflict can shape patterns of genetic diversity in certain sets of sex-biased genes across evolutionary time frames.

3.4 | Conflict over reproductive potential is greatest in sexually dimorphic species

To investigate the relationship between sexual conflict and levels of genetic diversity across the genome, we conducted a phylogenetically controlled comparative analysis of Tajima's *D* across species that vary in mating system and sexual dimorphism. Specifically, we used phylogenetic generalized least squares (PGLS) from the R package CAPER (Orme et al., 2013) to test the relationship between Tajima's *D* and measures of sexual dimorphism, while accounting for the observed level of phylogenetic signal in the data. For each species, we quantified median Tajima's *D* for spleen- and gonad-biased genes relative to non-tissue-biased genes. Tajima's *D* cannot be compared directly across species or populations, as demographic history has a major influence on genetic diversity, and therefore on Tajima's *D* estimation. Calculating a relative measure of Tajima's *D* makes it possible to circumvent problems arising from demographic changes in population size. There are a number of phenotypic indices of sexual conflict, including degree of sexual dichromatism, sperm number and residual testes weight, that are widely used indicators of post-copulatory sexual selection and therefore a measure of variance in male mating success in birds (Birkhead & Moller, 1998; Moller, 1991; Pitcher, Dunn, & Whittingham, 2005). We recovered a significant and positive relationship between relative Tajima's *D* in the gonad and sexual dichromatism ($r^2 = 0.890$, $p = 0.003$) after correcting for phylogeny, and marginally nonsignificant positive associations with both sperm number ($r^2 = 0.491$, $p = 0.073$) and residual testes weight ($r^2 = 0.298$, $p = 0.152$).

The proportion of sex-biased genes varies with mating system across these species (Harrison et al., 2015), which together with the fact that sex-biased genes have distinct patterns of Tajima's *D* (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Wright et al., 2018) and are subject to different selective pressures relative to unbiased genes (Ellegren & Parsch, 2007; Harrison et al., 2015), may confound the pattern we observe. We therefore repeated the analyses using relative median Tajima's *D* calculated using only unbiased genes in each tissue. In doing so, we found that relative Tajima's *D* in the gonad becomes significantly and positively correlated with sexual dichromatism ($r^2 = 0.788$, $p = 0.011$), and sperm number ($r^2 = 0.679$, $p = 0.027$) after correcting for phylogenetic relationships (Figure 3), and marginally nonsignificantly associated with residual testes weight ($r^2 = 0.446$, $p = 0.089$). In contrast, there was no significant association with Tajima's *D* in the spleen and measures of sexual dimorphism (Figure S4).

Interestingly, we found no significant relationship between Tajima's *D* and phenotypic sexual conflict for Z-linked genes in either tissue (Figure S6). Given there are fewer genes on the Z chromosome relative to the autosomes, this pattern might simply be a consequence of smaller sample sizes and therefore greater uncertainty around the median. To assess the role of gene number in our population genetic parameter estimates, we subsampled tissue-biased genes on the autosomes to the equivalent number of the Z-linked genes in each species 1,000 times. The Pearson's correlation coefficients for the relationship between Tajima's *D* and sexual dichromatism, testes weight and sperm number for gonad-biased Z-linked genes are smaller relative to the subsampled data set ($p = 0.027$, $p = 0.048$, $p = 0.168$). The slope of the regression is also smaller than the subsampled data ($p = 0.024$, $p = 0.058$, $p = 0.121$). This indicates that our failure to observe a significant relationship between Tajima's *D* and sexual conflict on the Z chromosome is not a consequence of reduced gene numbers relative to the autosomes.

4 | DISCUSSION

The manifestation, resolution and consequences of intralocus sexual conflict have been the subject to considerable recent debate. To address this, we exploited natural variation in the magnitude of sexual conflict across the body plan within individuals, and across mating systems between species, in a clade of birds that diverged 90 million years ago.

The role of regulatory variation between males and females in the resolution of sexual conflict has received substantial attention in recent literature, with population genomic studies suggesting that sex-biased genes are subject to ongoing sexual antagonism (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018) and

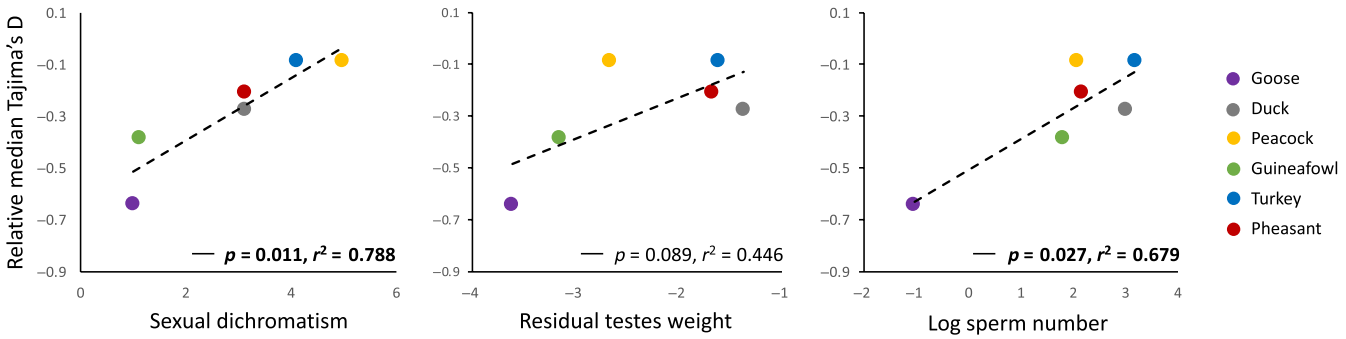

FIGURE 3 Phylogenetically controlled regression between proxies of sperm competition and Tajima's *D* in the gonad. Relative *D* is shown for autosomal genes with unbiased expression between males and females in the gonad. Relative *D* is calculated as the difference between median *D* for tissue-biased genes compared to non-tissue-biased genes. Tissue-biased genes were identified from male expression data. We tested the relationship between Tajima's *D* and measures of sexual dimorphism, while accounting for the observed level of phylogenetic signal in the data [Colour figure can be viewed at wileyonlinelibrary.com]

others indicating that they represent resolved conflict (Innocenti & Morrow, 2010; Wright et al., 2018). Sex-biased genes in the guppy tail, particularly male-biased genes, resolve conflict arising over reproduction through the evolution of separate sex-specific genetic architectures (Wright et al., 2018). However, as this tissue is heavily implicated in female mate choice and therefore primarily affects male reproductive fitness, it is possible that the relative importance of male versus female expression is unusual in this tissue and that sex-biased genes play equal roles in most species. Contrary to this, Dutoit et al. (2018) suggest that ongoing sexual antagonism is more prevalent in male- than female-biased genes in the gonad, potentially hinting at an important role for female-biased expression in conflict resolution. However, without a direct comparison between sex-biased and unbiased genes, the relationship remains unclear. Finally, both male- and female-biased genes in humans show elevated F_{ST} measures (Cheng & Kirkpatrick, 2016), although it is not clear how much of this signal is due to somatic versus gonadal expression, or whether this was associated with elevated Tajima's *D*.

Here, we find that balancing selection is weaker in sex-biased genes relative to unbiased genes, consistent with an important role for sex-biased expression in the resolution of sexual conflict. Lower Tajima's *D* in sex-biased genes is consistent with the rapid rates of evolution in this class of genes observed across many species (Ellegren & Parsch, 2007; Mank, 2017; Parsch & Ellegren, 2013; Rowe et al., 2018), either through positive selection (Meiklejohn et al., 2003; Pröschel et al., 2006; Zhang et al., 2007) or relaxed purifying selection (Dapper & Wade, 2016; Dutoit et al., 2018; Gershoni & Pietrokovski, 2014; Harrison et al., 2015). Balancing selection, which slows the fixation of alleles, is inconsistent with accelerated rates of sequence evolution observed for sex-biased genes (Harrison et al., 2015; Wright & Mank, 2013). In contrast, resolved conflict, which results in sex-specific selection and separate male and female genetic architectures suggested by our data, is expected to lead to the higher levels of standing diversity and faster rates of evolution observed across sex-biased genes in a broad array of taxa (Dapper & Wade, 2016).

Whereas identifying the mechanisms responsible for the resolution of genomic sexual conflict has received considerable attention, the consequences for phenotypic evolution have been comparatively understudied. This is in part due to the difficulties in identifying specific loci subject to sexual conflict and establishing their phenotypic effects from genome scans alone. Our study adds considerably to this goal by using different levels of dimorphism within the body plan and across related species to determine the relationship between population genetic and phenotypic measures of sexual conflict.

Relative to the spleen, the gonad is more phenotypically sexually dimorphic, has higher levels of sex-biased gene expression, and has evolved many sex-specific functions. If sexual dimorphism represents resolved sexual conflict, we might expect gonad-biased genes to have lower levels of balancing selection than spleen-biased genes and loci expressed similarly in both tissues. Consistent with this prediction, we find reduced balancing selection in the gonad, indicative of lower levels of ongoing sexual conflict. This supports the theory that resolved sexual conflict facilitates the evolution of phenotypic sex differences. It is plausible that the large numbers of sex-biased genes in the gonad relative to somatic tissue act to resolve conflict through regulatory decoupling of male and female expression and the evolution of sex-specific architecture.

While we found that intralocus sexual conflict is resolved in the gonad, we found a significant and positive correlation between the magnitude of sexual conflict, arising from differences in mating system, and balancing selection in the gonad but not the spleen. Whilst this may appear initially contradictory, this relationship is in fact consistent with an ephemeral nature of sexual antagonism and rapid turnover of sexual conflict loci. This is in line with previous work showing that sex-biased genes exhibit rapid rates of evolution and turnover (Harrison et al., 2015; Zhang et al., 2007). Our results suggest that unbiased genes are the locus of ongoing sexual conflict due to mating system, and that increasing levels of sexual conflict over reproduction result in elevated levels of genetic diversity across a greater proportion of genes. In contrast, relative Tajima's *D* in spleen-biased genes is not associated with any phenotypic measure of sexual conflict, suggesting that sexual conflict over reproduction has the greatest potential to contribute significantly to variation in the maintenance of genetic diversity across species. This has important consequences for understanding the relationship between sexual conflict and adaptation, where higher levels of conflict promote genetic diversity and provide genetic fuel for adaptive opportunities (Candolin & Heuschele, 2008; Chenoweth, Appleton, Allen, & Rundle, 2015; Jacomb, Marsh, & Holman, 2016; Lumley et al., 2015).

In contrast, we observed no significant relationship between mating system and balancing selection on the Z chromosome. Previously, we showed that the adaptive potential of the Z chromosome is compromised by increasing sexual selection, which decreases the relative effective population size of the Z chromosome compared to autosomes (Wright et al., 2015), leading to increased levels of genetic drift. This means that Z-linked genes in sexually dimorphic species are subject to higher levels of genetic drift (Wright & Mank, 2013). Our results indicate that the potential for sexual conflict to shape patterns of genetic diversity on the Z chromosome might be counteracted by the depleting forces of genetic drift, and that sexual conflict may not play a disproportionally greater role in Z chromosome evolution compared to the rest of the genome.

Negative Tajima's *D* can be interpreted in the context of positive selection, where selective sweeps can result in lower estimates. A greater frequency of selective sweeps in sex-biased genes could therefore explain our finding that Tajima's *D* is lower in the gonad than in the spleen. Furthermore, the positive correlation between Tajima's *D* and sexual dimorphism we observe in the gonad could also be due to more intense positive selection in species with less sexual dimorphism. However, elevated positive selection is unlikely to explain our results, as previous research on the same data set found no significant evidence for positive selection acting on sex-biased genes in the gonad, or any evidence for variation in the magnitude of

positive selection across species based on mating system (Harrison et al., 2015). Therefore, we conclude that lower Tajima's *D* is indicative of lower levels of balancing selection and resolved intralocus conflict, probably mediated by the evolution of sex-biased gene expression.

Population genomic measures of intersexual F_{ST} and Tajima's *D* can be influenced by a number of demographic events, not just sexual conflict, including sex-biased migration, sex-biased predation and changes in population size (Hartl & Clark, 2006). By conducting comparisons of population genomic statistics within each species, instead of directly comparing across species, we controlled for the effect of population contractions or expansions, and our use of captive populations further minimizes the effects of sex-biased migration or predation. Furthermore, samples were taken from all individuals during their first breeding season, effectively controlling for age differences that can confound measures of intersexual F_{ST} or lead to high levels of regulatory variation. However, we note that due to statistical noise, probably due to low sample sizes, we could not reliably identify specific loci subject to sexual conflict, and instead compare large groups of genes to determine broad trends across tissues and species. Our analyses of intersexual F_{ST} are particularly limited by sample size and therefore we urge caution when interpreting these in the light of sexual conflict. However, while we do find loci with elevated intersexual F_{ST}, which has previously been interpreted as evidence for ongoing sexual conflict (Cheng & Kirkpatrick, 2016; Dutoit et al., 2018; Lucotte et al., 2016), the number of loci with elevated F_{ST} do not appear to differ between the gonad and spleen, despite the obvious differences in function and role in survival between the two tissues.

Interestingly, our failure to detect differences in conflict over viability between the tissues is consistent with recent theoretical work (Kasimatis et al., 2017) suggesting that the magnitude of sexual conflict, and associated mortality load, required to generate patterns of intersexual F_{ST} across large numbers of loci is implausibly high. This suggests that they may be a result of alternative demographic processes or statistical noise arising from low sample sizes, instead of ongoing sexual conflict. Instead, our previous work indicates that divergence in allele frequencies between males and females in somatic tissue could instead be indicative of the evolution of sex-specific architectures, which would invoke weaker genetic loads.

In conclusion, our findings suggest that mating system can significantly increase standing diversity across the genome via sexual conflict. More importantly, our results suggest that sexual conflict is short-lived, and is resolved via the decoupling of male and female gene expression patterns. Our results are consistent both across a gradient of sexual dimorphism within the body plan and across species, and have important implications regarding the role of sexual selection in adaptive potential (Candolin & Heuschele, 2008; Chenoweth et al., 2015; Jacomb et al., 2016; Lumley et al., 2015), the persistence of sexual conflict over evolutionary timescales, and the role of dimorphism in facilitating sex-specific fitness optima.

ACKNOWLEDGEMENTS

This work was supported by a NERC Independent Research Fellowship to A.E.W., and the European Research Council (grant agreements 260233 and 680951) to J.E.M., who also gratefully acknowledges funding from a Canada 150 Research Chair. We thank Thorfinn Korneliussen for help with ANGSD and NGSTOOLS, Ida Koltke with NGSRELATE, Jon Slate, Kai Zeng and Toni Gossmann for productive discussions, and four anonymous reviewers for helpful comments. The authors acknowledge the use of the UCL Legion High Performance Computing Facility (Legion@UCL), and associated support services, in the completion of this work.

DATA AVAILABILITY STATEMENT

RNA-seq data are publicly available in the NCBI SRA (BioProject ID PRJNA271731). Transcriptome assemblies are available via Dryad (https://doi.org/10.5061/dryad.1v2d850). Statistics for autosomal genes in each species are available in Supporting Information data files.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Andersson, M. (1994). *Sexual selection*. Princeton, NJ: Princeton University Press

Barson, N. J., Aykanat, T., Hindar, K., Baranski, M., Bolstad, G. H., Fiske, P., …Primmer, C. R. . (2015). Sex-dependent dominance at a single locus maintains variation in age at maturity in salmon. *Nature*, *528*, 405–408.

Bhatia, G., Patterson, N., Sankararaman, S., & Price, A. L. (2013). Estimating and interpreting F_{ST}: The impact of rare variants. *Genome Research*, *23*, 1514–1521. https://doi.org/10.1101/gr.154831.113

Birkhead, T., & Moller, A. P. (1998). *Sperm competition and sexual selection*. San Diego, CA: Academic Press Inc.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Candolin, U., & Heuschele, J. (2008). Is sexual selection beneficial during adaptation to environmental change? *Trends in Ecology & Evolution*, *23*, 446–452. https://doi.org/10.1016/j.tree.2008.04.008

Charlesworth, D. (2006). Balancing selection and its effects on sequences in nearby genome regions. *PLoS Genetics*, *2*, e64. https://doi.org/10.1371/journal.pgen.0020064

Cheng, C., & Kirkpatrick, M. (2016). Sex-specific selection and sex-biased gene expression in humans and flies. *PLoS Genetics*, *12*, e1006170. https://doi.org/10.1371/journal.pgen.1006170

Chenoweth, S. F., Appleton, N. C., Allen, S. L., & Rundle, H. D. (2015). Genomic evidence that sexual selection impedes adaptation to a novel environment. *Current Biology*, *25*, 1860–1866. https://doi.org/10.1016/j.cub.2015.05.034

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences USA*, *98*, 1671–1675. https://doi.org/10.1073/pnas.98.4.1671

Connallon, T., & Knowles, L. L. (2005). Intergenomic conflict revealed by patterns of sex-biased gene expression. *Trends in Genetics*, *21*, 495–499. https://doi.org/10.1016/j.tig.2005.07.006

Czorlich, Y., Aykanat, T., Erkinaro, J., Orell, P., & Primmer, C. R. (2018). Rapid sex-specific evolution of age at maturity is shaped by genetic

architecture in Atlantic salmon. *Nature Ecology & Evolution*, *2*, 1800–1807. https://doi.org/10.1038/s41559-018-0681-5

Dapper, A. L., & Wade, M. J. (2016). The evolution of sperm competition genes: The effect of mating system on levels of genetic variation within and between species. *Evolution*, *70*, 502–511. https://doi.org/10.1111/evo.12848

Delcourt, M., Blows, M. W., & Rundle, H. D. (2009). Sexually antagonistic genetic variance for fitness in an ancestral and a novel environment. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 2009–2014. https://doi.org/10.1098/rspb.2008.1459

Dutoit, L., Mugal, C. F., Bolívar, P., Wang, M., Nadachowska-Brzyska, K., Smeds, L., … Ellegren, H. (2018). Sex-biased gene expression, sexual antagonism and levels of genetic diversity in the collared flycatcher (*Ficedula albicollis*) genome. *Molecular Ecology*, *27*, 3572–3581.

Ellegren, H., & Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene expression. *Nature Reviews Genetics*, *8*, 689. https://doi.org/10.1038/nrg2167

Foerster, K., Coulson, T., Sheldon, B. C., Pemberton, J. M., Clutton-Brock, T. H., & Kruuk, L. E. B. (2007). Sexually antagonistic genetic variation for fitness in red deer. *Nature*, *447*, 1107–1110. https://doi.org/10.1038/nature05912

Fumagalli, M., Vieira, F. G., Linderoth, T., & Nielsen, R. (2014). ngsTools: Methods for population genetics analyses from next-generation sequencing data. *Bioinformatics*, *30*, 1486–1487. https://doi.org/10.1093/bioinformatics/btu041

Gershoni, M., & Pietrokovski, S. (2014). Reduced selection and accumulation of deleterious mutations in genes exclusively expressed in men. *Nature Communications*, *5*, 4438. https://doi.org/10.1038/ncomms5438

Ghosh, R., Andersen, E. C., Shapiro, J. A., Gerke, J. P., & Kruglyak, L. (2012). Natural variation in a chloride channel subunit confers avermectin resistance in *C. elegans*. *Science*, *335*, 574–578. https://doi.org/10.1126/science.1214318

Gilks, W. P., Abbott, J. K., & Morrow, E. H. (2014). Sex differences in disease genetics: Evidence, evolution, and detection. *Trends in Genetics*, *30*, 453–463. https://doi.org/10.1016/j.tig.2014.08.006

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*, 644. https://doi.org/10.1038/nbt.1883

Harrison, P. W., Wright, A. E., Zimmer, F., Dean, R., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences USA*, *112*, 4393–4398. https://doi.org/10.1073/pnas.1501339112

Hartl, D. L., & Clark, A. G. (2006). *Principles of population genetics*. Sunderland, MA: Sinauer Associates Inc.

Hawkes, M. F., Gamble, C. E., Turner, E. C. R., Carey, M. R., Wedell, N., & Hosken, D. J. (2016). Intralocus sexual conflict and insecticide resistance. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20161429. https://doi.org/10.1098/rspb.2016.1429

Hedrick, P. W. (2011). Population genetics of malaria resistance in humans. *Heredity*, *107*, 283–304. https://doi.org/10.1038/hdy.2011.16

Ingleby, F. C., Flis, I., & Morrow, E. H. (2015). Sex-biased gene expression and sexual conflict throughout development. *Cold Spring Harbor Perspectives in Biology*, *7*, a017632.

Innocenti, P., & Morrow, E. H. (2010). The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS Biology*, *8*, e1000335. https://doi.org/10.1371/journal.pbio.1000335

Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., … Arnold, A. P. (2007). Dosage compensation is less effective in birds than in mammals. *Journal of Biology*, *6*, 2–2. https://doi.org/10.1186/jbiol53

Jacomb, F., Marsh, J., & Holman, L. (2016). Sexual selection expedites the evolution of pesticide resistance. *Evolution*, *70*, 2746–2751. https://doi.org/10.1111/evo.13074

Karp, N. A., Mason, J., Beaudet, A. L., Benjamini, Y., Bower, L., Braun, R. E., … White, J. K. (2017). Prevalence of sexual dimorphism in mammalian phenotypic traits. *Nature Communications*, *8*, 15475.

Kasimatis, K. R., Nelson, T. C., & Phillips, P. C. (2017). Genomic signatures of sexual conflict. *Journal of Heredity*, *108*, 780–790. https://doi.org/10.1093/jhered/esx080

Kasimatis, K. R., Ralph, P. L., & Phillips, P. C. (2019). Limits to genomic divergence under sexually antagonistic selection. *Biorxiv*, 591610. https://doi.org/10.1101/591610

Korneliussen, T. S., Albrechtsen, A., & Nielsen, R. (2014). ANGSD: Analysis of next generation sequencing data. *BMC Bioinformatics*, *15*, 356. https://doi.org/10.1186/s12859-014-0356-4

Korneliussen, T. S., & Moltke, I. (2015). NgsRelate: A software tool for estimating pairwise relatedness from next-generation sequencing data. *Bioinformatics*, *31*, 4009–4011.

Lewontin, R. C., & Krakauer, J. (1973). Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics*, *74*, 175–195.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Genome Project Data Processing S (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Lonn, E., Koskela, E., Mappes, T., Mokkonen, M., Sims, A. M., & Watts, P. C. (2017). Balancing selection maintains polymorphisms at neurogenetic loci in field experiments. *Proceedings of the National Academy of Sciences USA*, *114*, 3690–3695. https://doi.org/10.1073/pnas.1621228114

Lucotte, E. A., Laurent, R., Heyer, E., Ségurel, L., & Toupance, B. (2016). Detection of allelic frequency differences between the sexes in humans: A signature of sexually antagonistic selection. *Genome Biology and Evolution*, *8*, 1489–1500. https://doi.org/10.1093/gbe/evw090

Lumley, A. J., Michalczyk, Ł., Kitson, J. J. N., Spurgin, L. G., Morrison, C. A., Godwin, J. L., … Gage, M. J. G. (2015). Sexual selection protects against extinction. *Nature*, *522*, 470–473. https://doi.org/10.1038/nature14419

Mank, J. E. (2009). Sex chromosomes and the evolution of sexual dimorphism: Lessons from the genome. *The American Naturalist*, *173*, 141–150. https://doi.org/10.1086/595754

Mank, J. E. (2017). Population genetics of sexual conflict in the genomic era. *Nature Reviews Genetics*, *18*, 721–730. https://doi.org/10.1038/nrg.2017.83

Mank, J. E., & Ellegren, H. (2008). All dosage compensation is local: Gene-by-gene regulation of sex-biased expression on the chicken Z chromosome. *Heredity*, *102*, 312–320.

Meiklejohn, C. D., Parsch, J., Ranz, J. M., & Hartl, D. L. (2003). Rapid evolution of male-biased gene expression in *Drosophila*. *Proceedings of the National Academy of Sciences USA*, *100*, 9894–9899. https://doi.org/10.1073/pnas.1630690100

Mokkonen, M., Kokko, H., Koskela, E., Lehtonen, J., Mappes, T., Martiskainen, H., & Mills, S. C. (2011). Negative frequency-dependent selection of sexually antagonistic alleles in *Myodes glareolus*. *Science*, *334*, 972–974. https://doi.org/10.1126/science.1208708

Moller, A. P. (1991). Sperm compeition, sperm depletion, paternal care, and relative testis size in birds. *The American Naturalist*, *137*, 882–906.

Mostafavi, H., Berisa, T., Day, F. R., Perry, J. R. B., Przeworski, M., & Pickrell, J. K. (2017). Identifying genetic variants that affect viability in large cohorts. *PLoS Biology*, *15*, e2002458. https://doi.org/10.1371/journal.pbio.2002458

Nielsen, R., Korneliussen, T., Albrechtsen, A., Li, Y., & Wang, J. (2012). SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data. *PLoS ONE*, *7*, e37558.

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., & Pearse, W. (2013). caper: comparative analyses of phylogenetics and evolution in R. R package version 0.5.2.

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *353*, 261–274. https://doi.org/10.1098/rstb.1998.0208

Parsch, J., & Ellegren, H. (2013). The evolutionary causes and consequences of sex-biased gene expression. *Nature Reviews Genetics*, *14*, 83–87. https://doi.org/10.1038/nrg3376

Pitcher, T. E., Dunn, P. O., & Whittingham, L. A. (2005). Sperm competition and the evolution of testes size in birds. *Journal of Evolutionary Biology*, *18*, 557–567. https://doi.org/10.1111/j.1420-9101.2004.00874.x

Pröschel, M., Zhang, Z., & Parsch, J. (2006). Widespread adaptive evolution of *Drosophila* genes with sex-biased expression. *Genetics*, *174*, 893–900. https://doi.org/10.1534/genetics.106.058008

Rice, W. R. (1984). Sex chromosomes and the evolution of sexual dimorphism. *Evolution*, *38*, 735–742. https://doi.org/10.1111/j.1558-5646.1984.tb00346.x

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*, 139–140. https://doi.org/10.1093/bioinformatics/btp616

Rowe, L., Chenoweth, S. F., & Agrawal, A. F. (2018). The genomics of sexual conflict. *The American Naturalist*, *192*, 274–286. https://doi.org/10.1086/698198

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., & Bergelson, J. (1999). Dynamics of disease resistance polymorphism at the Rpm1 locus of Arabidopsis. *Nature*, *400*, 667–671. https://doi.org/10.1038/23260

Stewart, A. D., & Rice, W. R. (2018). Arrest of sex-specific adaptation during the evolution of sexual dimorphism in Drosophila. *Nature Ecology & Evolution*, *2*, 1507–1513. https://doi.org/10.1038/s41559-018-0613-4

Wang, Y., Tang, H., DeBarry, J. D., Tan, X., Li, J., Wang, X., … Paterson, A. H. (2012). MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Research*, *40*, e49–e49. https://doi.org/10.1093/nar/gkr1293

Waterhouse, R. M., Seppey, M., Simão, F. A., Manni, M., Ioannidis, P., Klioutchnikov, G., … Zdobnov, E. M. (2018). BUSCO applications from quality assessments to gene prediction and phylogenomics. *Molecular Biology and Evolution*, *35*, 543–548. https://doi.org/10.1093/molbev/msx319

Wright, A. E., Fumagalli, M., Cooney, C. R., Bloch, N. I., Vieira, F. G., Buechel, S. D., … Mank, J. E. (2018). Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evolution Letters*, *2*, 52–61. https://doi.org/10.1002/evl3.39

Wright, A. E., Harrison, P. W., Zimmer, F., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Variation in promiscuity and sexual selection drives avian rate of Faster-Z evolution. *Molecular Ecology*, *24*, 1218–1235. https://doi.org/10.1111/mec.13113

Wright, A. E., & Mank, J. E. (2013). The scope and strength of sex-specific selection in genome evolution. *Journal of Evolutionary Biology*, *26*, 1841–1853. https://doi.org/10.1111/jeb.12201

Wright, A. E., Moghadam, H. K., & Mank, J. E. (2012). Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*, *192*, 1433–1445. https://doi.org/10.1534/genetics.112.145102

Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., … Flicek, P. (2018). Ensembl 2018. *Nucleic Acids Research*, *46*, D754–D761. https://doi.org/10.1093/nar/gkx1098

Zhang, Y., Sturgill, D., Parisi, M., Kumar, S., & Oliver, B. (2007). Constraint and turnover in sex-biased gene expression in the genus Drosophila. *Nature*, *450*, 233–237. https://doi.org/10.1038/nature06323

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Wright AE, Rogers TF, Fumagalli M, Cooney CR, Mank JE. Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Mol Ecol.* 2019;28:2860–2871. https://doi.org/10.1111/mec.15115

Received: 1 July 2019 | Revised: 5 September 2019 | Accepted: 13 September 2019

DOI: 10.1111/mec.15245

INVITED REVIEWS AND SYNTHESES

MOLECULAR ECOLOGY WILEY

How to identify sex chromosomes and their turnover

Daniela H. Palmer[1] | Thea F. Rogers[1] | Rebecca Dean[2] | Alison E. Wright[1]

[1]Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK

[2]Department of Genetics, Evolution and Environment, University College London, London, UK

Correspondence
Daniela H. Palmer, Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK.
Email: d.h.palmer@sheffield.ac.uk

Funding information
Marie Curie Actions, Grant/Award Number: 655392; Directorate for Biological Sciences, Grant/Award Number: 1812164; Natural Environment Research Council, Grant/Award Number: NE/N013948/1

Abstract

Although sex is a fundamental component of eukaryotic reproduction, the genetic systems that control sex determination are highly variable. In many organisms the presence of sex chromosomes is associated with female or male development. Although certain groups possess stable and conserved sex chromosomes, others exhibit rapid sex chromosome evolution, including transitions between male and female heterogamety, and turnover in the chromosome pair recruited to determine sex. These turnover events have important consequences for multiple facets of evolution, as sex chromosomes are predicted to play a central role in adaptation, sexual dimorphism, and speciation. However, our understanding of the processes driving the formation and turnover of sex chromosome systems is limited, in part because we lack a complete understanding of interspecific variation in the mechanisms by which sex is determined. New bioinformatic methods are making it possible to identify and characterize sex chromosomes in a diverse array of non-model species, rapidly filling in the numerous gaps in our knowledge of sex chromosome systems across the tree of life. In turn, this growing data set is facilitating and fueling efforts to address many of the unanswered questions in sex chromosome evolution. Here, we synthesize the available bioinformatic approaches to produce a guide for characterizing sex chromosome system and identity simultaneously across clades of organisms. Furthermore, we survey our current understanding of the processes driving sex chromosome turnover, and highlight important avenues for future research.

KEYWORDS
bioinformatics, next-generation sequencing, sex chromosome turnover, sex chromosomes

Glossary: Achiasmy Complete recombination suppression in one sex.
Coverage Number of DNA-seq reads that represent a given nucleotide in a reference genome. For autosomal regions, coverage can be calculated as $N \times L/G$, where N is the number of reads, L is read length, and G is the length of the reference genome.
Dosage compensation A mechanism to maintain ancestral expression levels of the X or Z chromosome relative to the autosomes in the heterogametic sex. This is thought to evolve in response to degeneration of the sex-limited chromosome and subsequent unequal gene dose between males and females.
Heteromorphic sex chromosome Sex chromosomes that are karyotypically highly distinct from each other. The X and Y (or Z and W) chromosomes are diverged and show differences in gene content and size.
Homomorphic sex chromosome Sex chromosomes that are nearly identical in gene content and size. They are more challenging to identify from cytogenetic data alone.
k-mer All possible subsequences of a given length *k* within a genome.
Pseudoautosomal region (PAR) Homologous region of the sex chromosomes that continues to recombine between the X and Y (or Z and W).
Restriction site-associated DNA (RAD) sequencing A restriction site-associated DNA sequencing technique. A restriction enzyme is used to digest genomic DNA into fragments which are then ligated to adapters that will bind to an Illumina flow cell. Both ends of these fragments are then sequenced using next-generation methods.
Stratum Region where recombination between the sex chromosomes has been halted.
Synteny Conserved collinear regions. Conservation of gene order across two sets of chromosomes that are being compared to each other.

1 | INTRODUCTION

Sexual reproduction is a fundamental feature of eukaryotes, yet the mechanisms by which sex is determined are highly diverse (Bachtrog et al., 2014; Beukeboom & Perrin, 2014; Bull, 1983). This variation is apparent even among closely related species, or populations of the same species (Tree of Sex Consortium, 2014). In many organisms, sex chromosomes are associated with male or female development, and in many groups, including birds (Zhou et al., 2014), eutherian mammals (Cortez et al., 2014) and certain insects (Fraïsse, Picard, & Vicoso, 2017), the sex chromosome system is stable and highly conserved. However, it is apparent that sex chromosomes often evolve rapidly in many lineages, and the chromosome pair that determines sex can change rapidly over time (Pennell, Mank, & Peichel, 2018). In addition to turnover in the chromosome pair recruited to determine sex, transitions between different sex chromosome systems (e.g., XY to ZW, or ZW to XY) are also well documented across numerous clades. This diversity is particularly pronounced in certain groups of reptiles (Gamble et al., 2015; Pokorná & Kratochvíl, 2009), amphibians (Jeffries et al., 2018), fish (Darolti et al., 2019; Kitano & Peichel, 2012; Mank, Promislow, & Avise, 2006), insects (Blackmon & Demuth, 2014; Vicoso & Bachtrog, 2015) and plants (Balounova et al., 2019; Martin et al., 2019; Tennessen et al., 2018), where turnover between male (XY) and female (ZW) heterogamety is common over relatively short evolutionary time periods (Pennell et al., 2018). While recent efforts, including those of the Tree of Sex Consortium, have focused on characterizing the tremendous diversity of sex chromosomes across species, it is clear that we currently have an incomplete understanding of the variation in sex determination mechanisms across the tree of life (Bachtrog et al., 2014; Tree of Sex Consortium, 2014).

Despite the growing awareness that sex chromosomes have evolved independently many times throughout eukaryotes, our understanding of the processes driving the formation and turnover of new sex chromosome systems is limited and many unanswered questions remain. A large body of theoretical work outlines predictions for when and why sex chromosome transitions occur (Beukeboom & Perrin, 2014), including genetic drift (Bull & Charnov, 1977; Saunders, Neuenschwander, & Perrin, 2018), mutation load on the sex-limited chromosomes (Blaser, Grossen, Neuenschwander, & Perrin, 2013; Blaser, Neuenschwander, & Perrin, 2014), selection on sex ratio (Jaenike, 2001; Werren & Beukeboom, 1998) and sexually antagonistic selection (van Doorn & Kirkpatrick, 2007, 2010), yet attempts to empirically test these have been restricted to a few clades (Blackmon & Demuth, 2014; Jeffries et al., 2018; Kitano & Peichel, 2012; Wright et al., 2017). Identifying the evolutionary and genomic mechanisms predicted to drive sex chromosome turnover is a major priority, which in turn will shed light on why sex determination is labile in some taxa and not in others. Furthermore, differences in transmission pattern between male and female heterogametic sex chromosome systems (Beukeboom & Perrin, 2014) are predicted to have important consequences for adaptation (Mank, Vicoso, Berlin, & Charlesworth, 2010; Wright et al., 2015), sexual dimorphism (van Doorn & Kirkpatrick, 2010; Mullon, Wright, Reuter, Pomiankowski, & Mank, 2015; Muralidhar, 2019), and ultimately speciation (Irwin, 2018; Mank et al., 2010). Efforts to rigorously test predictions about the causes and consequences of sex chromosome evolution have been largely hampered by our incomplete knowledge of the diversity of sex chromosomes across a broad taxonomic range and limited power to identify convergent trends across independently evolved sex chromosomes. Traditionally, cytogenetic methods have been used to identify sex chromosome systems and turnover events (Valenzuela, Adams, & Janzen, 2003). However, while there have been recent improvements that facilitate sex chromosome identification using these approaches (Ezaz et al., 2005; Iannucci et al., 2019; Kawai et al., 2007), identifying homomorphic sex chromosomes, where the pair are nearly identical in gene content and size, is still challenging. This might disproportionately affect the identification of ZW systems as W chromosomes are predicted to evolve more slowly than Y chromosomes (Bachtrog et al., 2011), resulting in the underestimation of turnover events. To address how, when, and why sex chromosomes evolve (Wright, Dean, Zimmer, & Mank, 2016) we require far more information on sex chromosomes in diverse clades.

Recently, new bioinformatic methods are making it possible to identify and characterize sex chromosomes in a diverse array of non-model species using next generation sequencing data. In combination with comparative phylogenetic analyses, it is now possible to rigorously test theoretical predictions for sex chromosome formation and turnover. However, despite the diversity of newly developed methods to identify sex chromosomes, there have been limited attempts to synthesize them into a comprehensive guide applicable to a wide range of organisms (but see Muyle, Shearn, & Marais, 2017). This is key because the effectiveness of different approaches is influenced by a number of factors. In particular, the degree of sequence divergence between the sex chromosomes is an important element to consider. Sex chromosomes evolve from a pair of identical autosomes as recombination between the X and Y (or Z and W) is suppressed (Charlesworth, Charlesworth, & Marais, 2005). Recombination cessation catalyzes sequence divergence between the sex chromosomes, which can ultimately lead to heterogametic chromosomes that show major differences in size and gene content with severely degenerated W or Y chromosomes (Charlesworth & Charlesworth, 2000). In contrast, homogametic sex chromosomes are almost identical and exhibit few differences from each other in gene content. It is important to note that homogamety and heterogamety are not discrete states and instead represent two extremes on a continuum of sex chromosome divergence (Figure 1). Certain bioinformatic approaches to identify sex chromosomes are more effective for species at different points on this continuum. In addition, while sex chromosomes across species exhibit variation in the degree of heterogamety, different regions of the same sex chromosome can also fall at different points along this continuum (Figure 1). This is because recombination is often suppressed in a stepwise process, resulting in strata of different ages (Charlesworth et al., 2005; Lahn & Page, 1999; Wright, Moghadam, & Mank, 2012). Therefore,

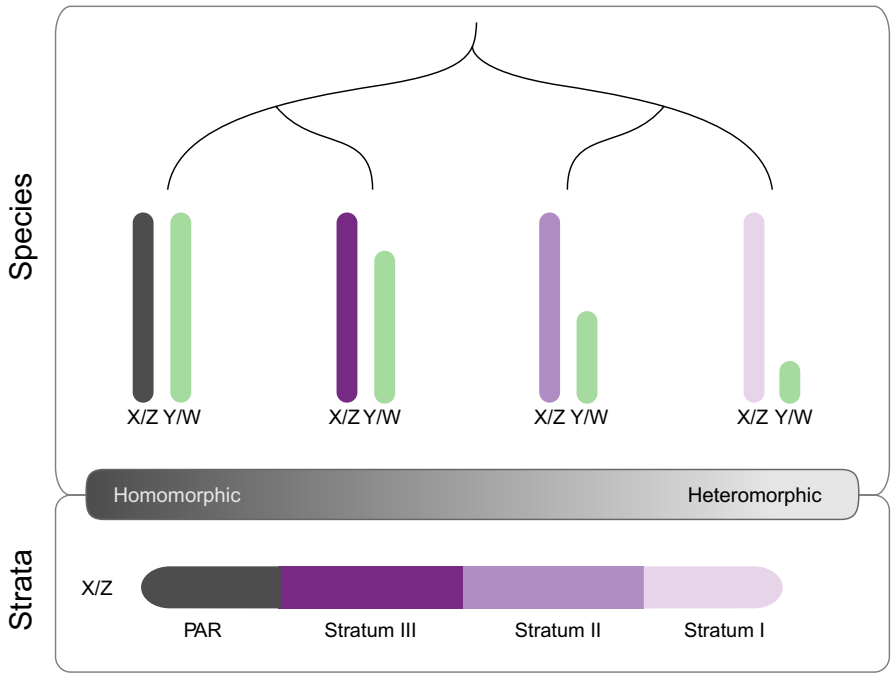

FIGURE 1 Illustration of the homomorphic-heteromorphic sex chromosome continuum. Sex chromosomes can range from heteromorphic, where the X and Y (or Z and W) chromosomes are diverged and highly distinct, to homomorphic, where pairs are nearly identical in gene content and size. However, sex chromosomes can vary in their degree of sequence differentiation not just among species (top panel) but also among strata within a species (bottom panel). Strata are regions of the chromosome where recombination between the sex chromosomes has been halted independently and therefore are of different ages. Different methods for identifying sex-linked loci will be appropriate for species/strata at different points on this continuum. Purple scale indicates sequence differentiation between chromosomes or strata, where lighter purple shows greater divergence

a combination of different, complementary methods is often necessary to identify sex chromosomes, and sex-linked regions, among species.

Here, we review the range of available approaches to identify sex chromosomes and fill in gaps across the tree of life, highlighting the strengths and weaknesses of each. We do not cover methods for high resolution sequencing of sex-limited chromosomes, as these have been discussed elsewhere (Tomaszkiewicz, Medvedev, & Makova, 2017), but instead focus on producing a guide for characterizing sex chromosome system and identity across diverse clades. In turn, we discuss future priorities in sex chromosome research and suggest how to use this growing data set to test, highlighting the strengths and weaknesses of each, how and why sex chromosomes evolve.

2 | GUIDE FOR IDENTIFYING SEX CHROMOSOMES

2.1 | Genomic coverage approach

A common approach to identify sex chromosomes is based on genome coverage from next-generation sequencing data. This approach exploits the difference in sex chromosome ploidy between males and females. In XY systems, X-linked genes show half the number of genomic reads in males relative to females, and Y-linked reads are absent in females (Figure 2a). This can be easily applied to ZW systems, where instead the W is absent in males, and females have only one copy of the Z. Since this approach is based on sex differences in genomic coverage, it is only effective when there is substantial sequence divergence between the sex chromosomes. Therefore, while it can be used to identify heteromorphic sex chromosomes or old, diverged strata, this method will misclassify pseudoautosomal regions, homomorphic sex chromosomes, or young strata as autosomal.

There are three main methods that employ genome coverage to distinguish sex chromosomes from autosomes. In the subtraction-based method, DNA-seq data from the homogametic sex are aligned to a reference genome generated from a heterogametic individual. As male and female genomes differ only by the Y (or W) chromosome, scaffolds with low coverage can be inferred as Y-linked (or W-linked). Whilst this approach can effectively identify sex-limited scaffolds, and therefore establish whether the sex chromosome system is male or female heterogametic, it has limited potential for identifying the X or Z. This step is key for establishing the identity of the sex chromosome pair via synteny-based approaches with other species (see Box 1), as sex-limited chromosomes are often highly degenerated which hinders attempts to infer orthology. Alternatively, the ratio of male to female reads aligned to a reference genome can be used to directly distinguish X from autosomal scaffolds (Darolti et al., 2019; Vicoso & Bachtrog, 2011, 2013; Vicoso, Emerson, Zektser, Mahajan, & Bachtrog, 2013). For example, in an XY system, the male to female coverage ratio for autosomal and X scaffolds should be roughly 1

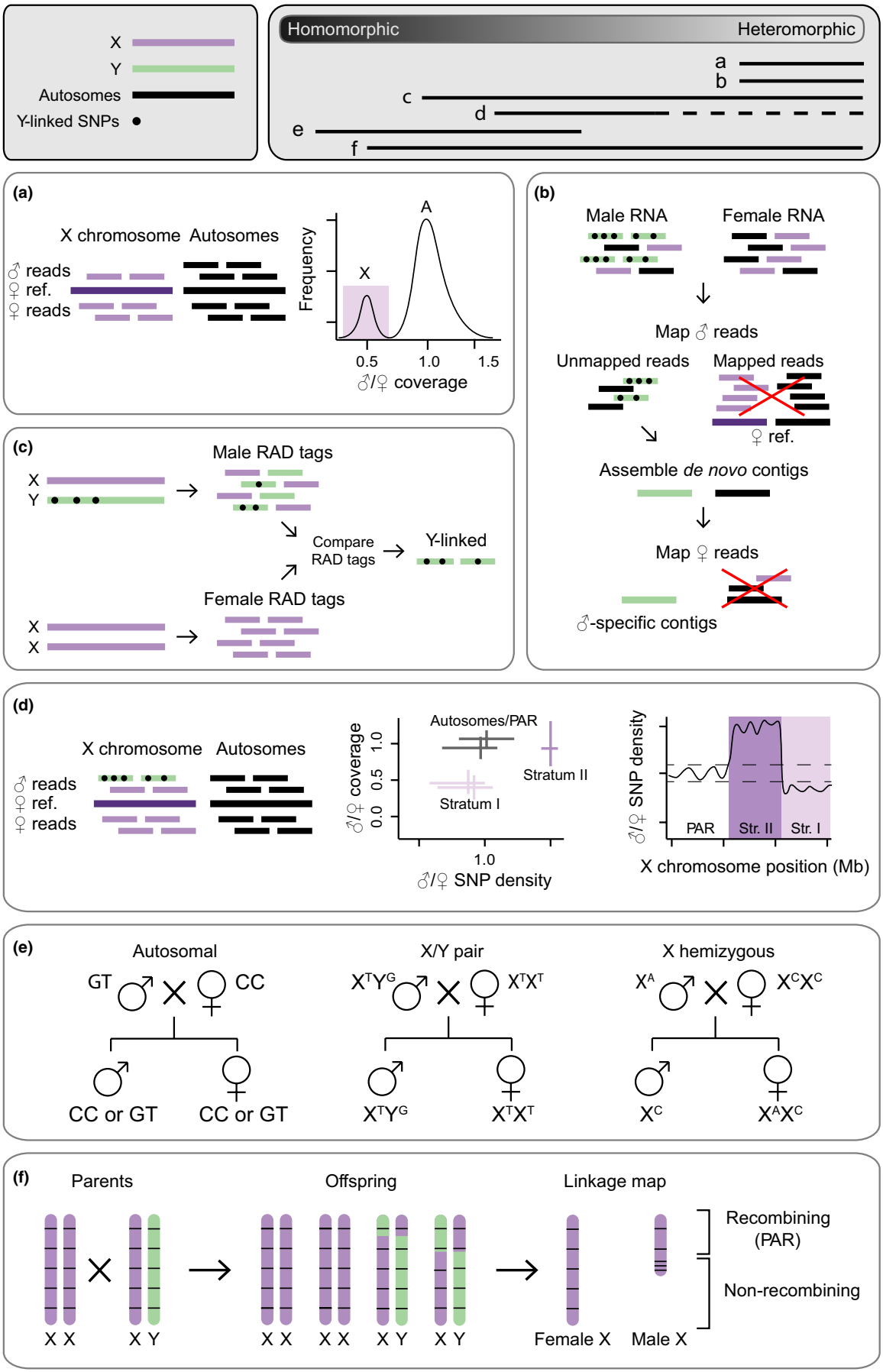
X
Y
Autosomes
Y-linked SNPs
Homomorphic
Heteromorphic
a
b
c
d
e
f
(a)
X chromosome
Autosomes
♂ reads
♀ ref.
♀ reads
Frequency
A
X
0.5
1.0
1.5
♂/♀ coverage
(b)
Male RNA
Female RNA
Map ♂ reads
Unmapped reads
Mapped reads
♀ ref.
Assemble de novo contigs
Map ♀ reads
♂-specific contigs
(c)
Male RAD tags
X
Y
Compare RAD tags
Y-linked
Female RAD tags
X
X
(d)
X chromosome
Autosomes
♂ reads
♀ ref.
♀ reads
♂/♀ coverage
0.0
0.5
1.0
Autosomes/PAR
Stratum II
Stratum I
1.0
♂/♀ SNP density
♂/♀ SNP density
PAR
Str. II
Str. I
X chromosome position (Mb)
(e)
Autosomal
GT
CC
CC or GT
CC or GT
X/Y pair
XTYG
XTXT
XTYG
XTXT
X hemizygous
XA
XCXC
XC
XAXC
(f)
Parents
Offspring
Linkage map
X X
X Y
X X
X X
X Y
X Y
Female X
Male X
Recombining (PAR)
Non-recombining

FIGURE 2 Overview of bioinformatic methods available for sex chromosome identification. This figure is based on XY sex chromosomes, but all methods can be inverted for ZW systems. Top left panel shows the key. Top right panel solid bars show which methods are most effective along different points of the sex chromosome divergence continuum. Dashed bar indicates that the method is partially effective. (a) Genomic coverage approach: in nonrecombining regions of sex chromosomes, where the Y has degenerated, males have only one X chromosome, and thus show a reduced genomic coverage relative to females. (b) Expression-based approach: male RNA-seq reads are mapped to a female reference. Unmapped reads are assembled into de novo contigs to identify putative Y-linked sequences. Re-mapping female transcripts to these contigs can be used to verify male-limitation. (c) Association-based approach: male and female RAD-tags are compared to isolate male-specific RAD loci. (d) SNP density approach: in younger regions of the sex chromosomes, which still retain high sequence similarity between the X and the Y, we expect an increase in male SNP density compared to females, as Y reads, carrying Y-specific SNPs, still map to the homologous X regions. This SNP density pattern is not expected in old strata with substantial Y degeneration, as the X is effectively hemizygous in males. Contrasting sex differences in coverage and SNP density is a powerful approach to identify sex-linked regions. (e) Segregation analysis approach: SNP data obtained from parents and progeny are analyzed in a statistical framework to assess the likelihood of autosomal versus sex-linked segregation patterns. (f) Linkage mapping approach: recombination patterns of parents and offspring are compared, and regions with no recombination between males and females indicate putative sex-linked regions

and 0.5 respectively. A variant of this method is called the chromosome quotient (CQ) approach (Hall et al., 2013). Due to noise in mapping reads to a genome, the male to female coverage ratio is typically a continuum, where there are two overlapping normal distributions of sex differences in coverage, one for the X or Z chromosome and the other for autosomal scaffolds (Figure 2a). Identifying the equidistant point between the maximum of these two peaks can help minimize the error in identifying sex-linked regions, and has been employed successfully across a number of species (Huylmans, Toups, Macon, Gammerdinger, & Vicoso, 2019; Vicoso & Bachtrog, 2015). Lastly, the *k*-mer counting approach (Akagi, Henry, Tao, & Comai, 2014; Carvalho & Clark, 2013; Li et al., 2018; Morris, Darolti, Bloch, Wright, & Mank, 2018; Pucholt, Wright, Conze, Mank, & Berlin, 2017) is based on similar underlying principles. Male and female genomes are broken up into *k*-mers, counted computationally, and autosomal, Y-, and X-linked *k*-mers are identified on the basis of read coverage. This method is unaffected by differences in filtering and read length and can be useful for identifying sex chromosomes across species where next-generation sequencing data sets are of varying quality (Morris et al., 2018). Additionally, *k-mer* analyses have been used to provide insight into the amount of repetitive elements accumulating on recently evolved Y chromosomes (Carvalho & Clark, 2013; Morris et al., 2018; Pucholt et al., 2017). Finally, in combination with next-generation sequencing data obtained from flow-sorted Y chromosomes, *k-mer* approaches can filter contaminant autosomal and X-linked sequences, thus improving the quality of the downstream Y chromosome assembly (Rangavittal et al., 2018).

However, there are a number of important caveats to consider. Coverage approaches are heavily sensitive to the algorithms used to map reads to a reference genome. This is because heteromorphic sex chromosomes still retain sequence orthology between the X and Y, and incorrectly mapped reads can mask coverage differences between the sexes and lead to the misclassification of sex-linked sequences as autosomal. Stringent mapping parameters are recommended to minimize false negatives, with a maximum mismatch of 0 or 1 (Carvalho & Clark, 2013; Hall et al., 2013; Smeds et al., 2015; Vicoso et al., 2013), as well as the filtering of non-uniquely mapped reads (Vicoso & Bachtrog, 2015). Furthermore, repetitive regions of DNA should be masked prior to implementing these approaches to remove repeats shared by the sex-limited chromosome and the autosomes (Carvalho & Clark, 2013; Hall et al., 2013; Smeds et al., 2015; Vicoso & Bachtrog, 2015). A similar caveat applies to the *k*-mer approach, where *k*-mer size can dramatically affect the number of inferred sex-linked scaffolds. In principle, a large *k* ensures that identical *k*-mers rarely result from sequencing errors and increases the probability that sequences encompass sex-limited sites. However, if *k* is too large then *k-mer* depth may be too low to detect statistical sex differences. In contrast, very short *k-mers* are likely to be overrepresented in the data set, leading to low resolution to identify sex-limited regions (Kelley, Schatz, & Salzberg, 2010). The choice of optimal *k*-mer size can range from 15–31 bp depending on genome size of the organism (Carvalho & Clark, 2013; Morris et al., 2018). Coverage-based approaches have been used to identify sex chromosomes from DNA-seq data obtained from only one individual from each sex (Vicoso & Bachtrog, 2013) but read depth must be reasonably high to avoid confounding effects of sequencing errors (see Box 1) (>20-fold; Carvalho & Clark, 2013; Hall et al., 2013; Smeds et al., 2015; Vicoso & Bachtrog, 2015). In practice, multiple individuals of each sex are required to avoid falsely identifying rare SNP variants as sex-linked contigs, the probability of which will depend on the genetic diversity of the population (see Box 1).

2.2 | Expression-based approach

This approach leverages sex differences in gene expression to identify sex-limited transcripts originating from the Y or W chromosome. RNA-seq reads from the heterogametic sex are mapped to a reference generated from the homogametic sex. Successfully mapped reads originate from regions of the genome that are shared between the sexes whereas unmapped reads represent sex-limited regions (Cortez et al., 2014; Moghadam, Pointer, Wright, Berlin, & Mank, 2012). These unmapped reads can be assembled de novo into potential Y- or W-linked contigs. Mapping RNA-seq reads from the homogametic sex onto these putative contigs can be used to validate sex-limitation (Cortez et al., 2014) (Figure 2b).

This approach is similar to subtraction-based methods employed using DNA-seq data and is best optimized for systems with

Box 1 Overarching challenges in identifying sex chromosomes

Identifying homomorphic sex chromosomes

Homomorphic sex chromosomes, or recently diverged strata, are challenging to identify as there is limited sequence divergence between chromosome pairs. Crucially, because homomorphic sex chromosomes can be the result of high sex chromosome turnover (Wright et al., 2016), they are precisely the systems needed to understand the mechanisms underlying the evolution of sex determination (Bachtrog et al., 2014; Beukeboom & Perrin, 2014).

A number of approaches are more suited to detecting homomorphic sex chromosomes than others. Because SNP variation accumulates before sex chromosome decay, differences in heterozygosity between males and females can be detected even when regions have not diverged sufficiently to show coverage differences (Pucholt et al., 2017). Similarly, segregation analysis approaches, such as SEX-DETector (Muyle et al., 2016) perform optimally when X and Y chromosomes coassemble in the reference genome and are therefore best suited to detecting homomorphic sex chromosomes. Since linkage mapping directly measures recombination, this approach can also be used to identify intermediately diverged sex chromosomes; however, depending on the recombination frequency, this may have limited success in defining strata boundaries (Wright et al., 2019).

Bioinformatic margins of error

It is crucial to independently verify candidate sex-linked regions, especially those identified using measures of sequence divergence or other proxies for arrested recombination. Although many of the methods we discuss can be implemented with small sample sizes, using fewer individuals increases the likelihood that candidate loci meet screening criteria by chance or due to sequencing artifacts. PCR amplification of candidates is a simple and widely used method of verification, however, while it is an inexpensive and straightforward method of verification, it can be prohibitively labour-intensive for large-scale studies. Additionally, PCR validation might fail for some loci that are surrounded by conserved sequence (Fowler & Buonaccorsi, 2016; Gamble, 2016), thus requiring additional steps toward verification. Estimating the false positive rate using computational methods can be a complementary and alternative approach to validating sex-linked loci. Permutation tests that shuffle sex assignments among sampled individuals are essential for generating null distributions against which to assess the validity of candidate loci (Huylmans et al., 2019; Jeffries et al., 2018; Morris et al., 2018; Scharmann, Grafe, Metali, & Widmer, 2017; Wright et al., 2017). For example, in an XY system, identifying the number of loci conforming to ZW expectations is essential to estimate the false positive rate and distinguish true sex-linkage from stochastic noise. Alternatively, directly verifying the presence of fixed differences between males and females can be used to validate sex-linkage of genes (Hough, Hollister, Wang, Barrett, & Wright, 2014). Bioinformatic approaches to validation such as these will be of increasing importance as data sets grow.

Depth of next-generation sequencing

An important point to consider when designing an experiment to identify sex chromosomes is the sequencing depth. Clearly there is a trade-off between number of individuals, which improves the likelihood of identifying sex-linked regions particularly if the population from which they are sampled is genetically diverse, and the depth of sequencing. Deeper sequencing reduces the chances of sequencing errors leading to the misidentification of sex-linked regions (Davey et al., 2013; Liu et al., 2012; Mastretta-Yanes et al., 2015; Nielsen, Paul, Albrechtsen, & Song, 2011). However, the majority of approaches rely on sequencing both the homogametic sex, where the sex chromosomes will have equal depth to the autosomes, and the heterogametic sex, where the X and Y (or Z and W) chromosomes will have half the sequencing depth. For example, our recommendation of >20-fold sequencing depth for coverage- and heterozygosity-based approaches (Carvalho & Clark, 2013; Hall et al., 2013; Smeds et al., 2015; Vicoso & Bachtrog, 2015) ensures sex chromosomes are sequenced 10-fold in the heterogametic sex.

Population genetic diversity

Approaches that rely on identifying consistent genetic differences between males and females (e.g., genomic coverage, SNP density, expression and RAD-seq methods) to identify sex chromosomes are most accurate when inbred populations are used. This is because in outbred populations, males and females will differ by chance at polymorphic sites across the genome, making it difficult to identify sex-linked regions, particularly when only a few individuals are sampled. In contrast, approaches that rely on patterns of SNP segregation (e.g., linkage mapping) perform optimally on outbred populations where genetic diversity is maximized. However, care must be taken if sampling across populations, as it is possible that individuals from different populations will have independently evolved sex chromosome systems which can confound the results of these approaches (discussed in Jeffries et al., 2018).

Box 1 (Continued)

Determining the identity of the sex chromosome pair

Once sex-linked loci are found, it is necessary to determine the identity of the sex chromosome pair in order to identify potential turnover events. This can be achieved by searching for orthologous sequences in an outgroup species with a chromosomal-level genome assembly. This is often challenging and highly dependent on conservation of synteny across clades. However, a number of different methods are available for this purpose, including the Reference-Assisted Chromosome Assembly (RACA) algorithm (Kim et al., 2013) as used in Darolti et al. (2019), or a custom approach developed by Jeffries et al. (2018), involving the generation of linkage maps from RAD-seq data to anchor scaffolds to an outgroup reference genome. The importance of these algorithms, as well as the importance of generating chromosomal-level genome assemblies in multiple species, will be a priority in order to estimate the diversity of sex chromosomes in many undersampled clades.

sufficiently diverged sex chromosomes or strata where there is sex-specificity among RNA-seq reads. Furthermore, this approach may underperform in systems where the sex chromosomes are starting to decay, as the loss of gene expression from genes on the Y or W chromosome has been shown to precede sequence degeneration (Bachtrog, 2013). Autosomal genes with sex-limited expression may also lead to erroneous results. Therefore, while sufficient data can be obtained from as little as one male and one female, prior knowledge of when sex-limited genes are expressed, and in which tissue, is essential to ensure detection of their associated transcripts. Typically, in heteromorphic systems, W and Y-linked genes tend to be expressed primarily in reproductive tissue (Moghadam et al., 2012; Skaletsky et al., 2003).

2.3 | Association-based approach

Several approaches exist to identify sex-linked regions using sex-specific genetic association. While whole-genome sequencing offers the most complete resolution for these analyses, reduced representation methods may also be employed if genotyping is sufficiently dense. Restriction site-associated DNA sequencing (RAD-seq) is a powerful tool to identify sex-limited loci and has been used to infer sex chromosome systems across a number of species (Gamble et al., 2015; Jeffries et al., 2018). RAD-seq markers are compared between males and females, and markers present in one sex and absent in the other are kept as candidate loci (Y-specific or W-specific; Figure 2c). Recently, this approach has been expanded to screen for variants with sex differences in allele frequency and heterozygosity (Brelsford, Lavanchy, Sermier, Rausch, & Perrin, 2017; Jeffries et al., 2018). For example, a Y-linked allele should have a frequency of 0.5 in males versus 0 in females, and should be heterozygous in males yet homozygous in females. Therefore, this approach can be successfully applied to identify sex-specific markers on homomorphic sex chromosomes (Gamble & Zarkower, 2014).

The inference of ploidy from RAD-seq data can also be a fruitful avenue to identify sex-linked regions. DetSex is a Bayesian method that infers segregation type based on ploidy information in males and females, which is derived from genotyping data (Gautier, 2014). The X chromosome is diploid in females yet haploid in males, whereas autosomes are diploid in both sexes. However, this approach assumes sex chromosomes are old and that Y reads do not map onto the X reference, and is therefore optimized for heteromorphic sex chromosomes. Furthermore, this approach requires the sequencing of many individuals (20–50 individuals). Others have leveraged RAD-seq data to identify sex-linked regions using GWAS, treating sex as a binary case/control variable, and using sliding window F_{ST} analysis to identify regions of genetic differentiation between males and females (Dixon, Kitano, & Kirkpatrick, 2019; Franchini et al., 2018).

The primary advantages of the RAD-seq approach are that it relies on genomic DNA, is relatively cheap, and is highly effective for wild-caught samples, provided they are accurately sexed. It can be used in combination with certain bioinformatic approaches to identify both homomorphic and heteromorphic sex chromosome systems, and the choice of restriction enzyme can be tailored to cut more or less frequently if the size of the nonrecombining region is known. The main challenge faced when using reduced representation methods is the problem of missing data (Lowry et al., 2017). Sex-specific sequences are often detected in both sexes and are likely to represent false positives. A solution might be to increase sample size; however, the number of shared loci decreases with sample numbers in RAD-seq data (Mastretta-Yanes et al., 2015). Several studies have had success by sampling ~5–20 individuals per sex (Fowler & Buonaccorsi, 2016; Gamble et al., 2015; Gamble & Zarkower, 2014; Jeffries et al., 2018); however, false positives can also be problematic with very small numbers of males and females, and greater skew in sample sexes. Implementing and developing approaches to quantify the false positive rate of identifying sex-linked sequences is a future priority when using this approach (see Box 1).

2.4 | SNP density approach

While sex differences in genomic coverage or expression are indicative of diverged sex chromosomes with significant Y or W degeneration, differences in SNP density between males and females are expected in sex chromosomes at the earlier stages of divergence. In particular, elevated SNP density in the heterogametic sex can be used to infer sex-linked regions when mapped to a reference genome generated from the homogametic sex. For

example, in nascent sex chromosomes with limited Y chromosome degeneration, Y-linked genomic reads will map to the homologous region of the X in a female reference genome, resulting in elevated SNP density in males relative to females (Figure 2d). Therefore, elevated SNP density in the heterogametic sex can be used to infer sex-linked regions when mapped to a reference genome generated from the homogametic sex (Darolti et al., 2019; Vicoso et al., 2013; Wright et al., 2017). In contrast, in regions where the Y has largely degenerated, we expect SNP density to be lower in males when mapped to a female genome as the X is effectively hemizygous in males (Rovatsos, Farkačová, Altmanová, Johnson Pokorná, & Kratochvíl, 2019; Rovatsos, Rehák, Velenský, & Kratochvíl, 2019; Rovatsos, Vukić, & Kratochvíl, 2016). Therefore, an absence of SNPs in females can indicate X-linked sequences. Finally, scaffolds with limited sex differences in polymorphism are probably autosomal or pseudoautosomal. Together, this rationale can be used not only to identify sex chromosomes at the intermediate stages of divergence, but also strata of different ages along the chromosome (Darolti et al., 2019; Wright et al., 2017) (Figure 2d). Contrasting SNP density between males and females is therefore a powerful approach to identify sex chromosomes or strata at the intermediate stages of X and Y (or Z and W) divergence.

The primary drawback of the SNP-based approach is the difficulty in defining a threshold above which SNP density between males and females can be used to infer sex-linkage. This is because the magnitude of sex differences in SNP density is directly proportional to the degree of divergence between the sex chromosomes. Therefore, implementing these approaches in young sex chromosome systems should ideally be accompanied by information as to the location of the sex determining region. Often this information is not available and therefore a permutation approach to estimate the null distribution of sex differences in SNP density across the genome is essential to identify regions with significantly elevated SNP density in the heterogametic sex (see Box 1). This method is most successful when combined with the coverage approach (Figure 2d) so that multiple, independent lines of evidence can be used to identify sex-linked regions (Darolti et al., 2019; Shearn et al., 2019; Vicoso et al., 2013).

2.5 | Segregation analysis approach

Segregation analyses can be a powerful approach to identify sex-linked sequences (Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Muyle et al., 2016). For example, SNPs in X-linked genes will only be transmitted from the father to daughters but not sons, whereas SNPs in Y-linked genes are only transmitted to sons. Recently, a probabilistic framework (SEX-DETector) has been developed to infer autosomal and sex-linked genes using patterns of allelic segregation (Muyle et al., 2016). SEX-DETector uses genotypic data from parents and progeny to infer three segregation types: autosomal, X-linked with a Y-linked ortholog (X/Y pair) and those without (X-hemizygous) (Figure 2e). Each SNP is assigned a likelihood of these three states and the method can also estimate the type of sex chromosome system through a model comparison strategy. An important step is the generation of a de novo reference assembly where X and Y sequences co-assemble into one contig instead of separate X- and Y-linked sequences. This co-assembly makes it possible to identify X/Y SNPs and is essential for differentiating Y-linked sequences from autosomal genes with male-limited expression in the case of RNA-seq data. Therefore, the approach is best optimized to systems with low or intermediate level of sex chromosome divergence where X and Y sequences are most likely to coassemble in the reference assembly. However, SEX-DETector can still identify X-hemizygous contigs in old systems, but there is a risk that these are actually X/Y pairs whose sequences were so diverged that they assembled into separate contigs (see Muyle et al., 2018).

This method has been used to identify sex-linked regions in several plant species (Martin et al., 2019; Muyle et al., 2017, 2018; Veltsos et al., 2019; Zemp et al., 2016), but there are a number of important points to consider. This approach requires family data and is therefore limited to species for which pedigree information is available. Second, SEX-Detector has primarily been used to analyse RNA-seq derived genotyping data although it can also be used with genomic-based data instead, providing the data set is not too big (Muyle et al., 2016). Whilst RNA-seq data clearly has advantages, only genes that are expressed can be identified as sex-linked. However, using multiple tissues or tissues where many genes are expressed can circumvent this problem. Finally, the pipeline requires polymorphism data to infer certain types of sex-linkage and therefore is not optimized for inbred populations. Ideally, parents should be sampled from different populations in order to maximize the genetic diversity of the progeny and increase statistical power (but see Box 1). However, this only applies to X-hemizygous genes, whose identification relies on the presence of polymorphisms on the X copy. The detection of X/Y gene pairs is instead based on fixed X-Y substitutions and is therefore not affected by population levels of genetic diversity (Muyle et al., 2016, 2018). As a result, X-hemizygous genes are sometimes more difficult to detect using this approach (Blavet et al., 2015) and this ascertainment bias should be taken into account when estimating gene loss.

2.6 | Linkage mapping approach

Instead of using a proxy for arrested recombination, such as sequence divergence or the accumulation of sex-specific SNPs, sex chromosomes can be identified by finding regions of the genome where there is no recombination in males or females. Linkage maps measure recombination frequency between genetic makers and are a traditional method for sex chromosome discovery (Al-Dous et al., 2011; Charlesworth, 2018; Goldberg, Spigler, & Ashman, 2010; Hou et al., 2015). The first step of this process requires DNA collection from parents and offspring. Typically, large sample sizes are required (~100s to 1,000s of progeny) from multiple independent families, where the number of individuals will determine the number of

potential crossover events observed and therefore resolution to distinguish autosomal from sex-linked regions. Therefore, when recombination is rare, even larger families are needed (Bergero, Gardner, Bader, Yong, & Charlesworth, 2019; Wright et al., 2019). Next, informative genetic markers need to be identified that are evenly spread across the whole genome, or along the sex chromosome if strata and the pseudoautosomal region are being identified (e.g., Yazdi & Ellegren, 2018). Finally, linkage maps for males and females are constructed, and regions of the genome with no recombination indicate putative sex-linked loci (Figure 2f). Simultaneously, QTL analysis using a binary trait model could be used to quantify the number and size of the regions involved.

The advantage of linkage mapping is that it directly measures recombination rates rather than using a proxy for arrested recombination, and thus can be applied to species with homomorphic sex chromosomes. However, the necessity for samples from parents and offspring will limit which species this approach can be used on. Recombination frequency will also determine how successful this approach is. If the sex-determining locus arose in an area of the genome which already had low recombination, as is believed to have occurred in papaya (Wai, Moore, Paull, Ming, & Yu, 2012), then sex chromosome discovery using linkage mapping will be more challenging. Furthermore, when recombination events are rare, the boundary between the nonrecombining and the pseudoautosomal regions is more difficult to define (Bergero et al., 2019; Wright et al., 2019). This is because the probability of observing a recombination event near this boundary is limited by sample size. Large families, and correspondingly many recombination events, are necessary to achieve the power required to characterize nonrecombining regions on sex chromosomes. This approach also cannot be used in species with sex-limited recombination (e.g., several Diptera and Lepidoptera; see Satomura, Osada, & Endo, 2019 for a complete review).

3 | FUTURE DIRECTIONS & PERSPECTIVES

The diversity of independently evolved sex chromosome systems across eukaryotes is striking (Bachtrog et al., 2014; Beukeboom & Perrin, 2014), yet our current understanding of the ecological and genetic factors that drive changes in sex determination system is limited, despite a large body of theoretical predictions. The development of new bioinformatic methods to identify and characterize sex chromosomes across non-model species is fueling efforts to test these predictions. Indeed, several studies have recently provided important insight into the dynamics and drivers of turnover (Blackmon & Demuth, 2014; Jeffries et al., 2018; Kitano & Peichel, 2012). A large body of theoretical work outlines predictions for when and why sex chromosome transitions occur (Bachtrog et al., 2011; Beukeboom & Perrin, 2014), under the hypotheses of genetic drift (Bull & Charnov, 1977; Saunders et al., 2018), accumulation of deleterious mutation on the sex-limited chromosomes (Blaser et al., 2013, 2014), selection on sex ratio (Jaenike, 2001; Werren & Beukeboom, 1998) and sexually antagonistic selection (van Doorn & Kirkpatrick, 2007, 2010). Here, we highlight key predictions for each of the hypotheses to motivate future sex chromosome research.

3.1 | Genetic drift

Genetic drift has been theorized to underlie sex chromosome turnover in the absence of selection when a novel sex determining region arises of equal fitness to the established one (Bull & Charnov, 1977). The emergence of a new sex determination locus is thought to be followed by a period of multifactorial sex determination involving multiple genotypes for each sex. The two resulting sex chromosome systems are connected by a path of neutral equilibria that balance sex ratio at the population level, enabling drift to drive a transition to the new system (Bull & Charnov, 1977). Transitions that reverse patterns of heterogamety are characterized by a drift-induced selective force that favours the fixation of novel sex determining mutations (Veller, Muralidhar, Constable, & Nowak, 2017). However, the weakness of drift-induced selection (fixation probabilities on the order of $1/N$) calls into question its significance in mediating turnover given the potential for other selective forces to act on competing sex chromosome systems (Veller et al., 2017). Furthermore, the coexistence of multiple sex determining loci in a number of species (e.g., cichlids, housefly, zebrafish, seabass) suggests that multifactorial sex determination need not be unstable, provided the sex ratio is balanced (Liew et al., 2012; Meisel et al., 2016; Moore & Roberts, 2013; Roberts et al., 2016; Vandeputte, Dupont-Nivet, Chavanne, & Chatain, 2007; Wilson et al., 2014). Because sex operates as a threshold trait in which female or male development is triggered when genetic and/or environmental cues surpass some level (Bulmer & Bull, 1982; Roff, 1996), the presence of multiple sex determining loci may not necessarily indicate that a system is undergoing a sex chromosome turnover (Beukeboom & Perrin, 2014; Perrin, 2016; Rodrigues et al., 2017).

Drift-induced turnover has been studied almost entirely using computer simulations, and this work has generated a number of predictions to guide future research (Nishioka, Miura, & Saitoh, 1993; Saunders et al., 2018; Veller et al., 2017). First, drift-induced sex chromosome transitions that maintain patterns of heterogamety are predicted to be 2–4 times more likely than those which reverse heterogamety when the invading sex determining locus is dominant; however, this ratio is influenced by effective population size and mating system. This is because transitions that preserve heterogamety involve fixation of the ancestral X or Z chromosome, which have a higher frequency in the population, while transitions reversing heterogamety require fixation of the ancestral Y or W (Saunders et al., 2018). Comparative studies across independently evolved sex chromosomes offer the potential to test this directly, provided that the sampling resolution is sufficient and the identity of sex chromosome pairs is known. The preserved heterogamety patterns among Salmonid fish (Phillips, 2013), Varanid and Lacertid lizards (Ezaz, Sarre, O'Meally, Graves, & Georges, 2009; Pokorná & Kratochvíl,

2009), and Ranid frogs (Jeffries et al., 2018) are consistent with drift-induced turnover, but are difficult to distinguish from expectations under alternative scenarios such as mutation-load selection (Jeffries et al., 2018). However, the predictions of mutation-load models rely on explicitly accounting for mutation rates, which can be challenging to obtain. Second, while transitions that maintain heterogamety are unaffected by demographic parameters, transitions that reverse heterogamety are more likely as effective population size decreases and reproductive skew increases (Saunders et al., 2018; Veller et al., 2017). Specifically, transitions from an XY to a ZW system become more common when the number of breeding males is low (Saunders et al., 2018). Therefore, experimental and comparative approaches in species with multifactorial systems may present a window into an ongoing turnover event, and offer an excellent opportunity to explicitly test the role of drift in sex chromosome turnover. Under drift, multifactorial systems should be found more frequently in species with large effective population sizes because the fixation of an invading sex determiner will proceed more slowly in such species (Saunders et al., 2018; Veller et al., 2017). Natural or experimentally induced variation in demographic traits and mating systems, and thereby effective population size, across species can be used to probe the role of drift in driving turnovers. Finally, directly identifying invading sex determiners makes it possible to test the prediction that heterogamety-reversing transitions should involve dominant mutations (Nishioka et al., 1993; Veller et al., 2017).

3.2 | Accumulation of deleterious mutations

As recombination is suppressed between sex chromosomes, the sex-limited Y and W start to decay by a combination of neutral and adaptive processes. The accumulation of loss-of-function mutations on the nonrecombining sex chromosomes is predicted to drive the turnover and formation of a new sex chromosome system. This process is thought to be affected by the number and strength of deleterious mutations, sexually antagonistic selection, effective population size, and the size of the nonrecombining region (Blaser et al., 2013, 2014).

A number of predictions for sex chromosome turnover arise from the mutation accumulation hypothesis. First, patterns of heterogamety should be preserved, because a switch (e.g., from an XY to a ZW system) requires the fixation of the ancestral, degenerated sex-limited chromosome as an autosome (Blaser et al., 2014; van Doorn & Kirkpatrick, 2010; Jeffries et al., 2018; Scott, Osmond, & Otto, 2018). Second, factors associated with high loads of deleterious mutations, and therefore sex chromosome degeneration, should also be linked to high turnover rates. Many species exhibit heterochiasmy or achiasmy, where recombination is reduced or absent in one sex, which would in theory accelerate the accumulation of deleterious mutations on the nonrecombining sex chromosome and therefore promote turnover. This is consistent with transitions across Ranid frogs (Jeffries et al., 2018) but not with the stability of ZW chromosomes in Lepidoptera (Lenormand, 2003), both of which exhibit reduced or absent recombination in the heterogametic sex. Various life history traits can also be used as a proxy of mutation rate and therefore sex chromosome degeneration in a comparative framework. For example, species that are warm blooded, shorter lived, or have a smaller body size usually have higher metabolic rates (Galtier, Jobson, Nabholz, Glémin, & Blier, 2009). However, current studies find that many cold-blooded vertebrates including fish (Mank & Avise, 2009; Mank et al., 2006; Volff, Nanda, Schmid, & Schartl, 2007), reptile, and amphibian lineages (Ezaz et al., 2009; Jeffries et al., 2018) have undergone far more sex chromosome turnover than warm-blooded mammals. This contrast may reflect the confounding effects of other factors, such as differences in effective population size. In addition, organisms with a longer haploid phase will experience purifying selection to maintain gene activity on the Y chromosome during meiosis (Wright et al., 2016). Therefore, we might expect less frequent sex chromosome turnover in organisms where haploid selection is more persistent. However, whilst it was initially shown that organisms with a long haploid phase exhibit lower levels of sex chromosome divergence, including some algae (Ahmed et al., 2014) and plants (Bergero, Qiu, & Charlesworth, 2015; Chibalina & Filatov, 2011), a recent study using a larger data set of sex-linked genes found rapid degeneration of the *Silene latifolia* Y chromosome (Papadopulos, Chester, Ridout, & Filatov, 2015). This result, together with the observation that many plant clades exhibit turnover of sex chromosome systems (Balounova et al., 2019; Charlesworth, 2015; Martin et al., 2019; Moore, Harkess, & Weingartner, 2016; Tennessen et al., 2018), suggest that haploid selection might have a minimal effect on rates of Y degeneration.

Finally, the rate of turnover of XY versus ZW chromosomes is predicted to differ in light of mutation load. First, the evolution of complete dosage compensation, a mechanism that compensates for the degeneration and loss of expression of the W and Y chromosomes (Gu & Walters, 2017; Mank, 2013), is thought to reduce the power of purifying selection to maintain gene activity on these chromosomes (Engelstädter, 2008; Wright et al., 2016). Dosage compensation mechanisms are more frequently observed on XY relative to ZW chromosomes in the species studied so far (Gu & Walters, 2017; Mullon et al., 2015; Tables S1–S3), potentially leading to faster rates of Y chromosome decay. However, there have been several recent counter-examples to this trend (Hale, McKinney, Thrower, & Nichols, 2018; Huylmans et al., 2019), and as more sex chromosomes are identified it will be possible to test whether there is indeed a consistent relationship between dosage compensation status and sex chromosome system. Second, in several vertebrate and plant groups (Kirkpatrick & Hall, 2004; Whittle & Johnston, 2002), males have a higher mutation rate than females. Therefore, deleterious mutations are predicted to accumulate more quickly on the Y chromosome, meaning that XY sex chromosome systems may undergo turnover more often than ZW systems (Bachtrog et al., 2011; Naurin, Hansson, Bensch, & Hasselquist, 2010). Testing this directly will require detailed knowledge of the identity of the sex chromosome pair across multiple species.

3.3 | Selection on sex ratio

Selection on sex ratio is thought to promote the invasion of a novel sex determination locus in order to restore Fisherian sex ratio values when they are unbalanced (Beukeboom & Perrin, 2014; Bull, 1983; Mank, Hosken, & Wedell, 2014). This can arise commonly through intragenomic conflicts from selfish or meiotic drive elements, either autosomal or sex-linked. Endosymbionts can have a similar impact, as illustrated by the *Wolbachia* feminizing element in populations of woodlice (Cordaux, Bouchon, & Grève, 2011). Increasing numbers of theoretical models outline the scenarios in which we might expect sex ratio selection to drive the evolution of new sex chromosome systems (Kozielska, Weissing, Beukeboom, & Pen, 2010; Úbeda, Patten, & Wild, 2015) and there is growing support from a few taxa (Badawi, Moumen, Giraud, Grève, & Cordaux, 2018; Becking et al., 2017; Chebbi et al., 2019; Cordaux et al., 2011; Cordaux & Gilbert, 2017; Leclercq et al., 2016; Miura, 2007). Similarly, a recent study outlined the role of haploid selection via gametic competition and meiotic drive in increasing the lability of sex determination systems (Scott et al., 2018).

Given the prevalence of sex ratio distorters in nature (Hall, 2004; Jaenike, 2001), in particular sex-linked meiotic drivers (Helleu et al., 2016; Tao et al., 2007), sex ratio selection is likely to be a common driver in sex chromosome turnover events (see Scott et al., 2018), yet is probably one of the most difficult to detect due to its transient nature (Kozielska et al., 2010). This is because once the novel sex determination region is fixed, balanced sex ratios are restored and the original sex determining locus is often lost from the population. As a result, comparative phylogenetic approaches will have limited power to quantify the relative contribution of meiotic drive to turnover events. However, one signature of a recurrent arms race between successive sex ratio distorters and their modifiers is an increase in the length of the sex determination pathway, as novel sex determination factors are integrated into existing gene networks (Schartl, 2004; Wilkins, 1995). In support of this, downstream components of sex determination cascades are broadly conserved relative to upstream regulators (Beukeboom & Perrin, 2014). Alternatively, laboratory crosses between pairs of sister species can uncover the potential for sex ratio selection to act by uncoupling drivers and modifiers; however, such experiments are not feasible in many groups. Instead, experimental selection in species with polyfactorial sex determination, such as the housefly (Kozielska, Pen, Beukeboom, & Weissing, 2006; Meisel, Olafson, Guerrero, Konganti, & Benoit, 2019), have the greatest scope to quantify the role of sex ratio selection and meiotic drive in the evolution of sex determination.

3.4 | Sexually antagonistic selection

Sexually antagonistic selection, which occurs when a mutation is harmful to one sex but beneficial to the other, is predicted to drive sex chromosome turnover. For example, an autosomal gene with male benefit and female harm effects might become linked to a sex determining gene, either through the evolution of a novel locus or translocation of the existing determiner or antagonistic locus. If this neo-sex chromosome produces males with higher fitness than the ancestral Y chromosome, then it can replace the ancestral sex determination mechanism (van Doorn & Kirkpatrick, 2007, 2010).

There is some empirical support for this theory, including the invasion of a novel female sex determining locus in cichlids where there is sexual conflict over a female-benefit, male-harming colour pattern (Roberts, Ser, & Kocher, 2009). However, since we can only look at a snapshot in evolutionary time, and given that sex determination is dynamic and polygenic in cichlids (Ser, Roberts, & Kocher, 2010), we do not know whether the new sex chromosome predates, or evolved in response to, the coloration patterns. The discovery of a neo-sex chromosome in the three-spined stickleback also supports models of sex chromosome evolution driven by sexual antagonism (Kitano et al., 2009), however, the absence of recombination suppression between the sexually antagonistic locus and the sex determining gene casts doubt on this (Natri, Shikano, & Merilä, 2013). Finally, sexually antagonistic genes have accumulated close to a novel sex determining gene (Rice, 1992) and on a neo-sex chromosome in *Drosophila* (Zhou & Bachtrog, 2012). Despite these studies, we lack direct support for the relative importance of sexual antagonism in driving turnovers. One way around this is through experimental evolution, and an ambitious study, involving 100 generations of backcrossing between two species of *Xiphophorus*, directly illustrates the potential for sexual conflict to drive sex chromosome turnover (Franchini et al., 2018).

Much of the current work in this area involves species of fish, and we suggest future work should continue in these taxa due to the repeated origins of homomorphic sex chromosomes. Studying species or populations where there is variation in the extent of recombination suppression between sex chromosomes, as in Poeciliids (Bergero et al., 2019; Darolti et al., 2019; Wright et al., 2017), promises to be a fruitful avenue. A powerful approach would be targeting young sex chromosomes within a sex-specific evolution framework to test whether sexually antagonistic mutations accumulate prior to recombination suppression (Ponnikas, Sigeman, Abbott, & Hansson, 2018). Experimental evolution continuing the work of Rice (1992), investigating whether recombination suppression spreads between a new sex determining gene and a sexually antagonistic gene would be an insightful, although challenging, future avenue.

ACKNOWLEDGEMENTS

This work was supported by a NERC Independent Research Fellowship (Award number NE/N013948/1) to A.E.W., NSF Postdoctoral Fellowship in Biology (Award number 1812164) to D.H.P. and Marie Curie Actions (Award number 655392) to R.D. We thank Iulia Darolti, Judith Mank and Sean Stankowski for productive discussions, and the three anonymous reviewers for helpful comments.

AUTHOR CONTRIBUTIONS

All authors were responsible for writing the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Daniela H. Palmer https://orcid.org/0000-0003-1242-9632
Thea F. Rogers https://orcid.org/0000-0003-0198-0242
Alison E. Wright https://orcid.org/0000-0003-2479-5250

REFERENCES

Ahmed, S., Cock, J. M., Pessia, E., Luthringer, R., Cormier, A., Robuchon, M., … Coelho, S. M. (2014). A haploid system of sex determination in the brown alga *Ectocarpus* sp. *Current Biology*, *24*(17), 1945–1957. https://doi.org/10.1016/j.cub.2014.07.042

Akagi, T., Henry, I. M., Tao, R., & Comai, L. (2014). Plant genetics. A Y-chromosome-encoded small RNA acts as a sex determinant in persimmons. *Science*, *346*(6209), 646–650.

Al-Dous, E. K., George, B., Al-Mahmoud, M. E., Al-Jaber, M. Y., Wang, H., Salameh, Y. M., … Malek, J. A. (2011). De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). *Nature Biotechnology*, *29*(6), 521–527. https://doi.org/10.1038/nbt.1860

Bachtrog, D. (2013). Y-chromosome evolution: Emerging insights into processes of Y-chromosome degeneration. *Nature Reviews Genetics*, *14*(2), 113–124. https://doi.org/10.1038/nrg3366

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*(9), 350–357. https://doi.org/10.1016/j.tig.2011.05.005

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T.-L., … Tree of Sex Consortium (2014). Sex determination: Why so many ways of doing it? *PLOS Biology*, *12*(7), e1001899. https://doi.org/10.1371/journal.pbio.1001899

Badawi, M., Moumen, B., Giraud, I., Grève, P., & Cordaux, R. (2018). Investigating the molecular genetic basis of cytoplasmic sex determination caused by *Wolbachia* endosymbionts in terrestrial isopods. *Genes*, *9*(6), 290. https://doi.org/10.3390/genes9060290

Balounova, V., Gogela, R., Cegan, R., Cangren, P., Zluvova, J., Safar, J., … Janousek, B. (2019). Evolution of sex determination and heterogamety changes in section Otites of the genus *Silene*. *Scientific Reports*, *9*(1), 1045. https://doi.org/10.1038/s41598-018-37412-x

Becking, T., Giraud, I., Raimond, M., Moumen, B., Chandler, C., Cordaux, R., & Gilbert, C. (2017). Diversity and evolution of sex determination systems in terrestrial isopods. *Scientific Reports*, *7*(1), 1084. https://doi.org/10.1038/s41598-017-01195-4

Bergero, R., & Charlesworth, D. (2011). Preservation of the Y transcriptome in a 10-million-year-old plant sex chromosome system. *Current Biology*, *21*(17), 1470–1474. https://doi.org/10.1016/j.cub.2011.07.032

Bergero, R., Gardner, J., Bader, B., Yong, L., & Charlesworth, D. (2019). Exaggerated heterochiasmy in a fish with sex-linked male coloration polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(14), 6924–6931. https://doi.org/10.1073/pnas.1818486116

Bergero, R., Qiu, S., & Charlesworth, D. (2015). Gene loss from a plant sex chromosome system. *Current Biology*, *25*(9), 1234–1240. https://doi.org/10.1016/j.cub.2015.03.015

Beukeboom, L. W., & Perrin, N. (Eds.) (2014). *The evolution of sex determination*. Oxford, UK: Oxford University Press.

Blackmon, H., & Demuth, J. P. (2014). Estimating tempo and mode of Y chromosome turnover: Explaining Y chromosome loss with the fragile Y hypothesis. *Genetics*, *197*(2), 561–572. https://doi.org/10.1534/genetics.114.164269

Blaser, O., Grossen, C., Neuenschwander, S., & Perrin, N. (2013). Sex-chromosome turnovers induced by deleterious mutation load. *Evolution; International Journal of Organic Evolution*, *67*(3), 635–645. https://doi.org/10.1111/j.1558-5646.2012.01810.x

Blaser, O., Neuenschwander, S., & Perrin, N. (2014). Sex-chromosome turnovers: The hot-potato model. *The American Naturalist*, *183*(1), 140–146. https://doi.org/10.1086/674026

Blavet, N., Blavet, H., Muyle, A., Käfer, J., Cegan, R., Deschamps, C., … Marais, G. (2015). Identifying new sex-linked genes through BAC sequencing in the dioecious plant *Silene latifolia*. *BMC Genomics*, *16*, 546. https://doi.org/10.1186/s12864-015-1698-7

Brelsford, A., Lavanchy, G., Sermier, R., Rausch, A., & Perrin, N. (2017). Identifying homomorphic sex chromosomes from wild-caught adults with limited genomic resources. *Molecular Ecology Resources*, *17*(4), 752–759. https://doi.org/10.1111/1755-0998.12624

Bull, J. J. (1983). *Evolution of sex determining mechanisms*. San Francisco, CA: Benjamin-Cummings Publishing Company.

Bull, J. J., & Charnov, E. L. (1977). Changes in the heterogametic mechanism of sex determination. *Heredity*, *39*(1), 1–14. https://doi.org/10.1038/hdy.1977.38

Bulmer, M. G., & Bull, J. J. (1982). Models of polygenic sex determination and sex ratio control. *Evolution*, *36*(1), 13–26.

Carvalho, A. B., & Clark, A. G. (2013). Efficient identification of Y chromosome sequences in the human and *Drosophila* genomes. *Genome Research*, *23*(11), 1894–1907.

Charlesworth, B., & Charlesworth, D. (2000). The degeneration of Y chromosomes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *355*(1403), 1563–1572. https://doi.org/10.1098/rstb.2000.0717

Charlesworth, D. (2015). Plant contributions to our understanding of sex chromosome evolution. *The New Phytologist*, *208*(1), 52–65. https://doi.org/10.1111/nph.13497

Charlesworth, D. (2018). The guppy sex chromosome system and the sexually antagonistic polymorphism hypothesis for Y chromosome recombination suppression. *Genes*, *9*(5), 264. https://doi.org/10.3390/genes9050264

Charlesworth, D., Charlesworth, B., & Marais, G. (2005). Steps in the evolution of heteromorphic sex chromosomes. *Heredity*, *95*(2), 118–128. https://doi.org/10.1038/sj.hdy.6800697

Chebbi, M. A., Becking, T., Moumen, B., Giraud, I., Gilbert, C., Peccoud, J., & Cordaux, R. (2019). The genome of *Armadillidium vulgare* (Crustacea, Isopoda) provides insights into sex chromosome evolution in the context of cytoplasmic sex determination. *Molecular Biology and Evolution*, *36*(4), 727–741.

Chibalina, M. V., & Filatov, D. A. (2011). Plant Y chromosome degeneration is retarded by haploid purifying selection. *Current Biology*, *21*, 1475–1479. https://doi.org/10.1016/j.cub.2011.07.045

Cordaux, R., Bouchon, D., & Grève, P. (2011). The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends in Genetics*, *27*(8), 332–341. https://doi.org/10.1016/j.tig.2011.05.002

Cordaux, R., & Gilbert, C. (2017). Evolutionary significance of *Wolbachia*-to-animal horizontal gene transfer: Female sex determination and the f element in the isopod *Armadillidium vulgare*. *Genes*, *8*(7), 186. https://doi.org/10.3390/genes8070186

Cortez, D., Marin, R., Toledo-Flores, D., Froidevaux, L., Liechti, A., Waters, P. D., … Kaessmann, H. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, *508*(7497), 488–493.

Darolti, I., Wright, A. E., Sandkam, B. A., Morris, J., Bloch, N. I., Farré, M., … Mank, J. E. (2019). Extreme heterogeneity in sex chromosome differentiation and dosage compensation in livebearers. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(38), 19031–19036. https://doi.org/10.1073/pnas.1905298116

Davey, J. W., Cezard, T., Fuentes-Utrilla, P., Eland, C., Gharbi, K., & Blaxter, M. L. (2013). Special features of RAD Sequencing data: Implications for genotyping. *Molecular Ecology*, *22*(11), 3151–3164. https://doi.org/10.1111/mec.12084

Dixon, G., Kitano, J., & Kirkpatrick, M. (2019). The origin of a new sex chromosome by introgression between two stickleback fishes. *Molecular Biology and Evolution*, *36*(1), 28–38. https://doi.org/10.1093/molbev/msy181

Engelstädter, J. (2008). Muller's ratchet and the degeneration of Y chromosomes: A simulation study. *Genetics*, *180*(2), 957–967. https://doi.org/10.1534/genetics.108.092379

Ezaz, T., Quinn, A. E., Miura, I., Sarre, S. D., Georges, A., & Marshall Graves, J. A. (2005). The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Research*, *13*(8), 763–776. https://doi.org/10.1007/s10577-005-1010-9

Ezaz, T., Sarre, S. D., O'Meally, D., Graves, J. A. M., & Georges, A. (2009). Sex chromosome evolution in lizards: Independent origins and rapid transitions. *Cytogenetic and Genome Research*, *127*(2–4), 249–260. https://doi.org/10.1159/000300507

Fowler, B. L. S., & Buonaccorsi, V. P. (2016). Genomic characterization of sex-identification markers in *Sebastes carnatus* and *Sebastes chrysomelas* rockfishes. *Molecular Ecology*, *25*(10), 2165–2175.

Fraïsse, C., Picard, M. A. L., & Vicoso, B. (2017). The deep conservation of the Lepidoptera Z chromosome suggests a non-canonical origin of the W. *Nature Communications*, *8*, 1486. https://doi.org/10.1038/s41467-017-01663-5

Franchini, P., Jones, J. C., Xiong, P., Kneitz, S., Gompert, Z., Warren, W. C., … Schartl, M. (2018). Long-term experimental hybridisation results in the evolution of a new sex chromosome in swordtail fish. *Nature Communications*, *9*(1), 5136. https://doi.org/10.1038/s41467-018-07648-2

Galtier, N., Jobson, R. W., Nabholz, B., Glémin, S., & Blier, P. U. (2009). Mitochondrial whims: Metabolic rate, longevity and the rate of molecular evolution. *Biology Letters*, *5*(3), 413–416. https://doi.org/10.1098/rsbl.2008.0662

Gamble, T. (2016). Using RAD-seq to recognize sex-specific markers and sex chromosome systems. *Molecular Ecology*, *25*(10), 2114–2116. https://doi.org/10.1111/mec.13648

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D. P., & Zarkower, D. (2015). Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Molecular Biology and Evolution*, *32*(5), 1296–1309. https://doi.org/10.1093/molbev/msv023

Gamble, T., & Zarkower, D. (2014). Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. *Molecular Ecology Resources*, *14*(5), 902–913. https://doi.org/10.1111/1755-0998.12237

Gautier, M. (2014). Using genotyping data to assign markers to their chromosome type and to infer the sex of individuals: A Bayesian model-based classifier. *Molecular Ecology Resources*, *14*(6), 1141–1159. https://doi.org/10.1111/1755-0998.12264

Goldberg, M. T., Spigler, R. B., & Ashman, T.-L. (2010). Comparative genetic mapping points to different sex chromosomes in sibling species of wild strawberry (*Fragaria*). *Genetics*, *186*(4), 1425–1433. https://doi.org/10.1534/genetics.110.122911

Gu, L., & Walters, J. R. (2017). Evolution of sex chromosome dosage compensation in animals: A beautiful theory, undermined by facts and bedeviled by details. *Genome Biology and Evolution*, *9*(9), 2461–2476. https://doi.org/10.1093/gbe/evx154

Hale, M. C., McKinney, G. J., Thrower, F. P., & Nichols, K. M. (2018). Evidence of sex-bias in gene expression in the brain transcriptome of two populations of rainbow trout (*Oncorhynchus mykiss*) with divergent life histories. *PLoS ONE*, *13*(2), e0193009. https://doi.org/10.1371/journal.pone.0193009

Hall, A. B., Qi, Y., Timoshevskiy, V., Sharakhova, M. V., Sharakhov, I. V., & Tu, Z. (2013). Six novel Y chromosome genes in *Anopheles* mosquitoes discovered by independently sequencing males and females. *BMC Genomics*, *14*, 273. https://doi.org/10.1186/1471-2164-14-273

Hall, D. W. (2004). Meiotic drive and sex chromosome cycling. *Evolution*, *58*(5), 925–931. https://doi.org/10.1111/j.0014-3820.2004.tb00426.x

Helleu, Q., Gérard, P. R., Dubruille, R., Ogereau, D., Prud'homme, B., Loppin, B., & Montchamp-Moreau, C. (2016). Rapid evolution of a Y-chromosome heterochromatin protein underlies sex chromosome meiotic drive. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(15), 4110–4115. https://doi.org/10.1073/pnas.1519332113

Hou, J., Ye, N., Zhang, D., Chen, Y., Fang, L., Dai, X., & Yin, T. (2015). Different autosomes evolved into sex chromosomes in the sister genera of *Salix* and *Populus*. *Scientific Reports*, *5*, 9076. https://doi.org/10.1038/srep09076

Hough, J., Hollister, J. D., Wang, W., Barrett, S. C. H., & Wright, S. I. (2014). Genetic degeneration of old and young Y chromosomes in the flowering plant *Rumex hastatulus*. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(21), 7713–7718. https://doi.org/10.1073/pnas.1319227111

Huylmans, A. K., Toups, M. A., Macon, A., Gammerdinger, W. J., & Vicoso, B. (2019). Sex-biased gene expression and dosage compensation on the *Artemia franciscana* Z-chromosome. *Genome Biology and Evolution*, *11*(4), 1033–1044.

Iannucci, A., Altmanová, M., Ciofi, C., Ferguson-Smith, M., Milan, M., Pereira, J. C., … Johnson Pokorná, M. (2019). Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity*, *123*(2), 215–227. https://doi.org/10.1038/s41437-018-0179-6

Irwin, D. E. (2018). Sex chromosomes and speciation in birds and other ZW systems. *Molecular Ecology*, *27*(19), 3831–3851.

Jaenike, J. (2001). Sex chromosome meiotic drive. *Annual Review of Ecology and Systematics*, *32*, 25–49. https://doi.org/10.1146/annurev.ecolsys.32.081501.113958

Jeffries, D. L., Lavanchy, G., Sermier, R., Sredl, M. J., Miura, I., Borzée, A., … Perrin, N. (2018). A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. *Nature Communications*, *9*(1), 4088. https://doi.org/10.1038/s41467-018-06517-2

Kawai, A., Nishida-Umehara, C., Ishijima, J., Tsuda, Y., Ota, H., & Matsuda, Y. (2007). Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenetic and Genome Research*, *117*(1–4), 92–102. https://doi.org/10.1159/000103169

Kelley, D. R., Schatz, M. C., & Salzberg, S. L. (2010). Quake: Quality-aware detection and correction of sequencing errors. *Genome Biology*, *11*(11), R116. https://doi.org/10.1186/gb-2010-11-11-r116

Kim, J., Larkin, D. M., Cai, Q., Asan, Zhang, Y., Ge, R.-L., … Ma, J. (2013). Reference-assisted chromosome assembly. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(5), 1785–1790. https://doi.org/10.1073/pnas.1220349110

Kirkpatrick, M., & Hall, D. W. (2004). Male-biased mutation, sex linkage, and the rate of adaptive evolution. *Evolution*, *58*(2), 437–440. https://doi.org/10.1111/j.0014-3820.2004.tb01659.x

Kitano, J., & Peichel, C. L. (2012). Turnover of sex chromosomes and speciation in fishes. *Environmental Biology of Fishes*, *94*(3), 549–558. https://doi.org/10.1007/s10641-011-9853-8

Kitano, J., Ross, J. A., Mori, S., Kume, M., Jones, F. C., Chan, Y. F., … Peichel, C. L. (2009). A role for a neo-sex chromosome in stickleback speciation. *Nature*, *461*(7267), 1079–1083.

Kozielska, M., Pen, I., Beukeboom, L. W., & Weissing, F. J. (2006). Sex ratio selection and multi-factorial sex determination in the housefly: A dynamic model. *Journal of Evolutionary Biology*, *19*(3), 879–888. https://doi.org/10.1111/j.1420-9101.2005.01040.x

Kozielska, M., Weissing, F. J., Beukeboom, L. W., & Pen, I. (2010). Segregation distortion and the evolution of sex-determining mechanisms. *Heredity*, *104*(1), 100–112. https://doi.org/10.1038/hdy.2009.104

Lahn, B. T., & Page, D. C. (1999). Four evolutionary strata on the human X chromosome. *Science*, *286*(5441), 964–967.

Leclercq, S., Thézé, J., Chebbi, M. A., Giraud, I., Moumen, B., Ernenwein, L., … Cordaux, R. (2016). Birth of a W sex chromosome by horizontal transfer of *Wolbachia* bacterial symbiont genome. *Proceedings of the National Academy of Sciences*, *113*, 15036–15041.

Lenormand, T. (2003). The evolution of sex dimorphism in recombination. *Genetics*, *163*(2), 811–822.

Li, S., Ajimura, M., Chen, Z., Liu, J., Chen, E., Guo, H., … Mita, K. (2018). A new approach for comprehensively describing heterogametic sex chromosomes. *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes*, *25*(4), 375–382. https://doi.org/10.1093/dnares/dsy010

Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R., & Orban, L. (2012). Polygenic sex determination system in zebrafish. *PLoS ONE*, *7*(4), e34397. https://doi.org/10.1371/journal.pone.0034397

Liu, Q., Guo, Y., Li, J., Long, J., Zhang, B., & Shyr, Y. (2012). Steps to ensure accuracy in genotype and SNP calling from Illumina sequencing data. *BMC Genomics*, *13*(Suppl 8), S8.

Lowry, D. B., Hoban, S., Kelley, J. L., Lotterhos, K. E., Reed, L. K., Antolin, M. F., & Storfer, A. (2017). Breaking RAD: An evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation. *Molecular Ecology Resources*, *17*, 142–152. https://doi.org/10.1111/1755-0998.12635

Mank, J. E. (2013). Sex chromosome dosage compensation: Definitely not for everyone. *Trends in Genetics*, *29*, 677–683. https://doi.org/10.1016/j.tig.2013.07.005

Mank, J. E., & Avise, J. C. (2009). Evolutionary diversity and turn-over of sex determination in teleost fishes. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation*, *3*(2–3), 60–67. https://doi.org/10.1159/000223071

Mank, J. E., Hosken, D. J., & Wedell, N. (2014). Conflict on the sex chromosomes: Cause, effect, and complexity. *Cold Spring Harbor Perspectives in Biology*, *6*(12), a017715. https://doi.org/10.1101/cshperspect.a017715

Mank, J. E., Promislow, D. E. L., & Avise, J. C. (2006). Evolution of alternative sex-determining mechanisms in teleost fishes. *Biological Journal of the Linnean Society*, *87*, 83–93. https://doi.org/10.1111/j.1095-8312.2006.00558.x

Mank, J. E., Vicoso, B., Berlin, S., & Charlesworth, B. (2010). Effective population size and the Faster-X effect: Empirical results and their interpretation. *Evolution; International Journal of Organic Evolution*, *64*(3), 663–674. https://doi.org/10.1111/j.1558-5646.2009.00853.x

Martin, H., Carpentier, F., Gallina, S., Godé, C., Schmitt, E., Muyle, A., … Touzet, P. (2019). Evolution of young sex chromosomes in two dioecious sister plant species with distinct sex determination systems. *Genome Biology and Evolution*, *11*(2), 350–361. https://doi.org/10.1093/gbe/evz001

Mastretta-Yanes, A., Arrigo, N., Alvarez, N., Jorgensen, T. H., Piñero, D., & Emerson, B. C. (2015). Restriction site-associated DNA sequencing, genotyping error estimation and de novo assembly optimization for population genetic inference. *Molecular Ecology Resources*, *15*(1), 28–41.

Meisel, R. P., Davey, T., Son, J. H., Gerry, A. C., Shono, T., & Scott, J. G. (2016). Is multifactorial sex determination in the house fly, *Musca domestica* (L.), stable over time? *The Journal of Heredity*, *107*(7), 615–625.

Meisel, R. P., Olafson, P. U., Guerrero, F. D., Konganti, K., & Benoit, J. B. (2019). High rate of sex chromosome turnover in muscid flies. *bioRxiv*, 655845. https://doi.org/10.1101/655845

Miura, I. (2007). An evolutionary witness: The frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation*, *1*(6), 323–331.

Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S., & Mank, J. E. (2012). W chromosome expression responds to female-specific selection. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(21), 8207–8211. https://doi.org/10.1073/pnas.1202721109

Moore, E. C., & Roberts, R. B. (2013). Polygenic sex determination. *Current Biology*, *23*, R510–R512. https://doi.org/10.1016/j.cub.2013.04.004

Moore, R. C., Harkess, A. E., & Weingartner, L. A. (2016). How to be a seXY plant model: A holistic view of sex-chromosome research. *American Journal of Botany*, *103*(8), 1379–1382. https://doi.org/10.3732/ajb.1600054

Morris, J., Darolti, I., Bloch, N. I., Wright, A. E., & Mank, J. E. (2018). Shared and species-specific patterns of nascent Y chromosome evolution in two guppy species. *Genes*, *9*(5), 238. https://doi.org/10.3390/genes9050238

Mullon, C., Wright, A. E., Reuter, M., Pomiankowski, A., & Mank, J. E. (2015). Evolution of dosage compensation under sexual selection differs between X and Z chromosomes. *Nature Communications*, *6*, 7720. https://doi.org/10.1038/ncomms8720

Muralidhar, P. (2019). Mating preferences of selfish sex chromosomes. *Nature*, *570*, 376–379. https://doi.org/10.1038/s41586-019-1271-7

Muyle, A., Käfer, J., Zemp, N., Mousset, S., Picard, F., & Marais, G. A. (2016). SEX-DETector: A probabilistic approach to study sex chromosomes in non-model organisms. *Genome Biology and Evolution*, *8*(8), 2530–2543. https://doi.org/10.1093/gbe/evw172

Muyle, A., Shearn, R., & Marais, G. A. (2017). The evolution of sex chromosomes and dosage compensation in plants. *Genome Biology and Evolution*, *9*(3), 627–645. https://doi.org/10.1093/gbe/evw282

Muyle, A., Zemp, N., Fruchard, C., Cegan, R., Vrana, J., Deschamps, C., … Marais, G. A. B. (2018). Genomic imprinting mediates dosage compensation in a young plant XY system. *Nature Plants*, *4*(9), 677–680. https://doi.org/10.1038/s41477-018-0221-y

Natri, H. M., Shikano, T., & Merilä, J. (2013). Progressive recombination suppression and differentiation in recently evolved neo-sex chromosomes. *Molecular Biology and Evolution*, *30*, 1131–1144. https://doi.org/10.1093/molbev/mst035

Naurin, S., Hansson, B., Bensch, S., & Hasselquist, D. (2010). Why does dosage compensation differ between XY and ZW taxa? *Trends in Genetics*, *26*(1), 15–20. https://doi.org/10.1016/j.tig.2009.11.006

Nielsen, R., Paul, J. S., Albrechtsen, A., & Song, Y. S. (2011). Genotype and SNP calling from next-generation sequencing data. *Nature Reviews Genetics*, *12*, 443. https://doi.org/10.1038/nrg2986

Nishioka, M., Miura, I., & Saitoh, K. (1993). Sex chromosomes of *Rana rugosa* with special reference to local differences in sex-determining mechanism. *Scientific Report of the Laboratory for Amphibian Biology, Hiroshima University*, *12*, 55–81.

Papadopulos, A. S. T., Chester, M., Ridout, K., & Filatov, D. A. (2015). Rapid Y degeneration and dosage compensation in plant sex chromosomes. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(42), 13021–13026. https://doi.org/10.1073/pnas.1508454112

Pennell, M. W., Mank, J. E., & Peichel, C. L. (2018). Transitions in sex determination and sex chromosomes across vertebrate species. *Molecular Ecology*, *27*(19), 3950–3963.

Perrin, N. (2016). Random sex determination: When developmental noise tips the sex balance. *BioEssays*, *38*, 1218–1226. https://doi.org/10.1002/bies.201600093

Phillips, R. B. (2013). Evolution of the sex chromosomes in salmonid fishes. *Cytogenetic and Genome Research*, *141*(2–3), 177–185. https://doi.org/10.1159/000355149

Pokorná, M., & Kratochvíl, L. (2009). Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? *Zoological Journal of the Linnean Society*, *156*, 168–183. https://doi.org/10.1111/j.1096-3642.2008.00481.x

Ponnikas, S., Sigeman, H., Abbott, J. K., & Hansson, B. (2018). Why do sex chromosomes stop recombining? *Trends in Genetics*, *34*(7), 492–503. https://doi.org/10.1016/j.tig.2018.04.001

Pucholt, P., Wright, A. E., Conze, L. L., Mank, J. E., & Berlin, S. (2017). Recent sex chromosome divergence despite ancient dioecy in the willow *Salix viminalis*. *Molecular Biology and Evolution*, *34*(8), 1991–2001. https://doi.org/10.1093/molbev/msx144

Rangavittal, S., Harris, R. S., Cechova, M., Tomaszkiewicz, M., Chikhi, R., Makova, K. D., & Medvedev, P. (2018). RecoverY: K-mer-based read classification for Y-chromosome-specific sequencing and assembly. *Bioinformatics*, *34*(7), 1125–1131. https://doi.org/10.1093/bioinformatics/btx771

Rice, W. R. (1992). Sexually antagonistic genes: Experimental evidence. *Science*, *256*(5062), 1436–1439.

Roberts, N. B., Juntti, S. A., Coyle, K. P., Dumont, B. L., Stanley, M. K., Ryan, A. Q., … Roberts, R. B. (2016). Polygenic sex determination in the cichlid fish *Astatotilapia burtoni*. *BMC Genomics*, *17*(1), 835. https://doi.org/10.1186/s12864-016-3177-1

Roberts, R. B., Ser, J. R., & Kocher, T. D. (2009). Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science*, *326*(5955), 998–1001.

Rodrigues, N., Studer, T., Dufresnes, C., Ma, W.-J., Veltsos, P., & Perrin, N. (2017). Dmrt1 polymorphism and sex-chromosome differentiation in *Rana temporaria*. *Molecular Ecology*, *26*(19), 4897–4905.

Roff, D. A. (1996). The evolution of threshold traits in animals. *The Quarterly Review of Biology*, *71*(1), 3–35. https://doi.org/10.1086/419266

Rovatsos, M., Farkačová, K., Altmanová, M., Johnson Pokorná, M., & Kratochvíl, L. (2019). The rise and fall of differentiated sex chromosomes in geckos. *Molecular Ecology*, *28*(12), 3042–3052. https://doi.org/10.1111/mec.15126

Rovatsos, M., Rehák, I., Velenský, P., & Kratochvíl, L. (2019). Shared ancient sex chromosomes in varanids, beaded lizards, and alligator lizards. *Molecular Biology and Evolution*, *36*(6), 1113–1120. https://doi.org/10.1093/molbev/msz024

Rovatsos, M., Vukić, J., & Kratochvíl, L. (2016). Mammalian X homolog acts as sex chromosome in lacertid lizards. *Heredity*, *117*(1), 8–13. https://doi.org/10.1038/hdy.2016.18

Satomura, K., Osada, N., & Endo, T. (2019). Achiasmy and sex chromosome evolution. *Ecological Genetics and Genomics*, *13*, 100046. https://doi.org/10.1016/j.egg.2019.100046

Saunders, P. A., Neuenschwander, S., & Perrin, N. (2018). Sex chromosome turnovers and genetic drift: A simulation study. *Journal of Evolutionary Biology*, *31*(9), 1413–1419. https://doi.org/10.1111/jeb.13336

Scharmann, M., Grafe, T. U., Metali, F., & Widmer, A. (2017). Sex-determination and sex chromosomes are shared across the radiation of dioecious *Nepenthes* pitcher plants. *bioRxiv*. Retrieved from https://www.biorxiv.org/content/10.1101/240259v1.abstract

Schartl, M. (2004). Sex chromosome evolution in non-mammalian vertebrates. *Current Opinion in Genetics & Development*, *14*(6), 634–641. https://doi.org/10.1016/j.gde.2004.09.005

Scott, M. F., Osmond, M. M., & Otto, S. P. (2018). Haploid selection, sex ratio bias, and transitions between sex-determining systems. *PLOS Biology*, *16*(6), e2005609. https://doi.org/10.1371/journal.pbio.2005609

Ser, J. R., Roberts, R. B., & Kocher, T. D. (2010). Multiple interacting loci control sex determination in lake Malawi cichlid fish. *Evolution*, *64*(2), 486–501.

Shearn, R., Lecompte, E., Mousset, S., Regis, C., Penel, S., Douay, G., … Marais, G. (2019). Evolutionary stasis of the pseudoautosomal boundary in strepsirrhine primates. *bioRxiv*, 445072.

Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P. J., Cordum, H. S., Hillier, L., Brown, L. G., … Page, D. C. (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, *423*(6942), 825–837.

Smeds, L., Warmuth, V., Bolivar, P., Uebbing, S., Burri, R., Suh, A., … Ellegren, H. (2015). Evolutionary analysis of the female-specific avian W chromosome. *Nature Communications*, *6*, 7330. https://doi.org/10.1038/ncomms8330

Tao, Y., Araripe, L., Kingan, S. B., Ke, Y., Xiao, H., & Hartl, D. L. (2007). A sex-ratio meiotic drive system in *Drosophila simulans*. II: An X-linked distorter. *PLOS Biology*, *5*(11), e293.

Tennessen, J. A., Wei, N., Straub, S. C. K., Govindarajulu, R., Liston, A., & Ashman, T.-L. (2018). Repeated translocation of a gene cassette drives sex-chromosome turnover in strawberries. *PLOS Biology*, *16*(8), e2006062. https://doi.org/10.1371/journal.pbio.2006062

Tomaszkiewicz, M., Medvedev, P., & Makova, K. D. (2017). Y and W chromosome assemblies: Approaches and discoveries. *Trends in Genetics*, *33*(4), 266–282. https://doi.org/10.1016/j.tig.2017.01.008

Tree of Sex Consortium (2014). Tree of Sex: A database of sexual systems. *Scientific Data*, *1*, 140015.

Úbeda, F., Patten, M. M., & Wild, G. (2015). On the origin of sex chromosomes from meiotic drive. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1798), 20141932. https://doi.org/10.1098/rspb.2014.1932

Valenzuela, N., Adams, D. C., & Janzen, F. J. (2003). Pattern does not equal process: Exactly when is sex environmentally determined? *The American Naturalist*, *161*(4), 676–683. https://doi.org/10.1086/368292

van Doorn, G. S., & Kirkpatrick, M. (2007). Turnover of sex chromosomes induced by sexual conflict. *Nature*, *449*(7164), 909–912.

van Doorn, G. S., & Kirkpatrick, M. (2010). Transitions between male and female heterogamety caused by sex-antagonistic selection. *Genetics*, *186*(2), 629–645. https://doi.org/10.1534/genetics.110.118596

Vandeputte, M., Dupont-Nivet, M., Chavanne, H., & Chatain, B. (2007). A polygenic hypothesis for sex determination in the European sea bass *Dicentrarchus labrax*. *Genetics*, *176*(2), 1049–1057.

Veller, C., Muralidhar, P., Constable, G. W. A., & Nowak, M. A. (2017). Drift-Induced selection between male and female heterogamety. *Genetics*, *207*(2), 711–727. https://doi.org/10.1534/genetics.117.300151

Veltsos, P., Ridout, K. E., Toups, M. A., González-Martínez, S. C., Muyle, A., Emery, O., … Pannell, J. R. (2019). Early sex-chromosome evolution in the diploid dioecious plant *Mercurialis annua*. *Genetics*, *212*(3), 815–835.

Vicoso, B., & Bachtrog, D. (2011). Lack of global dosage compensation in *Schistosoma mansoni*, a female-heterogametic parasite. *Genome Biology and Evolution*, *3*, 230–235. https://doi.org/10.1093/gbe/evr010

Vicoso, B., & Bachtrog, D. (2013). Reversal of an ancient sex chromosome to an autosome in *Drosophila*. *Nature*, *499*, 332–335. https://doi.org/10.1038/nature12235

Vicoso, B., & Bachtrog, D. (2015). Numerous transitions of sex chromosomes in *Diptera*. *PLoS Biology*, *13*(4), e1002078. https://doi.org/10.1371/journal.pbio.1002078

Vicoso, B., Emerson, J. J., Zektser, Y., Mahajan, S., & Bachtrog, D. (2013). Comparative sex chromosome genomics in snakes: Differentiation, evolutionary strata, and lack of global dosage compensation. *PLOS Biology*, *11*(8), e1001643. https://doi.org/10.1371/journal.pbio.1001643

Volff, J.-N., Nanda, I., Schmid, M., & Schartl, M. (2007). Governing sex determination in fish: regulatory putsches and ephemeral dictators. *Sexual Development*, *1*, 85–99. https://doi.org/10.1159/000100030

Wai, C. M., Moore, P. H., Paull, R. E., Ming, R., & Yu, Q. (2012). An integrated cytogenetic and physical map reveals unevenly distributed recombination spots along the papaya sex chromosomes. *Chromosome Research*, *20*, 753–767. https://doi.org/10.1007/s10577-012-9312-1

Werren, J. H., & Beukeboom, L. W. (1998). Sex determination, sex ratios, and genetic conflict. *Annual Review of Ecology and Systematics*, *29*, 233–261. https://doi.org/10.1146/annurev.ecolsys.29.1.233

Whittle, C. A., & Johnston, M. O. (2002). Male-driven evolution of mitochondrial and chloroplastidial DNA sequences in plants. *Molecular Biology and Evolution*, *19*(6), 938–949. https://doi.org/10.1093/oxfordjournals.molbev.a004151

Wilkins, A. S. (1995). Moving up the hierarchy: A hypothesis on the evolution of a genetic sex determination pathway. *BioEssays*, *17*, 71–77. https://doi.org/10.1002/bies.950170113

Wilson, C. A., High, S. K., McCluskey, B. M., Amores, A., Yan, Y.-L., Titus, T. A., … Postlethwait, J. H. (2014). Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains. *Genetics*, *198*(3), 1291–1308. https://doi.org/10.1534/genetics.114.169284

Wright, A. E., Darolti, I., Bloch, N. I., Oostra, V., Sandkam, B., Buechel, S. D., … Mank, J. E. (2017). Convergent recombination suppression suggests role of sexual selection in guppy sex chromosome formation. *Nature Communications*, *8*, 14251. https://doi.org/10.1038/ncomms14251

Wright, A. E., Darolti, I., Bloch, N. I., Oostra, V., Sandkam, B. A., Buechel, S. D., … Mank, J. E. (2019). On the power to detect rare recombination events. *Proceedings of the National Academy of Sciences of the United States of America*, *116*, 12607–12608. https://doi.org/10.1073/pnas.1905555116

Wright, A. E., Dean, R., Zimmer, F., & Mank, J. E. (2016). How to make a sex chromosome. *Nature Communications*, *7*, 12087. https://doi.org/10.1038/ncomms12087

Wright, A. E., Harrison, P. W., Zimmer, F., Montgomery, S. H., Pointer, M. A., & Mank, J. E. (2015). Variation in promiscuity and sexual selection drives avian rate of Faster-Z evolution. *Molecular Ecology*, *24*(6), 1218–1235. https://doi.org/10.1111/mec.13113

Wright, A. E., Moghadam, H. K., & Mank, J. E. (2012). Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*, *192*, 1433–1445. https://doi.org/10.1534/genetics.112.145102

Yazdi, H. P., & Ellegren, H. (2018). A genetic map of ostrich Z chromosome and the role of inversions in avian sex chromosome evolution. *Genome Biology and Evolution*, *10*(8), 2049–2060. https://doi.org/10.1093/gbe/evy163

Zemp, N., Tavares, R., Muyle, A., Charlesworth, D., Marais, G. A. B., & Widmer, A. (2016). Evolution of sex-biased gene expression in a dioecious plant. *Nature Plants*, *2*(11), 16168. https://doi.org/10.1038/nplants.2016.168

Zhou, Q., & Bachtrog, D. (2012). Sex-specific adaptation drives early sex chromosome evolution in *Drosophila*. *Science*, *337*(6092), 341–345.

Zhou, Q., Zhang, J., Bachtrog, D., An, N., Huang, Q., Jarvis, E. D., … Zhang, G. (2014). Complex evolutionary trajectories of sex chromosomes across bird taxa. *Science*, *346*(6215), 1246338. https://doi.org/10.1126/science.1246338

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Palmer DH, Rogers TF, Dean R, Wright AE. How to identify sex chromosomes and their turnover. *Mol Ecol*. 2019;28:4709–4724. https://doi.org/10.1111/mec.15245

American Genetic Association

Journal of Heredity, 2021, 250–259
doi:10.1093/jhered/esab016
Original Article
Advance Access publication March 24, 2021

OXFORD

Original Article

Multi-Copy Gene Family Evolution on the Avian W Chromosome

Thea F. Rogers, Tommaso Pizzari, and Alison E. Wright

From the Department of Animal and Plant Sciences, University of Sheffield, UK (Rogers and Wright) and Edward Grey Institute, Department of Zoology, University of Oxford, UK (Pizzari)

Address correpondence to Thea F. Rogers at the address above, or tfrogers1@sheffield.ac.uk and Alison E. Wright at the address above, or a.e.wright@sheffield.ac.uk

Received November 11, 2020; First decision December 20, 2020; Accepted March 19, 2021.

Corresponding Editor: Melissa Wilson

Abstract

The sex chromosomes often follow unusual evolutionary trajectories. In particular, the sex-limited chromosomes frequently exhibit a small but unusual gene content in numerous species, where many genes have undergone massive gene amplification. The reasons for this remain elusive with a number of recent studies implicating meiotic drive, sperm competition, genetic drift, and gene conversion in the expansion of gene families. However, our understanding is primarily based on Y chromosome studies as few studies have systematically tested for copy number variation on W chromosomes. Here, we conduct a comprehensive investigation into the abundance, variability, and evolution of ampliconic genes on the avian W. First, we quantified gene copy number and variability across the duck W chromosome. We find a limited number of gene families as well as conservation in W-linked gene copy number across duck breeds, indicating that gene amplification may not be such a general feature of sex chromosome evolution as Y studies would initially suggest. Next, we investigated the evolution of HINTW, a prominent ampliconic gene family hypothesized to play a role in female reproduction and oogenesis. In particular, we investigated the factors driving the expansion of HINTW using contrasts between modern chicken and duck breeds selected for different female-specific selection regimes and their wild ancestors. Although we find the potential for selection related to fecundity in explaining small-scale gene amplification of HINTW in the chicken, purifying selection seems to be the dominant mode of evolution in the duck. Together, this challenges the assumption that HINTW is key for female fecundity across the avian phylogeny.

Subject Area: Molecular adaptation and selection
Key words: sex chromosomes, HINTW, Y chromosome, copy number evolution

Sex chromosomes are subject to unique evolutionary pressures due to their sex-limited inheritance and exhibit many unusual characteristics compared to the rest of the genome (Furman et al. 2020). They evolve when an autosome acquires a sex-determining locus followed by halting of recombination between the sex chromosome pairs (Charlesworth 1991; Bergero and Charlesworth 2009). This recombination suppression triggers a cascade of neutral and adaptive processes that cause the once identical chromosomes to diverge from each other, often leading to the evolution of heteromorphic sex chromosomes (Bachtrog 2013). These effects are most pronounced for the sex-limited Y and W chromosomes, which experience a reduction in the efficacy of selection, often resulting in rapid decay of gene content and activity due to processes such as Muller's ratchet, the Hill-Robertson effect, and genetic hitchhiking

(Charlesworth 1978; Rice 1996; Charlesworth and Charlesworth 2000; Bachtrog and Charlesworth 2002; Bachtrog 2008). In addition, because the Y and W chromosomes are haploid and only present in one sex, their effective population size is a fraction of that of the autosomes (Bachtrog and Charlesworth 2002; Haddrill et al. 2007), making them more susceptible to genetic drift. Indeed, many Y chromosomes often consist of very few functional genes (Mank 2012), however, intriguingly many of these genes have undergone massive gene amplification and persist as members of multi-copy gene families. For instance, the human Y chromosome harbors nine multi-copy ampliconic gene families which constitute the majority of protein-coding genes present on the Y (Skaletsky et al. 2003). Why these ampliconic gene families have evolved on heteromorphic sex chromosomes is an open question and their phenotypic consequences remain debated. It is also becoming increasingly apparent that copy number of these gene families can vary substantially, not only across closely related species but also individuals of the same species (Poznik et al. 2016; Brashear et al. 2018; Lucotte et al. 2018; Ye et al. 2018; Vegesna et al. 2019; Vegesna et al. 2020). Understanding the factors driving this variability can provide insight into the adaptability and functional importance of sex chromosomes more broadly.

It is widely assumed that the expansion of multi-copy ampliconic gene families is an adaptive response to lack of recombination between the sex chromosomes, where non-allelic homologous gene conversion between copies can escape Muller's ratchet and the accumulation of deleterious mutations (Charlesworth and Charlesworth 2000; Connallon and Clark 2010; Betrán et al. 2012). Indeed, gene conversion appears to be a common feature of amplicons on both the Y and W chromosome across multiple species (Backström et al. 2005; Davis et al. 2010; Geraldes et al. 2010; Rozen et al. 2003; Skov et al. 2017). Furthermore, many Y amplicons are expressed exclusively within the testes (Skaletsky et al. 2003; Mueller et al. 2008; Vegesna et al. 2020) and often implicated in spermatogenesis and male fertility in humans (Vogt et al. 1996; Lahn and Page 1997; Kuroda-Kawaguchi et al. 2001), leading to the hypothesis that selection on male fertility, often as a consequence of sperm competition, drives the expansion of multi-copy gene families. While there appears to be a positive relationship between copy number and expression level across some gene families (Vegesna et al. 2019), as well as with sperm mobility in humans (Yan et al. 2017), comparative approaches across species have failed to detect a significant correlation between copy number and intensity of sperm competition (Vegesna et al. 2020), although this may be due to the small number of species examined to date. Intriguingly, in several species, there has been rapid co-amplification of genes on both sex chromosomes, suggestive of genomic conflict during gametogenesis to bias the transmission of the X versus Y (Soh et al. 2014; Bachtrog et al. 2019; Hughes et al. 2020). Detailed molecular analysis of the Sly and Slx gene families in the mouse provides strong support for antagonistic interactions and segregation distortion as a major force in driving gene amplification (Larson et al. 2018; Cocquet et al. 2012). Similarly, meiotic drive has been implicated in the evolution of gene families on the *Drosophila* Y chromosome (Bachtrog et al. 2019). Finally, many amplicons appear to be evolving under relaxed purifying selection, consistent with the reduced efficacy of selection on the non-recombining Y (Ghenu et al. 2016; Vegesna et al. 2020). Thus, while a myriad of forces has been implicated in the amplification of gene families on the Y and W chromosomes, the relative importance of each remains unclear.

To date, our understanding of multi-copy ampliconic gene families is primarily based on Y chromosome studies across mammals and *Drosophila*, and the W chromosome has been largely overlooked. Although the W is in many ways comparable to the Y chromosome, as both are sex-limited and do not recombine, the W is only present in females and the Y is only present in males. Therefore, the W chromosome, unlike the Y chromosome, does not experience sperm competition and might be subject to weaker sexual selection than the Y (Bachtrog et al. 2011). Additionally, in polygynous mating systems where a small proportion of males in the population mate with multiple females, the effective population size of the Y relative to the autosomes is smaller than that of the W (Mank 2012; Wright and Mank 2013) As a result, the W chromosome may be less susceptible to genetic drift than the Y. Therefore, if multi-copy gene families are a consequence of random gene amplification due to genetic drift, they should be more pronounced on the Y chromosome rather than represent a general feature of heteromorphic sex chromosomes. It remains unclear whether W-linked amplicons have followed similar patterns of evolution to ampliconic genes on Y chromosomes, or whether gene amplification always occurs in parallel with sex chromosome degeneration.

A limited number of W-linked multi-copy gene families have been documented in a handful of species, primarily avian (Backström et al. 2005; Davis et al. 2010; Moghadam et al. 2012; Smeds et al. 2015; Zhou et al. 2020). The best studied is HINTW, an ampliconic gene family present on the avian W chromosome that is hypothesized to play a role in female reproduction and oogenesis (O'Neill et al. 2000; Ceplitis and Ellegren 2004), and was originally proposed as the avian sex-determining gene (Pace and Brenner 2003; Parks et al. 2004; Moriyama et al. 2006). While an initial study of HINTW indicated that large-scale amplification of copy number is conserved across avian non-ratites (Hori et al. 2000), a recent study suggested that HINTW is single-copy in the Pekin duck (Li et al. 2021). To date, there has been no comprehensive investigation into the abundance, variability, and evolution of multi-copy ampliconic gene families on the W chromosome both across and within species.

Here, we conduct a comparative analysis of copy number variation of W-linked genes across chicken and duck breeds. Multi-copy gene families are notoriously challenging to study due to their highly repetitive nature (Tomaszkiewicz et al. 2016). This problem is confounded on the sex-limited Y and W chromosomes where amplicons are often located in repeat-rich regions that are poorly annotated in reference genomes. We employ NanoString technology, which is based on fluorescent probes, to provide high-throughput fine-scale estimates of gene copy number and variability (Cui et al. 2014; Ahn et al. 2016). First, we quantify the frequency and variability of multi-copy gene families on the W across duck breeds and find a limited number of amplicons on the duck W as well as conservation in copy number of W-linked genes. Next, we investigate the role of selection for fecundity in driving the amplification of HINTW using contrasts between chicken and duck breeds selected for either egg-laying, male meat production, or male plumage. We find that although large scale amplification of HINTW is ancestral to land and waterfowl species, smaller-scale gene duplications have occurred independently across chicken breeds. Our results support a potential role of female-specific selection in driving amplification of the HINTW gene family in the chicken but not the duck, challenging the assumption that HINTW is key for female fecundity across the avian phylogeny.

Materials and Methods

Samples and DNA extraction

Our workflow is summarized in Figure S1. We obtained tissue samples from Khaki Campbell, Indian Runner, Aylesbury, and Cayuga duck breeds and their modern ancestor, the Mallard duck (*Anas platyrhynchos*) (Zhang et al. 2018). In addition, we sampled the White Leghorn, Black Minorca, Oxford Old English, and Black Sumatra chicken breeds and their main modern ancestor, the Red Junglefowl (*Gallus gallus*) (Frisby et al. 1979; Fumihito et al. 1996).

Samples were collected in accordance with national and ethical guidelines. Specifically, we obtained feathers from White Leghorn and Black Minorca. We also obtained 50 ml of Red Junglefowl blood in 1ml of absolute ethanol from a captive population at Oxford University (PPL P50402706). We obtained fertilized eggs from the following duck breeds; Mallard, Khaki Campbell, Cayuga, Aylesbury, Indian Runner, and the following chicken breeds; Oxford Old English and Black Sumatra. All eggs were kept under standard incubation conditions at The University of Sheffield. Samples were collected according to national and ethical guidelines and the liver was dissected at embryonic day 19 and 24 in chicken and duck breeds, respectively, then stored in 95% ethanol.

DNA was extracted from feather and embryonic liver samples using DNeasy blood and tissue kit (QIAGEN) using standard protocols. DNA was extracted from blood samples using the ammonium acetate precipitation method. In total, DNA was obtained for three female and two male samples from each of the domesticated breeds, and two female and two male samples from each of the modern ancestor breeds. Embryonic birds were sexed visually and feather and blood samples were sexed using published sexing primers (Fridolfsson and Ellegren 1999).

The majority of modern chicken breeds originated at the start of the 20th century (Rubin et al. 2010). Most modern chicken breeds are descended from the Red Junglefowl (Frisby et al. 1979; Fumihito et al. 1996) with some genes introgressed from the Grey Junglefowl and possibly other Junglefowl species (Eriksson et al. 2008). The Black Minorca and White Leghorn are layer breeds, which have been selected for female reproductive traits (e.g., fecundity), and the Oxford Old English and Black Sumatra chickens have been selected for male traits such as plumage for ornamentation purposes and aggression for cockfighting. The Oxford Old English and Black Sumatra lay fewer eggs than the two layer breeds and experience numerous female fecundity problems (Ekarius 2007; Lewis 2010). Importantly, the chicken breeds used in this study have independent origins (Moghadam et al. 2012) and so we can treat them as independent replicates of increased or relaxed female-specific selection. Most modern duck breeds are descended from the Mallard duck (Zhang et al. 2018). The Indian Runner and Khaki Campbell duck breeds have been subject to strong female-specific selection for egg-laying, and the Aylesbury and the Cayuga for meat production (Ashton et al. 1999). Selection for meat- and egg-purpose breeds occurred at the early stages of duck domestication (Zhang et al. 2018) and so it is unclear whether the two layer duck breeds in our study can be considered independent replicates of increased female-specific selection.

Identification of W-Linked Genes

Previously, we identified 26 W-linked genes in the duck reference genome (Wright et al. 2014) using a combination of phylogenetic analyses and polymerase chain reaction (PCR) validation in females. Some of these W genes share the same Z-linked ortholog, indicating they are either paralogs of a multi-copy gene family or fragments of the same gene which have been assembled into separate genic sequences in the reference genome. Genome assemblies of sex chromosomes can be unreliable due to their repetitive nature and low sequencing coverage (Tomaszkiewicz et al. 2017) and so the latter scenario is plausible. To distinguish between these two scenarios, we aligned W-linked coding sequences with their Z-linked ortholog using PRANK (Löytynoja 2014) and calculated pairwise distances. For the majority of cases, W-linked sequences shared no sequence similarity with each other, indicating they are fragments of the same gene that have been incorrectly assembled and annotated into separate genes. For subsequent analyses, we averaged data across fragments for these genes. Our results are quantitatively identical whether fragments are analyzed separately or combined (see Supplementary Tables). The exception was KCMF1 in which the two annotated W sequences in the reference align and have a low pairwise distance, where the proportion of nucleotide differences was 0.091, suggesting these are paralogs of the same multigene family.

However, HINTW is not annotated in the duck reference genome and a previous study only identified a short fragment of sequence (Hori et al. 2000). Therefore, we sequenced a 702 bp fragment of HINTW in the Mallard using Sanger sequencing at the Core Genomic Facility, University of Sheffield with primers designed for the black oystercatcher (*Haematopus bachmani*) (Guzzetti et al. 2008). Primers are listed in Table S1.

For each PCR reaction, the following volumes and concentrations of reagents were used: 4 µl multiplex PCR Master Mix (QIAGEN), 2 µl forward primer, 2 µl reverse primer (initial conc of each 0.2 µM) and 1 µl DNA (initial conc 15 ng/µl). In addition to this, 1 ul of nuclease free H_2O was added to reach a total volume of 10 µl per reaction. The PCR conditions were: initial denaturing stage of 95 °C for 15 min, then 35 cycles of the following three steps; 94 °C for 30 s, an annealing step at 57 °C for 90 s, and an extension at 72 °C for 90 s. This was then followed by a final extension at 72 °C for 10 min.

Identification of Autosomal Invariant Genes

The NanoString pipeline relies on the identification of invariant genes, autosomal single copy genes in that do not vary in copy number, as internal controls. We identified invariant genes in the duck and chicken separately using a genomic coverage approach. SOLiD DNA-seq data from nine chicken breeds were obtained from Rubin et al. (2010) and reads were aligned to the chicken reference genome (Gallus_Gallus-5.0, Zerbino et al. 2018) using SHRiMP v. 2.2.2 (Rumble et al. 2009). Mapped reads with a quality score of 10 or above were retained using SAMtools v. 1.8 (Li et al. 2009). Illumina DNA-seq reads from seven duck breeds (Zhang et al. 2018) were aligned to the duck reference genome (BGI_duck_1.0, Zerbino et al. 2018) using BWA v. 0.5.7 (Li and Durbin 2009) with the "mem" algorithm. Read depth for each gene was calculated for both the chicken and the duck using the depth function in SAMtools. For each species, we conducted pairwise regressions of read depth per gene across every breed. We ranked residuals and identified genes in the lowest 35% quantile across all pairwise comparisons, indicative of limited or no copy number variation. We then used single nucleotide polymorphism (SNP) data to test for nucleotide polymorphism across these genes, and we only called SNPs if the minor allele was present in one than one read. We chose genes with an absence of nucleotide polymorphism, and therefore an absence of multiple copies, as our invariant genes.

Quantification of Gene Copy Number using NanoString

Copy number was quantified using the NanoString nCounter platform at the NERC Biomolecular Analysis Facility (NBAF), University of Liverpool. NanoString nCounter technology uses fluorescent probes to estimate fine scale variation in gene copy number across samples (Cui et al. 2014; Ahn et al. 2016). Probes were designed for W-linked genes and invariant genes in the chicken and duck separately in accordance with NanoString protocol (Table S2). Specifically, two or three probes were designed for HINTW in the chicken and 26 W-linked genes in the duck. One or two probes were designed for each invariant gene.

We implemented a number of controls to ensure copy number was quantified for only W-linked genes and not their Z-linked orthologs. Genome assemblies of sex chromosomes are often unreliable due to their repetitive nature and low sequencing coverage (Tomaszkiewicz et al. 2017) and therefore accurately identifying W-specific regions can be problematic. Furthermore, given that the Z and W chromosome evolved from the same pair of autosomes, certain regions of W-linked genes have high sequence similarity to their Z-linked gametolog (Wright et al. 2012). First, we designed probes to W-linked exons with low sequence similarity to Z-linked orthologs. Second, we included male samples in the Copy Number Variation (CNV) CodeSet analysis, making it possible to identify and exclude probes that bind to the Z chromosome.

The NanoString nCounter assay was performed according to standard protocol. Briefly, at least 300 ng of DNA per sample was fragmented via AluI digestion and then hybridized to the custom CNV CodeSet. Samples included three females and two males from each of the selectively bred breeds, and two female and two male samples from each of the modern ancestor breeds. Samples were distributed randomly over the CNV CodeSets to avoid batch effects. The nCounter Digital Analyzer was used to count and quantify signals of reporter probes. Data analysis was performed using the nSolver Analysis Software.

We implemented a number of sanity checks as recommended by NanoString. First, we removed probes with count data above background noise in males and therefore affinity to the Z chromosome (Table S2). Background noise was calculated for each sample according to NanoString protocol as the average plus two standard deviations of the count number in the negative controls. We also removed one probe with count data below background noise in females, indicating low binding affinity. Second, as multiple probes were designed per W-linked gene, we calculated the coefficient of variation for copy number across probes. A high coefficient of variation is indicative of a probe that is not binding as predicted. As recommended by NanoString, we removed two probes from two different genes where the sum of the coefficient of variation across samples was >= 100 (Table S2). We averaged count data across all remaining probes of each gene in every individual.

Quantification of Gene Copy Number from SNP Data

We used polymorphism estimates from publicly available DNA-seq data to independently verify the results obtained from the NanoString nCounter assay in the Mallard duck. Given that we expect many gene copies to share identical sequences due to gene conversion (Backström et al. 2005), we can only use SNPs to estimate a minimum copy number.

Illumina data from nine unsexed Mallard ducks (Zhang et al. 2018) were quality trimmed to a minimum of 34 bp using Trimmomatic v. 0.36 (Bolger et al. 2014). Data were then aligned to the duck reference genome (BGI_duck_1.0, Zerbino et al. 2018), with the 702 bp sequenced fragment of HINTW added, using BWA v. 0.7.17 (Li and Durbin 2009) with the "aln" algorithm. Alignments were filtered for uniquely mapped reads by keeping only lines of the BAM files that matched the flag "XT:A:U." We used read coverage to sex individuals, where Z-linked genes should show half the number of reads in females relative to males. Read depth per gene was calculated using the depth function in SAMtools. To control for differences in overall sequencing depth between individuals we divided read depth on the Z chromosome by average autosomal read depth in each sample. Six females were identified and used in subsequent analyses.

BCFtools v. 1.9 (Narasimhan et al. 2016) was used to call SNPs at sites with a mapping quality >20. In order to classify a SNP that indicated copy number variation, both the major and minor allele had to be supported by at least four reads and be present in more than half the individuals. Minor allele read depth was also required to be supported by at least 10% of the number of reads that supported the major allele.

Results

Copy Number of Genes on the Mallard W Chromosome

We surveyed the copy number of 26 genes on the Mallard duck W chromosome using count data obtained from NanoString nCounter. First, count data for W genes were normalized to invariant genes, autosomal genes present in a single copy, following NanoString protocol to account for any differences across samples in genomic DNA input arising from pipetting error or inaccuracies in DNA quantitation. Specifically, in each individual separately, we calculated average counts across all 10 invariant genes and bootstrapped with 1000 replicates to obtain the 95% confidence intervals. We divided the confidence intervals by two to account for comparisons between autosomal genes, which are present in two copies, and W-linked genes, which are present at a minimum of one copy. We then divided count number for each W gene by invariant count values to obtain estimates of W copy number in each individual and 95% confidence intervals.

In the Mallard duck, most W genes are present in a single copy. We found that HINTW is ampliconic, present in approximately 18 copies. This is in contrast to recent work suggesting that HINTW is single-copy in the Pekin duck (Xu and Zhou 2020; Li et al. 2021). Furthermore, we found that KCMF1W is a multi-copy gene family present in 2–3 copies (Tables 1 and S3).

We independently verified copy number estimates using publicly available sequence data from Mallard individuals and nucleotide polymorphism analyses. No SNPs were found in any of the W genes with the exception of KCMF1W (ENSAPLG00000003106), where a single SNP was identified. This supports our finding that the majority of W-linked genes are present in a single copy in the Mallard. Although we verified that HINTW is ampliconic using NanoString data, we did not identify any nucleotide polymorphism across copies. This instead may indicate the occurrence of gene conversion across HINTW in the duck, which acts to homogenize gene sequence among variants and is consistent with previous results in galliform birds (Backström et al. 2005).

Table 1. Copy number of W-linked genes across duck breeds.

Gene name	Duck Ensembl ID	Average copy number					Δ Copy number	Coefficient of variation	Stratum^
		Mallard	Caguya	Aylesbury	Indian Runner	Khaki Campbell			
HINTW+	NA	18.03	16.35	16.57	17.22	16.83	1.68	0.04	1
CHD1W*	ENSAPLG05191 ENSAPLG02506	0.94	0.97	0.98	0.99	0.98	0.05	0.09	1
KCMF1W	ENSAPLG03026 ENSAPLG03106	2.43	2.59	2.65	2.63	2.63	0.20	0.10	2
RASA1W	ENSAPLG05611 ENSAPLG10611 ENSAPLG11371	0.64	0.69	0.70	0.69	0.70	0.06	0.11	2
ATP5A1W+	ENSAPLG09007	0.82	0.79	0.84	0.85	0.83	0.06	0.07	3
BTF3W	ENSAPLG04652	0.65	0.60	0.64	0.65	0.65	0.05	0.06	3
HNRPKW*+	ENSAPLG10986	0.97	1.00	1.09	1.02	1.05	0.12	0.11	3
MIER3W+	ENSAPLG10850	0.62	0.61	0.65	0.62	0.63	0.04	0.08	3
NIPBLW	ENSAPLG02953 ENSAPLG03022 ENSAPLG05315 ENSAPLG10290 ENSAPLG10560	0.67	0.69	0.71	0.69	0.70	0.04	0.09	3
SMAD2W	ENSAPLG04964	0.69	0.72	0.74	0.71	0.71	0.05	0.09	3
SPIN1W*	ENSAPLG02923	0.63	0.61	0.64	0.66	0.64	0.05	0.08	3
UBAP2W	ENSAPLG16004 ENSAPLG16155	0.61	0.58	0.59	0.60	0.60	0.03	0.06	3
UBE2R2W	ENSAPLG16000	0.76	0.74	0.76	0.78	0.76	0.04	0.07	3
VCPW+	ENSAPLG05806	0.91	0.84	0.90	0.91	0.90	0.07	0.06	3
ZFRW*	ENSAPLG15519	0.67	0.68	0.69	0.70	0.68	0.03	0.08	3
ZSWIM6W	ENSAPLG13555 ENSAPLG14338	0.77	0.80	0.80	0.83	0.80	0.06	0.09	3

* q-PCR analysis showed variation in copy number of ortholog across chicken breeds (Moghadam *et al.* 2012).
\+ SNP analysis showed chicken ortholog is multicopy (Moghadam *et al.* 2012).
^ Anseriform strata as defined by Wright *et al.* 2014. Strata 1 & 2 are conserved in chicken and duck but Stratum 3 evolved independently.
Note: six zeros have been removed from start of the digits in the Ensembl IDs.

Copy Number Variation Across Duck Breeds

We used the same approach to estimate the copy number of W-linked genes across the four duck breeds, with the exception of HINTW which we discuss separately below. Copy number was broadly conserved, as the majority of genes are present in a single copy across all breeds (Tables 1 and S3), with the exception of KCMF1W. This multi-copy gene family varies from 2 to 3 copies in some breeds to 3 to 4 copies in others, suggesting there may have been lineage-specific duplications in certain breeds (Tables S3 and S4).

In order to verify these results using a separate approach, we next estimated copy number in each breed relative to the Mallard duck. For each W-linked gene, normalized count data in each individual were divided by the average normalized count data for the Mallard to estimate relative copy number. We found that every W gene had a copy number ranging from 0.88 to 1.21 relative to the Mallard in all individuals, supporting our finding that there is limited copy number variation across duck breeds.

Finally, we estimated variation in copy numbers by calculating the coefficient of variance of raw count data across all individuals and breeds for each W-linked gene. Coefficient estimates ranged from 0.078 to 0.112 across individuals (Tables S5 and S6), and importantly no value exceeded the maximum coefficient of variation for invariant genes (mean COV = 0.131, max COV = 0.416), indicating limited variation in W-linked copy number. We repeated the analysis across breeds using average copy number in each breed and found a similar pattern, whereby coefficients of variation ranged from 0.043 to 0.106. No W gene exhibited higher variation across breeds than that observed across invariant genes (mean COV = 0.111, max COV = 0.356).

Copy Number Variation of Ampliconic HINTW Across Duck and Chicken Breeds

Next, using contrasts between modern chicken and duck breeds selected for different female-specific selection regimes and their wild ancestors, we investigated the factors driving the expansion of HINTW. First, we estimated the size of the ampliconic HINTW gene family across duck breeds and found limited differences, where the number of copies ranged from 15.49 to 18.49 across individuals (Figure 1A, Tables S3, and S4). In addition, the coefficient of variance of HINTW count data across individuals (mean COV = 0.080) and breeds (mean COV = 0.043) was not higher than variation across invariant genes (Tables S5 and S6). Importantly, there is no significant difference in average copy number between breeds (ANOVA; $p = 0.312$). This suggests that the copy number of HINTW is broadly conserved across duck individuals and breeds (Table S7), consistent with our predictions for purifying selection.

In contrast, we found notable variation in the size of the HINTW gene family across chicken breeds and individuals, ranging from 7.54 to 17.36 copies. The coefficient of variance for the chicken was

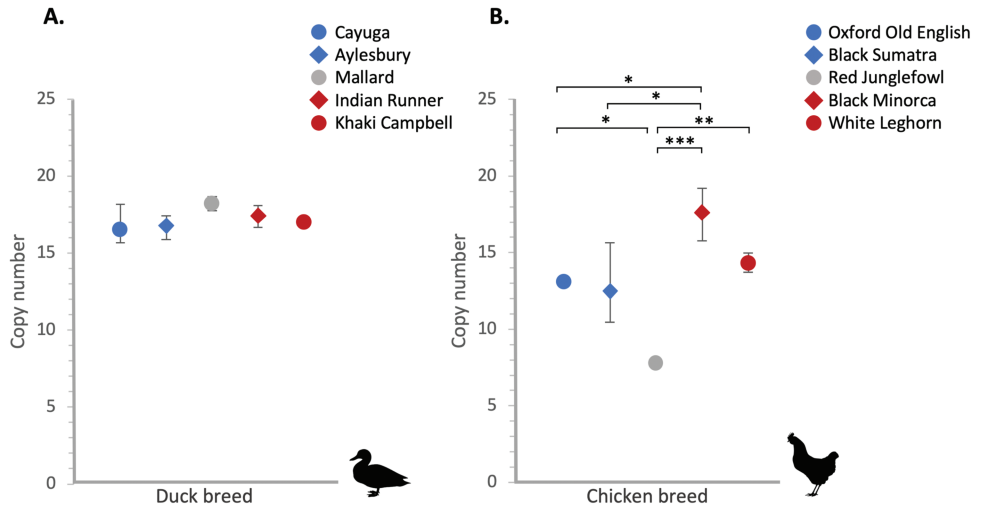

Figure 1. Copy number variation of HINTW across **(A)** duck and **(B)** chicken breeds. Copy number was estimated using the NanoString nCounter platform. Each circle or diamond represents the mean HINTW copy number per breed, and bars show the range of HINTW copy number across individuals. Blue markers represent breeds subject to relaxed female-specific selection, red markers represent breeds subject to increased female-specific selection, and grey markers denote the modern ancestor. Stars indicate pairwise significance values from Tukey multiple comparisons of means where *p < 0.05, **p < 0.01 and ***p < 0.001.

0.213 across individuals and 0.221 across breeds, both of which are higher than mean variation exhibited across invariant genes (mean COV = 0.151, max COV = 0.244 across individuals and mean COV = 0.116, max COV = 0.166 across breeds). Importantly, we found that the average size of HINTW gene family varied significantly between breeds (ANOVA; p = 0.001). Interestingly, all breeds have a higher copy number of HINTW than the Red Junglefowl, and this was significant for three breeds (Figure 1B), indicating that the early domestication of chicken breeds may have been associated with a period of female-specific selection, presumably for egg-laying. We find a general trend that breeds which have been selected for egg production via artificial female-specific selection (Kerje et al. 2003), had on the average higher number of copies relative to breeds that have been bred for male fighting and plumage and subject to relaxed female-specific selection (Ekarius 2007; Lewis 2010) (Figure 1B). However, this relationship was only significant for the Black Minorca and not the White Leghorn (Table S8).

Discussion

The sex-limited Y and W chromosomes exhibit a small but unusual gene content in many species compared to the rest of the genome. One striking feature is the existence of ampliconic gene families, arising from massive gene amplification of distinct classes of genes. Our understanding of how and why these ampliconic regions have evolved is primarily based on detailed Y chromosome studies across mammals and *Drosophila*, which have implicated a multitude of factors in the expansion of gene families, including meiotic drive, sperm competition, genetic drift, and gene conversion (Skaletsky et al. 2003; Ellis et al. 2011; Cocquet et al. 2012; Good 2012; Soh et al. 2014; Ghenu et al. 2016; Larson et al. 2018; Bachtrog et al. 2019; Vegesna et al. 2020). However, the evolution of multi-copy gene families on the W chromosome has been largely overlooked, with the exception of a handful of studies (Hori et al. 2000; Backström et al. 2005; Davis et al. 2010; Moghadam et al. 2012; Zhou et al. 2020). As a result, it remains unclear whether ampliconic genes are a fundamental feature of heteromorphic sex chromosome evolution or a peculiar quirk of Y chromosomes. Here, we conduct a comparative analysis to examine the abundance, variability, and evolution of ampliconic gene families on the avian W chromosome both across and within two avian species.

Our results show little evidence for gene amplification on the duck W chromosome. Of the 26 W-linked genes we studied, only two are present in multiple copies. One of these is HINTW, a large well-known ampliconic gene family, that has previously been characterized across a wide range of avian species (Hori et al. 2000; Backström et al. 2005). The fact that HINTW is ampliconic in the Mallard and four duck breeds is in contrast to recent work in the Pekin duck (Xu and Zhou 2020; Li et al. 2021). Moreover, our finding that W chromosomes in the Mallard and domesticated duck breeds are generally depauperate in multi-copy gene families is consistent with a growing body of avian literature, including studies in the chicken (Moghadam et al. 2012), flycatcher (Smeds et al. 2015), sparrow (Davis et al. 2010), songbirds (Xu et al. 2019), and Pekin duck (Li et al. 2021). Outside of birds, to our knowledge, there is only one report of a W-linked ampliconic gene family in the willow *Salix purpurea* (Zhou et al. 2020), though few W chromosomes have been studied in sufficient detail. This deficit of gene families on the W is in stark contrast to the Y chromosome in mammals and *Drosophila*, where there has been massive amplification of gene sets.

This emerging pattern is consistent with theoretical predictions for how we expect the W to evolve differently to the Y due to their contrasting inheritance patterns (Mank 2012; Bachtrog et al. 2014). First, as the W chromosome is maternally inherited it is not subject to sperm competition, a factor which has been hypothesized, with mixed empirical support, to drive the expansion of Y-linked gene families (Hughes et al. 2010; Vegesna et al. 2020). It should be noted that the lack of support Vegesna et al. (2020) find for this hypothesis could be due to the small number of species examined in their study. Second, genetic drift is predicted to be weaker on the W in comparison to the Y chromosome. In polygynous mating systems, where a small proportion of males in the population mate with several females, the effective population size of the Y relative to the autosomes is smaller than that of the W (Mank 2012; Wright and Mank 2013). Relaxed purifying selection has been invoked to explain amplification of certain gene families on the primate and human Y

chromosome, and the large variability in copy number across individuals and populations (Ghenu et al. 2016; Ye et al. 2018; Vegesna et al. 2020). Under drift, we expect variance in copy number to be approximately proportional to gene family size, where larger gene families will have a greater chance of gene duplication. Interestingly, we do not observe this pattern on the duck W chromosome where variability in the size of the HINTW gene family, present in ~18 copies, was similar to KFMC1, present in ~2 copies, across individuals and breeds. This is consistent with previous work showing evidence for purifying selection on the Mallard W (Wright et al. 2014).

Finally, Y and W chromosomes are exposed to different types of gametogenesis, where the W is subject to oogenesis and the Y to spermatogenesis. Importantly, these contrasting environments likely lead to differences in the potential for antagonistic coevolution between the sex chromosomes. Antagonistic coevolution is predicted to drive the co-amplification of X and Y-linked genes (Bachtrog 2020), but should be weaker during oogenesis than spermatogenesis. This is because the window for intragenomic conflict between chromosomes is restricted to the first meiotic division during oogenesis as only a single oocyte is produced containing either the Z or W (Bellott et al. 2017). Therefore, antagonistic coevolution between the Z and W will be limited to the first meiotic division. In contrast, competition between the X and Y can occur during meiosis I and II of spermatogenesis as both of these cell divisions produce viable gametes. As a result, we expect the meiotic drive to play a less prominent role in the evolution of the W compared to the Y, and might explain why meiotic drive has been heavily implicated in the amplification of gene families on the mouse and *Drosophila* Y chromosomes (Bachtrog et al. 2019; Ellis et al. 2011; Cocquet et al. 2012; Good 2012; Soh et al. 2014; Larson et al. 2018).

In addition, expression of the sex chromosomes is repressed during the post-meiotic stages of spermatogenesis, leading to intragenomic conflict between X- and Y-linked genes over transcriptional machinery and selection for gene amplification to maintain gene expression (Moretti et al. 2020). In contrast, no corresponding mechanism of sex chromosome repression in oogenesis has been reported thus far, and so we expect less co-amplification due to antagonistic coevolution in ZW systems. In support of these predictions, there is no evidence for co-amplification of HINTZ or KCMF1 on the avian Z chromosome (Bellott et al. 2010), indicating that antagonistic coevolution is unlikely to be a major factor influencing gene amplification on the W. Together, our results indicate that large scale expansions of gene families do not always occur in parallel with sex chromosome degeneration and so may not be such a general feature of sex chromosome evolution as Y studies would initially suggest.

Finally, as the W chromosome is maternally inherited it is not subject to sperm competition, a factor which has been hypothesized, with mixed empirical support, to drive the expansion of Y-linked gene families (Hughes et al. 2010; Vegesna et al. 2020). However, in theory, sex-specific selection for increased expression of genes associated with fecundity could drive amplification of gene families on the W chromosome, analogous to the hypothesized role of sperm competition on the Y chromosome (Hughes et al. 2005). In order to examine the factors driving the evolution of multi-copy gene families, we contrasted the copy number of HINTW across breeds of the duck and chicken. Specifically, we chose breeds that have been subject to stronger or relaxed female-specific selection. In theory, sex-specific selection for increased expression of genes associated with fecundity could drive amplification of gene families. This seems particularly relevant for HINTW, which is expressed in the developing ovaries (O'Neill et al. 2000) and hypothesized to play a role in female reproduction (O'Neill et al. 2000; Ceplitis and Ellegren 2004). Furthermore, the increased copy number of Y-linked genes has been shown to result in greater gene expression levels across primates, although this pattern is not universal across all gene families (Yan et al. 2017; Vegesna et al. 2019). However, in general, there is uncertainty over whether the W chromosome is subject to female-specific selection, and is enriched for female reproductive functions (Moghadam et al. 2012), or subject to purifying selection for dosage effects (Smeds et al. 2015; Bellott et al. 2017; Xu et al. 2019; Xu and Zhou 2020).

We find that HINTW copy number across duck breeds and individuals is remarkably conserved, in contrast to ampliconic gene families of equivalent size on the mammalian and *Drosophila* Y chromosomes (Bachtrog 2013). We were unable to identify any sequence polymorphism across copies of HINTW, indicative of persistent gene conversion. While gene conversion is unlikely to explain the origin of multi-copy gene families, because it acts at a scale of a few hundreds of bases as opposed to a much larger scale of whole gene duplicates (Chen et al. 2007; Connallon and Clark 2010; Marais et al. 2010), it has been proposed to select for the maintenance of ampliconic gene families and has been shown to operate across HINTW copies in a number of avian species (Backström et al. 2005). However, it is worth noting that the duck HINTW fragment in our study was only 702 bp, lowering the probability of finding an SNP in this gene and increasing our chances of inferring the action of gene conversion. Together, our results are inconsistent with the role of female-specific selection in driving the evolution of HINTW copy number in the duck. Instead, the conservation in copy number we observe across breeds suggests that the HINTW copy number is under strong purifying selection. This is consistent with a number of recent studies showing that the avian W chromosome evolves predominantly under purifying selection to maintain ancestral gene dosage (Wright et al. 2014; Smeds et al. 2015; Bellott et al. 2017; Bellott and Page 2021).

In contrast, in the chicken, we find notable variation in HINTW copy number across breeds. Breeds subject to increased female-specific selection tend to exhibit a greater number of HINTW copies. This is consistent with the prediction that the chicken HINTW plays a role in female fecundity (O'Neill et al. 2000; Ceplitis and Ellegren 2004). However, there is considerable variation in this trend, potentially indicating that female-limited selection is not the dominant force driving the evolution of HINTW.

The discrepancy between levels of variation in the size of the HINTW gene family in the chicken and duck is intriguing, particularly as large-scale gene amplification likely occurred in the ancestor of non-ratite birds (Hori et al. 2000). While evidence from the chicken indicates that HINTW plays a role in oogenesis (O'Neill et al. 2000; Ceplitis and Ellegren 2004), evidence for the functionality of HINTW in the duck is lacking. In fact, HINTW in the duck has been shown to lack the C-terminal 14 residues (Hori et al. 2000). HINTW forms a heterodimer with and inhibits HINTZ in the chicken (Hori et al. 2000), and it is possible that the deletion in the duck has altered its ancestral functionality. Alternatively, HINTW may have evolved differential gene expression across duck breeds without a corresponding increase in copy number. Consistent with this explanation, many W-linked genes have evolved increased expression in the chicken embryonic gonad in response to female-specific selection relative to the modern ancestor Red Junglefowl in the absence of copy number variation (Moghadam et al. 2012). It is also possible that the chicken has been subjected to stronger or more consistent sex-specific selection regimes than the duck, although evidence for this is currently lacking.

Similarly, it is possible that the timing of domestication differs between the duck and chicken breeds in our study, or that there are differences in the extent of interbreeding. Although the exact breed history of chicken and ducks is obscure, evidence indicates that duck breeds selected for egg-laying and meat production form two monophyletic groups that split early in duck domestication approximately 2000 years ago (Zhang et al. 2018). Therefore, we think that the lack of inter-breed copy number variation in the duck is unlikely to be a consequence of more recent origin or greater levels of interbreeding, although we cannot rule out this possibility.

In addition, we find that gene amplification has proceeded independently on the chicken or duck W chromosome (Van Tuinen and Hedges 2001). When we contrast copy number estimates from previous work for the chicken (Moghadam et al. 2012) with our study, we find that W genes tend to duplicate independently, albeit at low copy number, in each species separately (Table 1). This suggests that the W is not an inert genetic wasteland but seems to evolve dynamically even after recombination was halted between the sex chromosomes.

Finally, it is worth discussing the difficulties and limitations associated with studying copy number variation of ampliconic gene families. First, while our NanoString probe-based approach offers high-throughput fine-scale estimates of gene copy number and variability, we were not able to distinguish between functional and non-functional gene copies. This is particularly relevant for our conclusions surrounding the evolution of HINTW in the duck. Furthermore, it is not possible to detect gene copies with sequences that are substantially divergent from the probe sequences used. However, gene conversion should homogenize the sequence of gene copies, limiting the potential for this to confound our results. Finally, there is evidence that certain ampliconic genes on the Y are lineage-specific, for instance, Sly and Slx are specific to the mouse lineage (Moretti et al. 2020). The list of W-linked genes we included in our analyses is not exhaustive (Wright et al. 2014) due to the challenges of sequencing sex chromosomes. Expanding the scope of this work to test whether lineage-specific loci are more likely to undergo massive scale amplification would be an interesting future avenue.

Concluding Remarks

Massive gene amplification is a characteristic feature of Y chromosome evolution. However, until now, it has remained unclear whether gene duplication is as prevalent on the W chromosome. We reveal that on the duck W chromosome, only two out of 26 W-linked genes show evidence of gene duplication. We hypothesize that this may be because genetic drift is reduced on the W relative to Y chromosomes, and we find a limited variation of within-species gene copy number consistent with purifying selection. Contrary to this, we find some evidence that expansion of the HINTW gene family has evolved in response to female-specific selection for egg laying in the chicken but not the duck, calling into question the broad functionality of this prominent gene family. Taken together, our results suggest that in terms of gene duplication, the W chromosome follows a different evolutionary trajectory to that of the Y.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Funding

This work was supported by a Natural Environment Research Council (NERC) Independent Research Fellowship to A.E.W. (NE/N013948/1), a NERC ACCE Ph.D. studentship to T.F.R., and an EECG Research Award from the American Genetics Association to A.E.W.

Acknowledgements

We thank the following for providing avian samples; Edward Boothman (White Leghorn), Richard Windsor (Black Minorca), David Hackett (Oxford Old English), Charles Hardcastle (Mallard), Graham Mortimer (Khaki Campbell, Indian Runner), and Richard Hedges and Paul Hayes (Cayuga, Aylesbury, Black Sumatra). We thank Nicola Hemmings for helpful discussions on staging and incubating avian eggs, Geoff Parker for advice on chicken breeds and chicken breeders, The British Waterfowl Association and Rare Poultry Club for helping to find breeders, Judith Mank, Daniela Palmer, and Peter Price for productive discussions. The authors acknowledge the use of the Sheffield HPC Cluster, and associated support services, in the completion of this work. Data generation was carried out by the Centre for Genomic Research, which is based at the University of Liverpool.

Data Availability

The raw data underlying these analyses is available in Dryad, DOI: https://doi.org/10.5061/dryad.18931zcw9.

References

Ahn S, Hong M, Van Vrancken M, Lyou YJ, Kim ST, Park SH, Kang WK, Park YS, Jung SH, Woo M, *et al.* 2016. A nCounter CNV assay to detect HER2 amplification: a correlation study with immunohistochemistry and in situ hybridization in advanced gastric cancer. *Mol Diagn Ther*. 20:375–383.

Ashton C, Ashton M, Donner C. 1999. *British waterfowl standards*. UK: British Waterfowl Association.

Bachtrog D. 2008. The temporal dynamics of processes underlying Y chromosome degeneration. *Genetics*. 179:1513–1525.

Bachtrog D. 2013. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nat Rev Genet*. 14:113–124.

Bachtrog D. 2020. The Y chromosome as a battleground for intragenomic conflict. *Trends Genet*. 36:510–522.

Bachtrog D, Charlesworth B. 2002. Reduced adaptation of a non-recombining neo-Y chromosome. *Nature*. 416:323–326.

Bachtrog D, Kirkpatrick M, Mank JE, McDaniel SF, Pires JC, Rice W, Valenzuela N. 2011. Are all sex chromosomes created equal? *Trends Genet*. 27:350–357.

Bachtrog D, Mahajan S, Bracewell R. 2019. Massive gene amplification on a recently formed Drosophila Y chromosome. *Nat Ecol Evol*. 3:1587–1597.

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, *et al.*; Tree of Sex Consortium. 2014. Sex determination: why so many ways of doing it? *PLoS Biol*. 12:e1001899.

Backström N, Ceplitis H, Berlin S, Ellegren H. 2005. Gene conversion drives the evolution of HINTW, an ampliconic gene on the female-specific avian W chromosome. *Mol Biol Evol*. 22:1992–1999.

Bellott DW, Page DC. 2021. Dosage-sensitive functions in embryonic development drove the survival of genes on sex-specific chromosomes in snakes, birds, and mammals. *Genome Res*. 31(2):198–210.

Bellott DW, Skaletsky H, Pyntikova T, Mardis ER, Graves T, Kremitzki C, Brown LG, Rozen S, Warren WC, Wilson RK, *et al.* 2010. Convergent evolution of chicken Z and human X chromosomes by expansion and gene acquisition. *Nature*. 466(7306):612–616.

Bellott DW, Skaletsky H, Cho TJ, Brown L, Locke D, Chen N, Galkina S, Pyntikova T, Koutseva N, Graves T, *et al.* 2017. Avian W and mammalian Y chromosomes convergently retained dosage-sensitive regulators. *Nat Genet*. 49:387–394.

Bergero R, Charlesworth D. 2009. The evolution of restricted recombination in sex chromosomes. *Trends Ecol Evol*. 24:94–102.

Betrán E, Demuth JP, Williford A. 2012. Why chromosome palindromes? *Int J Evol Biol*. 2012:207958.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 30:2114–2120.

Brashear WA, Raudsepp T, Murphy WJ. 2018. Evolutionary conservation of Y Chromosome ampliconic gene families despite extensive structural variation. *Genome Res*. 28:1841–1851.

Ceplitis H, Ellegren H. 2004. Adaptive molecular evolution of HINTW, a female-specific gene in birds. *Mol Biol Evol*. 21:249–254.

Charlesworth B. 1978. Model for evolution of Y chromosomes and dosage compensation. *Proc Natl Acad Sci U S A*. 75:5618–5622.

Charlesworth B. 1991. The evolution of sex chromosomes. *Science*. 251:1030–1033.

Charlesworth B, Charlesworth D. 2000. The degeneration of Y chromosomes. *Philos Trans R Soc Lond B Biol Sci*. 355:1563–1572.

Chen JM, Cooper DN, Chuzhanova N, Férec C, Patrinos GP. 2007. Gene conversion: mechanisms, evolution and human disease. *Nat Rev Genet*. 8:762–775.

Cocquet J, Ellis PJ, Mahadevaiah SK, Affara NA, Vaiman D, Burgoyne PS. 2012. A genetic basis for a postmeiotic X versus Y chromosome intragenomic conflict in the mouse. *PLoS Genet*. 8:e1002900.

Connallon T, Clark AG. 2010. Gene duplication, gene conversion and the evolution of the Y chromosome. *Genetics*. 186:277–286.

Cui W, Cai Y, Wang W, Liu Z, Wei P, Bi R, Chen W, Sun M, Zhou X. 2014. Frequent copy number variations of PI3K/AKT pathway and aberrant protein expressions of PI3K subunits are associated with inferior survival in diffuse large B cell lymphoma. *J Transl Med*. 12:10.

Davis JK, Thomas PJ, Thomas JW; NISC Comparative Sequencing Program. 2010. A W-linked palindrome and gene conversion in New World sparrows and blackbirds. *Chromosome Res*. 18:543–553.

Ekarius C. 2007. *Storey's Illustrated Guide to Poultry Breeds*. Marceline, MO: Walsworth Publishing Company.

Ellis PJ, Bacon J, Affara NA. 2011. Association of Sly with sex-linked gene amplification during mouse evolution: a side effect of genomic conflict in spermatids? *Hum Mol Genet*. 20:3010–3021.

Eriksson J, Larson G, Gunnarsson U, Bed'hom B, Tixier-Boichard M, Strömstedt L, Wright D, Jungerius A, Vereijken A, Randi E, *et al.* 2008. Identification of the yellow skin gene reveals a hybrid origin of the domestic chicken. *PLoS Genet*. 4:e1000010.

Fridolfsson, A.-K., Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *J Avian Biol*. 30(1):116.

Frisby DP, Weiss RA, Roussel M, Stehelin D. 1979. The distribution of endogenous chicken retrovirus sequences in the DNA of galliform birds does not coincide with avian phylogenetic relationships. *Cell*. 17:623–634.

Fumihito A, Miyake T, Takada M, Shingu R, Endo T, Gojobori T, Kondo N, Ohno S. 1996. Monophyletic origin and unique dispersal patterns of domestic fowls. *Proc Natl Acad Sci U S A*. 93:6792–6795.

Furman BLS, Metzger DCH, Darolti I, Wright AE, Sandkam BA, Almeida P, Shu JJ, Mank JE. 2020. Sex Chromosome Evolution: So Many Exceptions to the Rules. *Genome Biol Evol*. 12:750–763.

Geraldes A, Rambo T, Wing RA, Ferrand N, Nachman MW. 2010. Extensive gene conversion drives the concerted evolution of paralogous copies of the SRY gene in European rabbits. *Mol Biol Evol*. 27:2437–2440.

Ghenu AH, Bolker BM, Melnick DJ, Evans BJ. 2016. Multicopy gene family evolution on primate Y chromosomes. *BMC Genomics*. 17:157.

Good JM. 2012. The conflict within and the escalating war between the sex chromosomes. *PLoS Genet*. 8:e1002955.

Guzzetti BM, Talbot SL, Tessler DF, Gill VA, Murphy EC. 2008. Secrets in the eyes of Black Oystercatchers: a new sexing technique. *J Field Ornithol*. 79(2):215–223.

Haddrill PR, Halligan DL, Tomaras D, Charlesworth B. 2007. Reduced efficacy of selection in regions of the Drosophila genome that lack crossing over. *Genome Biol*. 8:R18.

Hori T, Asakawa S, Itoh Y, Shimizu N, Mizuno S. 2000. Wpkci, encoding an altered form of PKCI, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. *Mol Biol Cell*. 11:3645–3660.

Hughes JF, Skaletsky H, Pyntikova T, Minx PJ, Graves T, Rozen S, Wilson RK, Page DC. 2005. Conservation of Y-linked genes during human evolution revealed by comparative sequencing in chimpanzee. *Nature*. 437:100–103.

Hughes JF, Skaletsky H, Pyntikova T, Graves TA, van Daalen SK, Minx PJ, Fulton RS, McGrath SD, Locke DP, Friedman C, *et al.* 2010. Chimpanzee and human Y chromosomes are remarkably divergent in structure and gene content. *Nature*. 463:536–539.

Hughes JF, Skaletsky H, Pyntikova T, Koutseva N, Raudsepp T, Brown LG, Bellott DW, Cho TJ, Dugan-Rocha S, Khan Z, *et al.* 2020. Sequence analysis in Bos taurus reveals pervasiveness of X-Y arms races in mammalian lineages. *Genome Res*. 30:1716–1726.

Kerje S, Carlborg O, Jacobsson L, Schütz K, Hartmann C, Jensen P, Andersson L. 2003. The twofold difference in adult size between the red junglefowl and White Leghorn chickens is largely explained by a limited number of QTLs. *Anim Genet*. 34:264–274.

Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, *et al.* 2001. The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat Genet*. 29:279–286.

Lahn BT, Page DC. 1997. Functional coherence of the human Y chromosome. *Science*. 278:675–680.

Larson EL, Kopania EEK, Good JM. 2018. Spermatogenesis and the evolution of mammalian sex chromosomes. *Trends Genet*. 34:722–732.

Lewis C. 2010. *The illustrated guide to chickens: how to choose them - how to keep them*. Oswestry, UK: Scotprint.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 25:1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R; 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 25:2078–2079.

Li J, Zhang J, Liu J, Zhou Y, Cai C, Xu L, Zhou Q. (2021). A new duck genome reveals conserved and convergently evolved chromosome architectures of birds and mammals. *GigaScience*. 10(1):1–15.

Löytynoja A. 2014. Phylogeny-aware alignment with PRANK. *Methods Mol Biol*. 1079:155–170.

Lucotte EA, Skov L, Jensen JM, Macià MC, Munch K, Schierup MH. 2018. Dynamic copy number evolution of X- and Y-linked ampliconic genes in human populations. *Genetics*. 209:907–920.

Mank JE. 2012. Small but mighty: the evolutionary dynamics of W and Y sex chromosomes. *Chromosome Res*. 20:21–33.

Marais GA, Campos PR, Gordo I. 2010. Can intra-Y gene conversion oppose the degeneration of the human Y chromosome? A simulation study. *Genome Biol Evol*. 2:347–357.

Moghadam HK, Pointer MA, Wright AE, Berlin S, Mank JE. 2012. W chromosome expression responds to female-specific selection. *Proc Natl Acad Sci U S A*. 109:8207–8211.

Moretti C, Blanco M, Ialy-Radio C, Serrentino ME, Gobé C, Friedman R, Battail C, Leduc M, Ward MA, Vaiman D, *et al.* 2020. Battle of the sex chromosomes: competition between X and Y chromosome-encoded proteins for partner interaction and chromatin occupancy drives multicopy gene expression and evolution in muroid rodents. *Mol Biol Evol*. 37:3453–3468.

Moriyama S, Ogihara J, Kato J, Hori T, Mizuno S. 2006. PKCI-W forms a heterodimer with PKCI-Z and inhibits the biological activities of PKCI-Z in vitro, supporting the predicted role of PKCI-W in sex determination in birds. *J Biochem*. 139:91–97.

Mueller JL, Mahadevaiah SK, Park PJ, Warburton PE, Page DC, Turner JM. 2008. The mouse X chromosome is enriched for multicopy testis genes showing postmeiotic expression. *Nat Genet*. 40:794–799.

Narasimhan V, Danecek P, Scally A, Xue Y, Tyler-Smith C, Durbin R. 2016. BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data. *Bioinformatics*. 32:1749–1751.

O'Neill M, Binder M, Smith C, Andrews J, Reed K, Smith M, Millar C, Lambert D, Sinclair A. 2000. ASW: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. *Dev Genes Evol*. 210:243–249.

Pace HC, Brenner C. 2003. Feminizing chicks: a model for avian sex determination based on titration of Hint enzyme activity and the predicted structure of an Asw-Hint heterodimer. *Genome Biol*. 4:R18.

Parks KP, Seidle H, Wright N, Sperry JB, Bieganowski P, Howitz K, Wright DL, Brenner C. 2004. Altered specificity of Hint-W123Q supports a role for Hint inhibition by ASW in avian sex determination. *Physiol Genomics*. 20:12–14.

Poznik GD, Xue Y, Mendez FL, Willems TF, Massaia A, Wilson Sayres MA, Ayub Q, McCarthy SA, Narechania A, Kashin S, *et al.*; 1000 Genomes Project Consortium. 2016. Punctuated bursts in human male demography inferred from 1,244 worldwide Y-chromosome sequences. *Nat Genet*. 48:593–599.

Rice WR. 1996. Evolution of the Y sex chromosome in animals. *BioScience*. 46(5):331–343

Rozen S, Skaletsky H, Marszalek JD, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Page DC. 2003. Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. *Nature*. 423:873–876.

Rubin CJ, Zody MC, Eriksson J, Meadows JR, Sherwood E, Webster MT, Jiang L, Ingman M, Sharpe T, Ka S, *et al.* 2010. Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature*. 464:587–591.

Rumble SM, Lacroute P, Dalca AV, Fiume M, Sidow A, Brudno M. 2009. SHRiMP: accurate mapping of short color-space reads. *PLoS Comput Biol*. 5:e1000386.

Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, *et al.* 2003. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*. 423:825–837.

Skov L, Schierup MH; Danish Pan Genome Consortium. 2017. Analysis of 62 hybrid assembled human Y chromosomes exposes rapid structural changes and high rates of gene conversion. *PLoS Genet*. 13:e1006834.

Smeds L, Warmuth V, Bolivar P, Uebbing S, Burri R, Suh A, Nater A, Bureš S, Garamszegi LZ, Hogner S, *et al.* 2015. Evolutionary analysis of the female-specific avian W chromosome. *Nat Commun*. 6:7330.

Soh YQ, Alföldi J, Pyntikova T, Brown LG, Graves T, Minx PJ, Fulton RS, Kremitzki C, Koutseva N, Mueller JL, *et al.* (2014). Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. *Cell*. 159(4):800–813. doi:10.1016/j.cell.2014.09.052. PMID: 25417157; PMCID: PMC4260969.

Tomaszkiewicz M, Medvedev P, Makova KD. 2017. Y and W chromosome assemblies: approaches and discoveries. *Trends Genet*. 33:266–282.

Tomaszkiewicz M, Rangavittal S, Cechova M, Campos Sanchez R, Fescemyer HW, Harris R, Ye D, O'Brien PC, Chikhi R, Ryder OA, *et al.* 2016. A time- and cost-effective strategy to sequence mammalian Y chromosomes: an application to the de novo assembly of Gorilla Y. *Genome Res*. 26:530–540.

van Tuinen M, Hedges SB. 2001. Calibration of avian molecular clocks. *Mol Biol Evol*. 18:206–213.

Vegesna R, Tomaszkiewicz M, Medvedev P, Makova KD. 2019. Dosage regulation, and variation in gene expression and copy number of human Y chromosome ampliconic genes. *PLoS Genet*. 15:e1008369.

Vegesna R, Tomaszkiewicz M, Ryder OA, Campos-Sánchez R, Medvedev P, DeGiorgio M, Makova KD. 2020. Ampliconic genes on the great ape Y chromosomes: rapid evolution of copy number but conservation of expression levels. *Genome Biol Evol*. 12:842–859.

Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Köhn FM, Schill WB, Farah S, Ramos C, *et al.* 1996. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet*. 5:933–943.

Wright AE, Harrison PW, Montgomery SH, Pointer MA, Mank JE. 2014. Independent stratum formation on the avian sex chromosomes reveals inter-chromosomal gene conversion and predominance of purifying selection on the W chromosome. *Evolution*. 68:3281–3295.

Wright AE, Mank JE. 2013. The scope and strength of sex-specific selection in genome evolution. *J Evol Biol*. 26:1841–1853.

Wright AE, Moghadam HK, Mank JE. 2012. Trade-off between selection for dosage compensation and masculinization on the avian Z chromosome. *Genetics*. 192:1433–1445.

Xu L, Auer G, Peona V, Suh A, Deng Y, Feng S, Zhang G, Blom MP, Christidis L, Prost S, *et al.* 2019. Dynamic evolutionary history and gene content of sex chromosomes across diverse songbirds. *Nat Ecol Evol*. 3(5):834–844. doi:10.1038/s41559-019-0850-1

Xu L, Zhou Q. 2020. The female-specific W chromosomes of birds have conserved gene contents but are not feminized. *Genes*. 11(10):1–14.

Yan Y, Yang X, Liu Y, Shen Y, Tu W, Dong Q, Yang D, Ma Y, Yang Y. 2017. Copy number variation of functional RBMY1 is associated with sperm motility: an azoospermia factor-linked candidate for asthenozoospermia. *Hum Reprod*. 32:1521–1531.

Ye D, Zaidi AA, Tomaszkiewicz M, Anthony K, Liebowitz C, DeGiorgio M, Shriver MD, Makova KD. 2018. High levels of copy number variation of ampliconic genes across major human Y haplogroups. *Genome Biol Evol*. 10:1333–1350.

Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, Billis K, Cummins C, Gall A, Girón CG, *et al.* 2018. Ensembl 2018. *Nucl Acids Res*. 46:D754–D761.

Zhang Z, Jia Y, Almeida P, Mank JE, van Tuinen M, Wang Q, Jiang Z, Chen Y, Zhan K, Hou S, *et al.* 2018. Whole-genome resequencing reveals signatures of selection and timing of duck domestication. *GigaScience*. 7(4):giy027.

Zhou R, Macaya-Sanz D, Carlson CH, Schmutz J, Jenkins JW, Kudrna D, Sharma A, Sandor L, Shu S, Barry K, *et al.* 2020. A willow sex chromosome reveals convergent evolution of complex palindromic repeats. *Genome Biol*. 21:38.

Sex-Specific Selection Drives the Evolution of Alternative Splicing in Birds

Thea F. Rogers ,* Daniela H. Palmer , and Alison E. Wright *

Department of Animal and Plant Sciences, University of Sheffield, Sheffield, United Kingdom

***Corresponding authors:** E-mails: tfrogers1@sheffield.ac.uk; a.e.wright@sheffield.ac.uk.

Associate editor: Irina Arkhipova

Abstract

Males and females of the same species share the majority of their genomes, yet they are frequently exposed to conflicting selection pressures. Gene regulation is widely assumed to resolve these conflicting sex-specific selection pressures, and although there has been considerable focus on elucidating the role of gene expression level in sex-specific adaptation, other regulatory mechanisms have been overlooked. Alternative splicing enables different transcripts to be generated from the same gene, meaning that exons which have sex-specific beneficial effects can in theory be retained in the gene product, whereas exons with detrimental effects can be skipped. However, at present, little is known about how sex-specific selection acts on broad patterns of alternative splicing. Here, we investigate alternative splicing across males and females of multiple bird species. We identify hundreds of genes that have sex-specific patterns of splicing and establish that sex differences in splicing are correlated with phenotypic sex differences. Additionally, we find that alternatively spliced genes have evolved rapidly as a result of sex-specific selection and suggest that sex differences in splicing offer another route to sex-specific adaptation when gene expression level changes are limited by functional constraints. Overall, our results shed light on how a diverse transcriptional framework can give rise to the evolution of phenotypic sexual dimorphism.

Key words: **sex-specific selection, alternative splicing, sexual dimorphism, transcriptome, sexual conflict.**

Introduction

Males and females of many species can have divergent evolutionary optima, and are often subject to conflicting selection pressures (Andersson 1994), yet they share an almost identical set of genes. As a result, when contradictory sex-specific selection pressures act on traits that have a shared genetic basis, significant amounts of sexual conflict can occur (Parker and Partridge 1998; Bonduriansky and Chenoweth 2009). Despite this, sex differences are common across a broad range of phenotypes, including morphology, physiology, behavior, and life history, and it is widely assumed that transcriptional dimorphism encodes these sexually dimorphic traits by breaking down intersexual correlations and facilitating sex-specific adaptation (Connallon and Knowles 2005; Connallon and Clark 2010; Innocenti and Morrow 2010; Mank 2017a). Genes with differences in expression level between males and females are pervasive across many species, and exhibit unique evolutionary properties, including faster rates of sequence and expression evolution (Ranz et al. 2003; Khaitovich et al. 2005; Ellegren and Parsch 2007; Harrison et al. 2015). Indeed, these genes have been the subject of considerable focus in understanding how selection can navigate the constraints imposed by a shared genome, and the consequences for sex-specific adaptation (Mank 2017a, 2017b).

Sex differences in alternative splicing, where different exons are spliced or shuffled in males and females to create distinct sex-specific sequences (Blekhman et al. 2010; Nilsen and Graveley 2010), have the potential to play key roles in sex-specific adaptation, yet they have been largely overlooked with the exception of a few studies (Blekhman et al. 2010; Brown et al. 2014; Gibilisco et al. 2016; Grantham and Brisson 2018). In particular, alternative splicing enables multiple transcripts to be generated from a single gene, increasing sex-specific proteome diversity (Matlin et al. 2005; Nilsen and Graveley 2010). In theory, this could act so that certain exons (e.g., those with sex-specific beneficial functions) are retained in one sex, and certain exons (e.g., those that have sex-specific detrimental effects) are excluded in the other sex, generating distinct sex-specific isoforms. There is mounting evidence that splicing varies substantially across species, sexes, and tissues (Su et al. 2008; Gibilisco et al. 2016), and has important phenotypic consequences for sex determination, disease, physiology, and development (Cline and Meyer 1996; Schütt and Nöthiger 2000; McIntyre et al. 2006; Kalsotra and Cooper 2011; Gerstein et al. 2014). Despite this, although certain isoforms have key cellular roles and mediate important phenotypes, the extent to which global patterns of splicing are functionally relevant is an important point of discussion (Blencowe 2017; Tress et al. 2017a, 2017b; Wan and Larson 2018). Many alternative splicing events are highly tissue-specific and patterns of splicing shift rapidly across species over evolutionary time (Pan et al. 2005; Barbosa-Morais

Article

et al. 2012; Merkin et al. 2012; Melé et al. 2015) but whether this reflects stochastic transcriptional noise, relaxed selection, or lineage-specific innovations remains unclear (Blencowe 2017; Tress et al. 2017a, 2017b; Wan and Larson 2018). Importantly, the contribution of sex-specific selection to the rapid turnover of sex differences in splicing across species has yet to be tested, as most studies exploring the link between transcriptional variation and sexual selection have not accounted for sex-specific patterns of alternative splicing.

Furthermore, the factors constraining the evolution of alternative splicing have yet to be investigated. There is growing evidence that pleiotropy, where a gene performs several functions and affects multiple traits, hinders the evolution of gene expression level and limits the response to sex-specific selection (Chen and Dokholyan 2006; Mank et al. 2008; Papakostas et al. 2014). Indeed, genes with broad expression patterns, a proxy for pleiotropy, are less likely to be differentially expressed between males and females (Mank et al. 2008). Alternative splicing could avoid these pleiotropic and functional constraints acting on expression level through the generation of distinct male and female isoforms, thereby acting as an alternate or complementary route to sex-specific adaptation.

Here, we characterize patterns of alternative splicing across males and females of three avian species in order to test the role of sex-specific selection in the evolution of alternative splicing and establish its role in sex-specific adaptation and sexual dimorphism. We identify hundreds of genes that exhibit significant sex-biased alternative splicing and show that sex differences in splicing are correlated with phenotypic sex differences. We find that patterns of sex-specific alternative splicing have evolved rapidly, likely as a product of sex-specific selection, and that genes that are differentially spliced exhibit genomic signatures consistent with sex-specific fitness effects. Broadly, our results provide insight into how, via a diverse transcriptional architecture, the same genome is selected to encode multiple phenotypes, and demonstrates the role of alternative splicing in the evolution of phenotypic complexity.

Results and Discussion

Alternative Splicing Is Widespread and Common across Birds

We quantified alternative splicing in males and females across multiple tissues in three avian species that diverged ~90 Ma (supplementary fig. S1, Supplementary Material online). Splicing was estimated as the relative proportion of two alternative isoforms at each splice site, otherwise referred to as percent spliced-in (PSI). A PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. Alternative splicing is common and widespread across all individuals, with an average of 21%, 17%, and 24% of autosomal genes undergoing at least one splice event in the duck, turkey, and guineafowl, respectively (supplementary table S1, Supplementary Material online). We identified five different types of alternative splicing events (supplementary fig. S2, Supplementary Material online); skipped exons (SE), where an exon is either excluded or included from the mRNA, mutually exclusive exons (MXE), where one exon is skipped and the other is retained or vice versa, alternative 5′ and 3′ splice site events (A5′SS and A3′SS), where the exon boundary on either the 5′- or 3′-end of the intron is extended or shortened, and retained intron events, where a whole intron is retained in the final transcript. A gene can exhibit multiple different types of splicing events. SE and MXE splicing events are the most common type of splicing across the three species, with the other types of splicing occurring at very low frequency (supplementary table S1, Supplementary Material online). Additionally, SE and MXE events are also more commonly associated with the generation of functional isoforms than other types of splicing (Weatheritt et al. 2016), and so we focus solely on these in subsequent analyses.

Tissues Exhibit Distinct Transcriptional Profiles

Next, we examined patterns of sex differences in splicing across tissues. Males and females undergo very similar rates of splicing (supplementary table S1, Supplementary Material online) in both the spleen and the gonad across the autosomes in each of the three species, and this finding is consistent across multiple filtering thresholds (supplementary table S2, Supplementary Material online). However, despite similarities in the total proportion of alternatively spliced genes, patterns of splicing vary substantially between the sexes (table 1 and supplementary table S4, Supplementary Material online).

Using hierarchical clustering, we found that both gonad and spleen samples cluster first by phylogenetic relatedness, where splicing is more similar between turkey and guineafowl, which diverged ~30 Ma, than with the duck which diverged ~90 Ma (supplementary fig. S1, Supplementary Material online). However, in each species, ovary and testis tissue cluster separately whereas the spleen shows no clustering among males and females (fig. 1*A* and *B*). Across all three species, we consistently identified far fewer genes with significant differential alternative splicing in the spleen relative to the gonad (table 1 and supplementary table S4, Supplementary Material online), consistent with results from *Drosophila* (Gibilisco et al. 2016). Our finding that ovaries and testes exhibit distinct transcriptional profiles mirrors patterns of sex differences in expression level (hereafter termed differential expression) across many species (Uebbing et al. 2016), where the gonad often exhibits significant differential expression between males and females for more than half of all expressed genes (Zhang et al. 2007; Mank et al. 2010) but somatic tissues show less differential expression (Yang et al. 2006; Mank et al. 2007; Harrison et al. 2015). This suggests that ovaries and testes are regulated by distinct sex-specific gene regulatory networks, and that sex-specific splice variants play a role in the construction of sex-specific genetic architecture (Mank et al. 2007; Wright et al. 2018). Interestingly, we observe far fewer genes exhibiting differential alternative splicing (3.3%, 1.1%, 2.8% of autosomal genes in the duck, turkey, and guineafowl gonad, respectively; supplementary table S4, Supplementary Material online) relative to differential expression (45.3%,

Table 1. Differential Alternative Splicing between Males and Females across Autosomal Splice Sites and Genes.

		Sex-Biased Alternative Splicing Events			Sex-Biased Alternatively Spliced Genes			
Species	Tissue	MXE[a]	SE[b]	Total	MXE[a]	SE[b]	Total	Proportion of Genes
Duck	Gonad	181	677	886	148	551	640	7.6%
Duck	Spleen	7	27	31	6	26	34	0.4%
Turkey	Gonad	91	481	579	78	421	475	5.2%
Turkey	Spleen	2	39	41	2	38	40	0.5%
Guineafowl	Gonad	219	720	977	174	596	701	7.4%
Guineafowl	Spleen	1	13	14	1	13	13	0.1%

[a]MXE denotes mutually exclusive exon events.
[b]SE denotes skipped exon events.

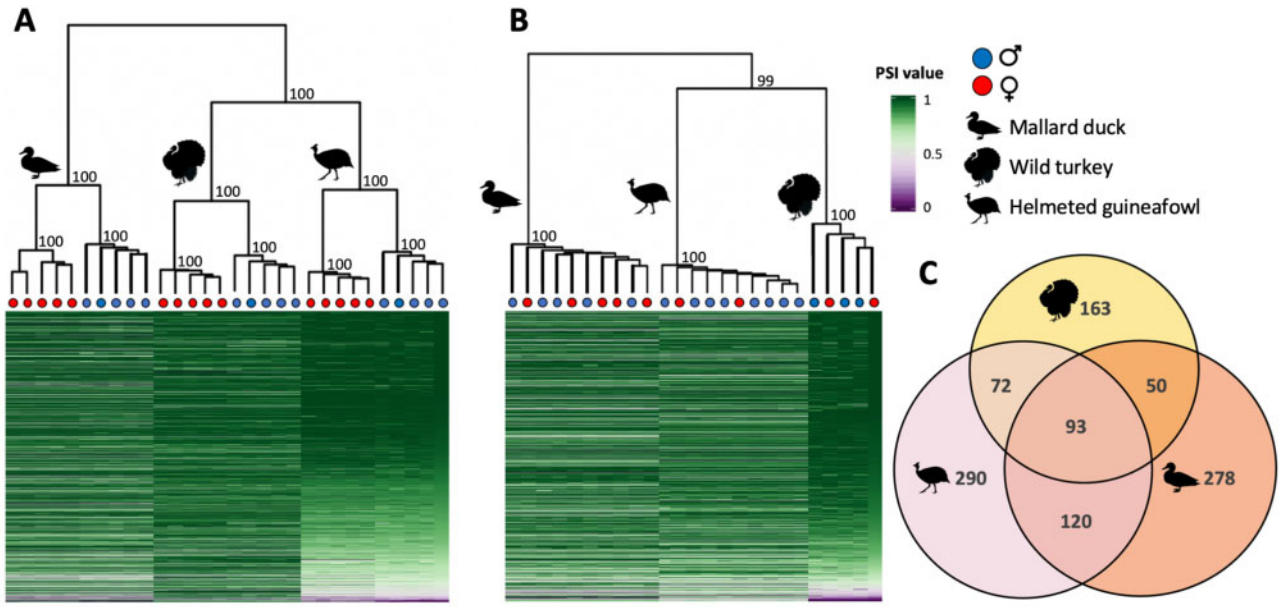

Fig. 1. Global patterns of alternative splicing. Panels (*A*) and (*B*) show heatmaps and hierarchical clustering of alternative splicing level in the gonad and spleen, respectively. Percent spliced-in values (PSI) refer to the proportion of alternative isoforms at a splice site, where a PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. If a gene undergoes multiple splice events, the average PSI is shown. Numbers on each branch represent the bootstrap probability values. Panel (*C*) shows orthologous genes with significant sex differences in splicing in the gonad that are shared among the duck (pink), turkey (yellow), and guineafowl (orange). We observe significant overlap ($P < 0.0001$, super exact test) of differentially spliced orthologs across the three species.

45.7%, 44.3% in the duck, turkey, and guineafowl gonad, respectively), calling into question the relative effect of splicing versus expression in sex-specific regulatory networks.

Sex Differences in Alternative Splicing Are Associated with Phenotypic Sexual Dimorphism

We have shown that patterns of splicing vary substantially between the sexes and across tissues (table 1 and supplementary table S4, Supplementary Material online). To test whether this sex-biased transcriptional variation (hereafter termed differential splicing) is associated with phenotypic sex differences, we contrasted patterns of splicing across a gradient of sexual dimorphism. Specifically, we employed contrasts across wild turkey individuals that represent a gradient in male secondary sexual characteristics. The wild turkey exhibits two male phenotypes in the forms of dominant and subordinate males. The species is strongly sexually dimorphic, with dominant males showing greater body size than females, along with a range of sexually selected traits including distinct plumage and mating behaviors (Buchholz 1995, 1997; Hill et al. 2005). Subordinate males develop fewer and less exaggerated sexually selected traits than dominant males, but are clearly male in phenotype, occupying an intermediate position on the continuum of sexual dimorphism.

Hierarchical clustering of autosomal genes showed that in the gonad, subordinate and dominant males cluster together with high confidence (supplementary fig. S3, Supplementary Material online), and are distinct from females, as opposed to being intersex. However, there were subtle differences in patterns of alternative splicing between dominant and subordinate males (fig. 2). For exons with significant differences in splicing between dominant males and females (table 1), we

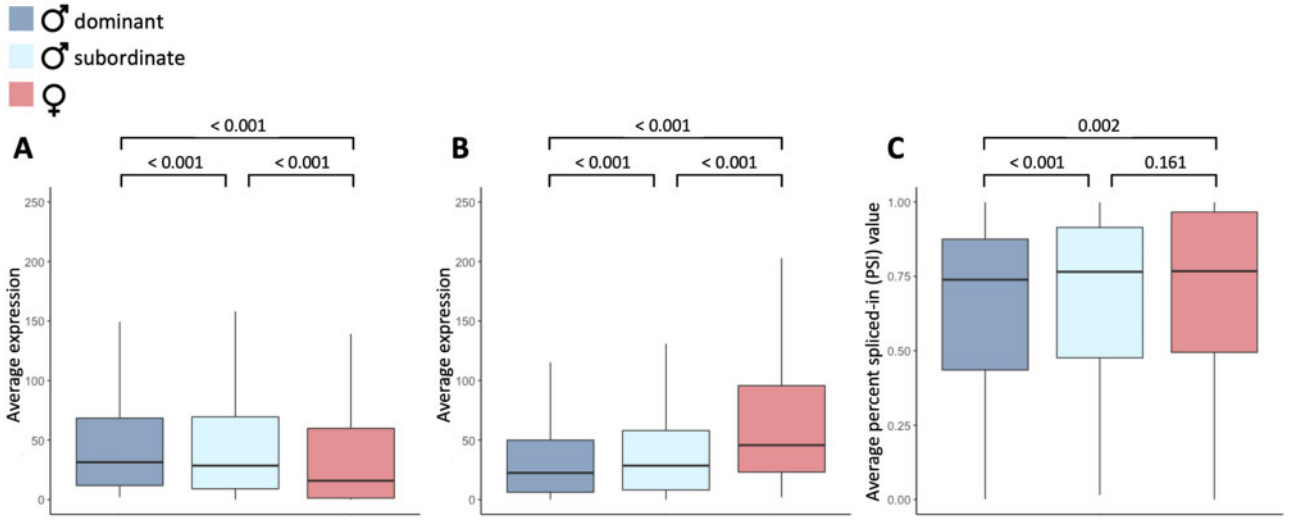

FIG. 2. Expression of sex-biased isoforms in dominant male turkeys (dark blue), subordinate male turkeys (light blue), and female turkeys (red). Panel (*A*) and (*B*) show average expression (read counts) of male- and female-biased isoforms, respectively and panel (*C*) is the average percent spliced-in value (PSI) of all sex-biased isoforms. Significance values were calculated using a paired Wilcoxon's signed-rank test.

classified the alternative isoforms as either male- or female-biased depending on whether they were expressed more highly in dominant males or females. We focused our analyses on the gonad as it exhibits the greatest magnitude of differential splicing, making it the tissue most likely to be influenced by sex-specific selection. Subordinate males express male-biased isoforms in the gonad at significantly lower levels than dominant males (paired Wilcoxon signed-rank test, $P \leq 0.001$), indicating that patterns of splicing are demasculinized in subordinate males (fig. 2*A*). Subordinate males also express female-biased isoforms at significantly higher levels than dominant males (paired Wilcoxon signed-rank test, $P < 0.001$) (fig. 2*B*), consistent with feminized splicing. Importantly, subordinate males exhibit intermediate patterns of splicing for all genes that exhibit differential splicing between dominant males and females (fig. 2*C*). These patterns are consistent with the phenotypic sex differences observed across morphs, where subordinate males occupy an intermediate position on the continuum of sexual dimorphism.

We tested whether this pattern was a result of regression toward the mean by randomizing samples 100 times. Each time, we randomly picked three dominant male and three female samples, identified genes with differential splicing, and then assessed the remaining dominant males, females, and subordinate males for the magnitude of splicing (PSI). We found that subordinate males had significantly higher PSI than dominant males for all 100 sample comparisons, and significantly lower PSI than females for the majority of the 100 sample combinations (79 significant comparisons). In contrast, significant differences were observed much less frequently between the randomly chosen dominant male samples (34 significant comparisons) or between female samples (6 significant comparisons), indicating that regression toward the mean is unlikely to explain our results. Gene expression level across turkey morphs has previously been shown to exhibit similar patterns of demasculinization and feminization (Pointer et al. 2013), consistent with a role of transcriptional dimorphism in encoding phenotypic sex differences. Our results suggest a previously overlooked link between genomic and phenotypic dimorphism, where differential alternative splicing works concurrently with differential expression level to produce the diverse transcriptional framework underpinning complex phenotypic sexual dimorphisms.

Sex-Specific Selection Acts on Isoforms That Are Differentially Expressed between Males and Females

We find that patterns of alternative splicing cluster strongly by species (fig. 1*A* and *B*), consistent with rapid rates of regulatory evolution within lineages. This pattern of clustering is contrary to that observed for gene expression level, including the ones in this study, which clusters first by sex in the gonad, then species (Harrison et al. 2015; Mank 2017a). Our finding that patterns of differential expression are more conserved than patterns of alternative splicing is a broad taxonomic trend (Barbosa-Morais et al. 2012; Merkin et al. 2012; Gibilisco et al. 2016), indicative of rapid turnover of alternative splicing across species. However, we observe significant overlap ($P < 0.001$, super exact test) of differentially alternatively spliced orthologs across the three species (fig. 1*C* and supplementary table S3, Supplementary Material online), indicating that although patterns of splicing evolve quickly, significant sex differences in splicing are limited to a core set of avian genes. To test whether this conserved set of genes is enriched for specific functions, we conducted a Gene Ontology analysis (Mi et al. 2019), but failed to find any significantly enriched terms ($P < 0.05$).

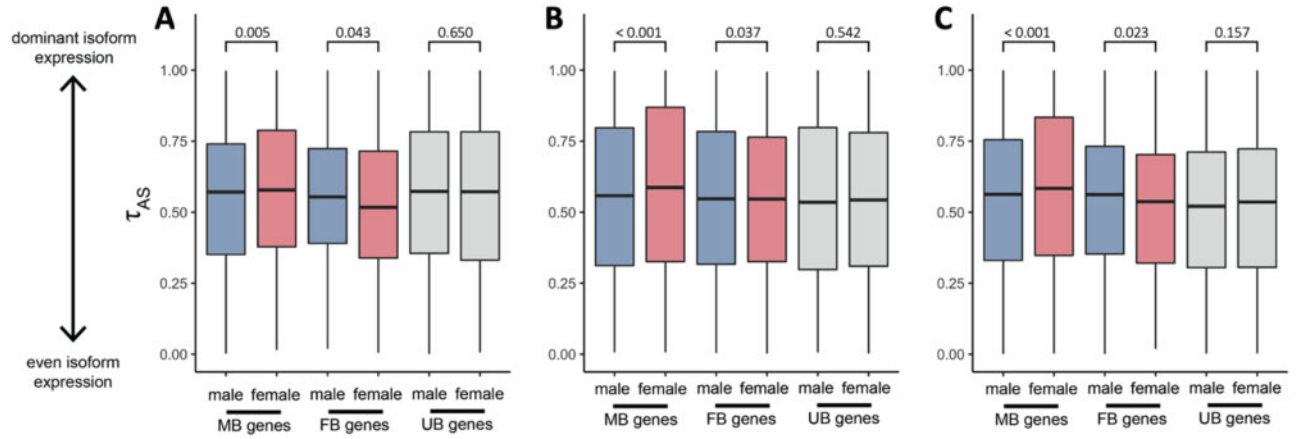

FIG. 3. Average male and female isoform specificity (τ_{AS}) across genes. τ_{AS} for genes with male-biased expression level, female-biased expression level, and unbiased expression between the sexes for the (*A*) duck, (*B*) turkey, and (*C*) guineafowl. Significance values were calculated using a paired Wilcoxon's signed-rank test.

We implemented an evolutionary framework, using regulatory variation as a proxy for selection, to test whether the rapid rate of regulatory evolution we observe is a product of sexual selection. Studies of regulatory variation have recently been implemented as a powerful approach to infer selection (Brawand et al. 2011; Gallego Romero et al. 2012; Moghadam et al. 2012; Dean et al. 2015), where selection on loci increases with expression level (Duret and Mouchiroud 1999; Pál et al. 2001; Drummond et al. 2006; Gout et al. 2010).

Applying this framework to alternative splicing, if purifying selection is the dominant evolutionary force acting on splice variants, we predict highly expressed genes to express fewer isoforms than lowly expressed genes, which might be spuriously transcribed and subject to weaker constraints. Furthermore, when expression level differs between the sexes, purifying selection would be strongest in the sex with the higher expression, resulting in the expression of fewer isoforms in that sex. For example, for male-biased genes, we would predict that males tend to have fewer isoforms than females.

However, if there is sexual selection for sex-specific isoforms, we expect the opposite relationship between isoform diversity and sex. Here, we predict the evolution of novel isoforms to be analogous to gene duplication with neofunctionalization, where the ancestral paralog retains its original function and expression pattern but the newly duplicated paralog evolves sex-specific functions and sex-biased expression (Connallon and Clark 2011). Applying this to splicing, we expect the ancestral splice variant to retain its ancestral expression pattern and function, but the novel sex-specific isoform to evolve sex-specific functions and expression. As a result, we expect a greater diversity of isoforms in the sex with higher expression, where selection for sex-specific isoforms is the greatest. Specifically, males should express more isoforms than females for male-biased genes, where novel male-specific isoforms are free to evolve male-specific functions, whereas isoforms expressed in both sexes are retained to perform their original function. We predict the opposite pattern for female-biased genes, which under sex-specific selection should exhibit a greater diversity of isoforms expressed in females.

These two scenarios generate opposing predictions for the expected patterns of isoform diversity in males and females. To distinguish between these selective regimes, we developed an isoform specificity index (τ_{AS}) to quantify variation in isoform abundance per gene. This metric is adapted from the tissue specificity index (Yanai et al. 2005), where high values show that a single isoform is always expressed and low values indicate an even representation of multiple isoforms.

We found a significant relationship between isoform specificity (τ_{AS}) and expression level across all genes, where highly expressed genes tend to express fewer isoforms than lowly expressed genes (supplementary fig. S5 and table S6, Supplementary Material online). This indicates that purifying selection acts on broad patterns of splicing across the genome, suggesting that global patterns of splicing are functionally relevant (Blencowe 2017; Tress et al. 2017a, 2017b; Wan and Larson 2018). However, we also recovered a significant association with sex, where isoform specificity (τ_{AS}) differs significantly between males and females for genes that are differentially expressed between the sexes, but not for those with similar expression levels (fig. 3 and supplementary fig. S5, Supplementary Material online). Importantly, this association is reversed between male- and female-biased genes, as we predicted. Specifically, males show significantly greater isoform diversity for male-biased genes, and females show greater isoform diversity for female-biased genes. There are no significant differences in isoform diversity between males and females for unbiased genes. This is consistent with our predictions of selection for sex-specific splice variants, and opposite to what we would expect if purifying selection were the dominant evolutionary force acting on splicing in males and females. These patterns are observed across all three species, which diverged 90 Ma, indicating that the role of sex-specific selection in splicing evolution is a broad taxonomic trend across birds.

If sex-specific isoforms are indeed under selection for sex-specific functions, then we expect these loci to affect fitness

differently in males relative to females. To test whether differential splicing has sex-specific effects, we used a population genomic approach across the three avian species, contrasting patterns of intersexual sequence differentiation and balancing selection (Wright et al. 2018). Recent theoretical work has indicated that patterns of elevated intersexual differentiation previously observed in the literature that have been attributed to ongoing sexual conflict would require implausibly large selective pressures and mortality loads (Kasimatis et al. 2017, 2019, 2020; Ruzicka et al. 2020). However, we do not use this approach to infer ongoing conflict, rather, sex-specific genetic architecture which invokes relatively lower genetic loads. Under sex-specific architecture, where loci exhibit sex differences in their phenotypic effects, we predict elevated intersexual differentiation but relaxed balancing selection (Mank 2017b).

Consistent with this prediction, we found that differentially alternatively spliced genes exhibited elevated intersexual F_{ST} and low Tajima's D in the duck gonad and guineafowl gonad (χ^2 test, $P = 0.003$ and $P = 0.059$, respectively; supplementary table S5, Supplementary Material online), consistent with differentially spliced genes affecting viability or survival in one sex but having little or no effect in the other. This pattern was not significant in the turkey gonad (χ^2 test, $P = 0.266$; supplementary table S5, Supplementary Material online), however, there are many fewer differentially spliced genes in turkey (table 1) which likely limits our power to test for any relationship in this species. Genes that were significantly differentially expressed between males and females were removed from this analysis as they have been shown previously to have sex-specific phenotypic effects (Wright et al. 2018). To confirm that these sex-specific effects are driven by sex-specifically expressed parts of genes, we extracted intersexual F_{ST} for sex-biased and unbiased exons. We found that F_{ST} was higher across sequences from sex-biased exons relative to unbiased exons in both the turkey and the guineafowl ($P = 0.014$, $P = 0.083$, turkey and guineafowl, respectively, paired Wilcoxon signed-rank test) but there was no significant difference in the duck ($P = 0.543$). This is the first statistical evidence, to our knowledge, that sex-specific selection acts on broad patterns of alternative splicing and that differentially spliced genes across the genome exhibit genomic signatures consistent with sex-specific effects.

Genes with Sex Differences in Splicing Are Subject to Greater Functional Constraints

Pleiotropy is thought to hinder the evolution of differential gene expression level and limit the response to sex-specific selection (Mank et al. 2008; Meisel 2011). Indeed, genes with broad expression patterns, a proxy for pleiotropy, are less likely to be differentially expressed (Mank et al. 2008). Alternative splicing might avoid pleiotropy and other constraints acting on expression level through the generation of distinct male and female isoforms. If so, we expect differential alternative splicing to be more common in genes with similar expression patterns between males and females. In line with our prediction, we found that while nonsignificant (duck $P = 0.06$, turkey $P = 0.55$, guineafowl $P = 0.49$, hypergeometric tests with Benjamini–Hochberg correction), there is less overlap than expected between differentially expressed and differentially spliced genes in the gonad ($RF < 1$; duck $RF = 0.83$, turkey $RF = 0.86$, guineafowl $RF = 0.94$, fig. 4A–C, and supplementary tables S7 and S8, Supplementary Material online). These results are consistent across multiple filtering thresholds and types of splicing events (supplementary table S8, Supplementary Material online).

Next, we explicitly tested whether genes under functional constraints are more predisposed to evolve differential splicing. First, we calculated a measure of tissue specificity (τ), a proxy for pleiotropy, where lower values indicate even expression distribution across tissues and larger values equate to greater levels of tissue specificity (Yanai et al. 2005). Measurements of τ were derived from the chicken UniGene database (Mank et al. 2008) and encompass expression patterns from nine tissues. Across all three species, we found that differentially spliced genes have significantly broader expression patterns relative to genes that are unbiased in expression, consistent with greater functional constraint (fig. 4D–F). This is in stark contrast to genes with differential expression level which, as previously observed (Mank et al. 2008; Meisel 2011), have greater tissue specificity than unbiased genes. Second, we employed contrasts of coding sequence evolution between genes that are unbiased, are exclusively differentially spliced or exclusively differentially expressed. Previously, differentially expressed genes have been shown to exhibit elevated rates of coding sequence evolution in a wide range of species as a consequence of relaxed evolutionary constraint and genetic drift (Gershoni and Pietrokovski 2014; Harrison et al. 2015). In contrast, we find that genes with differential splicing do not exhibit significantly elevated rates of sequence evolution in comparison to unbiased genes or genes that are differentially expressed between the sexes (fig. 4G–I), consistent with stronger purifying selection acting on coding sequences. This pattern is conserved when accounting for gene length and expression level, although the pattern then becomes nonsignificant in the duck (supplementary table S9, Supplementary Material online). Taken together, these results suggest that when genes are subject to functional constraints, the evolution of sex-specific isoforms may offer a more viable mechanism than changes in expression level to achieve sex-specific functions.

Concluding Remarks

Our results indicate that sex-specific selection acts on broad patterns of alternative splicing across the genome, which in turn may facilitate the evolution of sexually dimorphic phenotypes. Sex differences in alternative splicing and gene expression level are restricted to distinct sets of genes, where differential alternative splicing is limited to genes subject to strong purifying selection and functional constraint, indicating that splicing may function as an alternate route to sex-specific adaptation. However, it remains unclear whether dimorphism is a consequence of aggregate patterns of sex-biased splicing or large-effect loci, or how the magnitude of

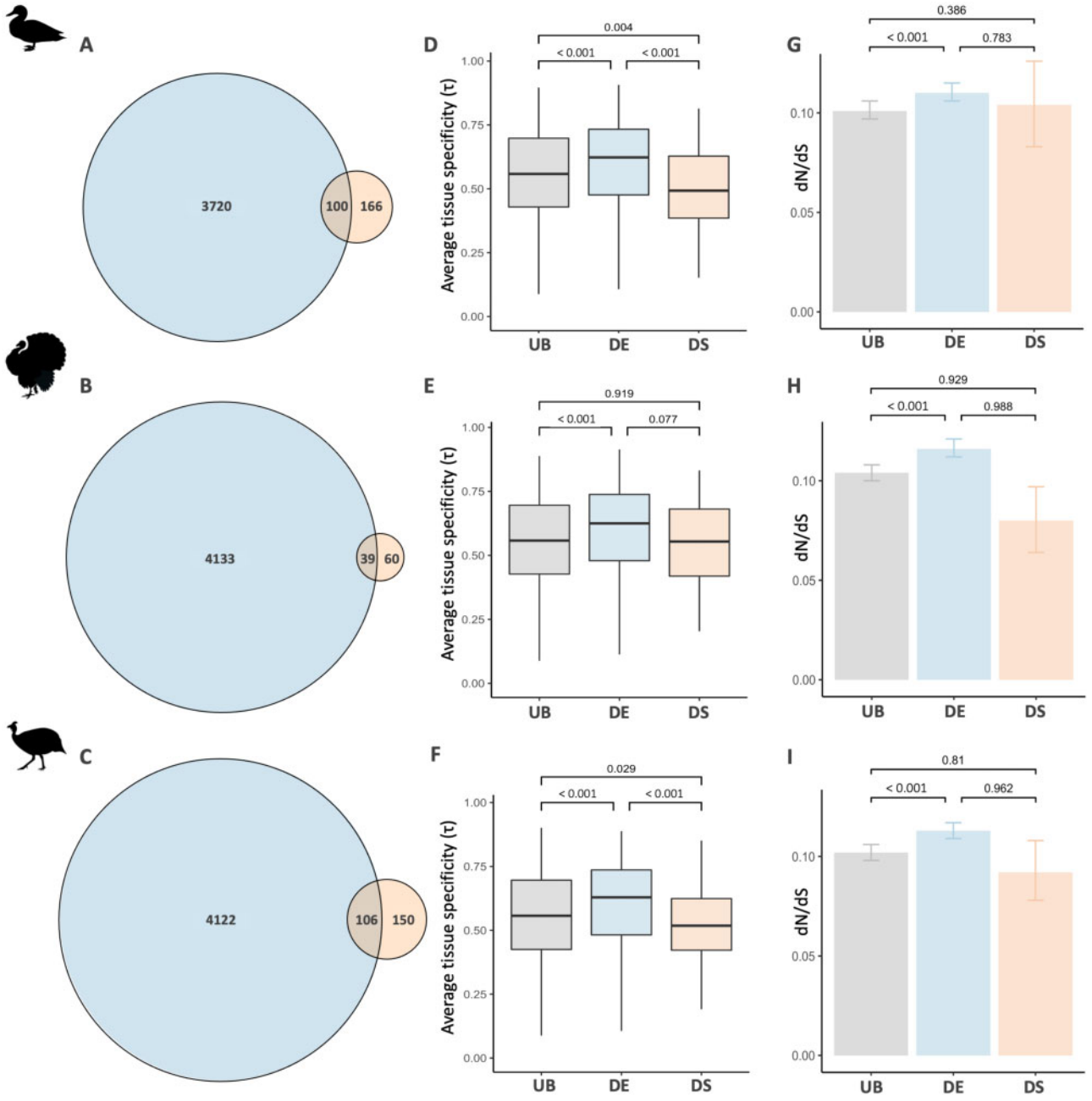

FIG. 4. Overlap, tissue specificity, and evolutionary rates of genes with sex differences in splicing and expression level in the duck (*A*, *D*, *G*), turkey (*B*, *E*, *H*), and guineafowl (*C*, *F*, *I*) gonad. Panels (*A*–*C*) show the overlap between differentially spliced (orange) and differentially expressed (blue) genes. Panels (*D*–*F*) show average tissue specificity (τ), where 0 denotes genes that are expressed ubiquitously and 1 means genes have tissue-specific expression. Panels (*G*–*I*) show the average ratio of nonsynonymous (d*N*) to synonymous (d*S*) substitutions for genes that are exclusively differentially spliced (orange), exclusively differentially expressed (blue), or unbiased (gray). In (*D*), (*E*), and (*F*), significance values were calculated with Wilcoxon's rank-sum test. In (*G*), (*H*), and (*I*), 95% confidence intervals and significance values were obtained from 1,000 bootstrap replicates.

splicing scales with phenotypic differences. Taken as a whole, our findings demonstrate how diverse patterns of transcriptional regulation can play an important role in phenotypic complexity.

Materials and Methods

Quality Filtering and Mapping

Previously, we obtained tissue samples, extracted, and sequenced RNA from semicaptive populations of the mallard duck (*Anas platyrhynchos*), wild turkey (*Meleagris gallopavo*), and helmeted guineafowl (*Numida meleagris*) (BioProject ID PRJNA271731, Harrison et al. 2015). The duck diverged from the guineafowl and turkey ~90 Ma, and the turkey and guineafowl diverged 30 Ma, providing medium- and long-term evolutionary comparison points for assessing divergence in splicing (supplementary fig. S1, Supplementary Material online). This includes RNA-seq data from five male and five female individuals of each species except for the turkey, where five dominant male, two subordinate male, and five female gonad samples were taken along with three dominant male

and two female spleen samples. RNA data were quality filtered using Trimmomatic v0.36 (Bolger et al. 2014). We filtered reads containing adapter sequences and trimmed reads if the sliding window average had a Phred score over four bases that was <15 or if the leading/trailing bases had a Phred score <3. The program used to quantify alternative splice events, rMATS (Shen et al. 2014), requires all reads to be equal length so reads were removed postfiltering if either read pair was <95 bp in length and all remaining reads were trimmed to 95 bp.

RNA-seq reads were mapped to respective reference genomes obtained from Ensembl (mallard duck; CAU_duck1.0; GCA_002743455.1, wild turkey; Turkey_2.01; GCA_000146605.1, helmeted guineafowl; NumMel1.0; GCA_002078875.2), using HISAT2 v.2.10 (Kim et al. 2015). We suppressed discordant and unpaired alignments for paired reads and excluded reads from the SAM output that failed to align. Reported alignments were tailored for transcript assemblers including StringTie. These alignments were used in downstream analyses to quantify both alternative splicing and gene expression levels to ensure accurate comparisons between patterns of splicing and gene expression levels.

Quantifying Alternative Splicing

We quantified alternative splicing in males and females in each species using rMATS v.4.0.3. Specifically, rMATS assesses annotated splice junctions in the reference genome for alternative splicing and detects differential splicing between two groups of samples. Splicing at each splice site is measured as the PSI, which indicates the proportion of two alternative isoforms at each splice site. A PSI value of 1 or 0 indicates that only one of the two alternative isoforms is always expressed and a value of 0.5 indicates equal expression of both isoforms. We detected alternative splicing events using $0 < \text{PSI} < 1$ in more than half of the individuals in each sample group. To compare splicing between groups of samples, rMATS calculates an inclusion difference (ΔPSI) (average PSI of male samples−average PSI of female samples), which ranges from 1 (the one isoform is only expressed in males) to −1 (the alternative isoform is only expressed in females). Therefore, ΔPSI of 0 means that patterns of splicing do not differ between males and females (i.e., the proportion of alternative isoforms for that splice site is the same between the sexes). rMATS uses a likelihood-ratio test to identify significant differences in ΔPSI between males and females. We identified differential splicing events using an FDR *P* value <0.05 and ΔPSI threshold of 0.1 following Grantham and Brisson (2018). The only exception was for analyses comparing patterns of differential splicing to differential expression where we used an FDR *P* value <0.05 and male: female $\log_2$-fold change PSI value of 1 to ensure equivalent thresholds were implemented. We calculated the significance of the overlap between differentially spliced orthologs using the SuperExactTest package (Wang et al. 2015) in R. Patterns of splicing were only quantified for autosomal genes as the Z chromosome is subject to unusual patterns of sex-specific selection due to its unequal inheritance pattern between males and females (Rice 1984). This workflow is summarized in supplementary figure S4, Supplementary Material online.

It has been suggested that many of the splicing events detected through next-generation sequencing approaches reflect stochastic transcriptional noise, however, this has been the subject of considerable recent debate (Melamud and Moult 2009; Tress et al. 2017a, 2017b; Wan and Larson 2018). We implemented a number of stringent filters to remove alternative splicing events that are likely nonfunctional noise. First, we evaluated splicing using only reads mapping to exon–exon boundaries that span splicing junctions to quantify splicing. Second, following Grantham and Brisson (2018), splicing sites were excluded if the number of reads supporting the inclusion and spliced exon junction was <20 in at least half the samples of both sexes in each tissue separately. Finally, although rMATS analyses different types of alternative splicing events, SE and MXE events are more commonly known to translate into functional isoforms (Weatheritt et al. 2016). These types of splicing comprise the majority of splice events we identified (supplementary table S1, Supplementary Material online) and so subsequent analyses were only performed on SE and MXE splicing events.

Cluster Analysis of Alternative Splicing Data

We assessed transcriptional similarity of splicing across samples, as measured by PSI, using the R package Pvclust (Suzuki and Shimodaira 2006). Hierarchical clustering with Euclidean distance was performed and the reliability of each of the trees produced was tested by bootstrap resampling (1,000 replicates).

Quantifying Gene Expression Level

SAM files generated from HISAT2 were coordinate sorted using SAMtools v1.9 (Li et al. 2009) and converted to BAM format. For each species, StringTie v1.3.5 (Pertea et al. 2015) was used to estimate gene expression level only for transcripts in the reference genome, ignoring novel transcripts, to ensure that expression was quantified for the same set of loci across all samples. We then extracted read count information directly from the StringTie output to generate count matrices for genes and transcripts as recommended by the StringTie pipeline. To ensure that our estimates of expression level were not biased by differences in alternative splicing across samples, we calculated gene expression level using only constitutively expressed exons (i.e., removing exons that are alternatively spliced or differentially alternatively spliced between males and females, FDR<0.05).

In each species, a minimum expression level threshold of 1 log CPM in at least half of the individuals of both sexes was imposed to remove lowly expressed genes in the gonad and spleen separately. Expression level was normalized using TMM (trimmed mean of *m* values) in EdgeR (Robinson et al. 2010). Genes were excluded from the analysis if they were single-exon or not located on annotated autosomal chromosomes. Sex-biased genes were identified using a standard $\log_2$-fold change value of 1 and FDR *P* value <0.05 (Assis et al. 2012; Harrison et al. 2015). This workflow is summarized in supplementary figure S4, Supplementary Material online.

Estimating Isoform Specificity (τ_{AS})

We developed an isoform specificity index to quantify variation in isoform abundance per gene. This is adapted from the tissue specificity index (τ) (Yanai et al. 2005), a commonly used metric that calculates whether expression is broadly expressed or localized in one tissue. Here, we instead use expression of each isoform to calculate isoform specificity, where a value of 0 indicates an even representation of isoform abundance and a value of 1 shows that a single isoform is always expressed. We call this measure τ_{AS}. For a given gene, τ_{AS} is defined as:

$$\tau_{AS} = \frac{\sum_{i=1}^{n} (1-\widehat{x}_i) + (1-\widehat{y}_i)}{n-1};$$

$$\widehat{x}_i = \frac{x_i}{\max_{1\le i\le n}(x_i,y_i)}, \quad \widehat{y}_i = \frac{y_i}{\max_{1\le i\le n}(x_i,y_i)},$$

where n is the total number of isoforms (assuming each splice site produces two isoforms), x_i is the read count supporting the inclusion of the exon in the gene product, and y_i is the read count supporting the exclusion of the exon from the gene product. We excluded splice sites that did not pass the coverage thresholds described above, and we excluded any exon that did not have a minimum read count of 20 in at least half of the individuals (within or between sexes) supporting both inclusion and exclusion of the exon. We then calculated male and female τ_{AS} for each gene. Importantly, power to detect isoform variation is limited by expression level so we reduced read counts in the sex with higher expression before calculating τ_{AS}. Specifically, read counts in the more highly expressed sex were scaled to the sex with the lower expression for each gene. This accounts for reduced power to detect splice events in samples with lower expression. In addition to this, to check that our results were not biased by variation in sequencing depth across samples, we normalized τ_{AS}, where read counts were divided by total library size in each sample. We tested for statistical differences between male and female τ_{AS} using a paired Wilcoxon's signed-rank test.

Estimating Population Genomic Statistics

For each individual, we merged spleen and gonad BAM files using SAMtools v1.9 (Li et al. 2009) with the exception of the turkey, where both tissues were not sequenced for all individuals so we used only gonad data for subsequent analyses. We used ANGSD (Korneliussen et al. 2014) to estimate population genetic summary statistics, following our previous approach (Wright et al. 2018, 2019) as ANGSD implements methods to account for uneven sequencing depth and is therefore appropriate for transcriptome data. We filtered BAM files to discard reads if they did not uniquely map, had a flag $\geq$256, had a mate that was not mapped, or had a mapping quality $<$20. Bases were filtered if base quality was $<$13 or if there were data in fewer than half the individuals. Mapping quality scores were adjusted for excessive mismatches and quality scores were adjusted around indels to rule out false single-nucleotide polymorphisms. We identified and removed related individuals (two wild turkey samples) from our analyses using NGSRELATE (Korneliussen and Moltke 2015) to avoid violating Hardy–Weinberg assumptions.

We calculated sample allele frequency likelihoods at each site from genotype likelihoods with the SAMtools model in ANGSD. Next, we estimated the overall unfolded site frequency spectrum for each species (Nielsen et al. 2012). Specifically, at each site we randomly sampled an allele frequency according to its likelihood, as calculated by ANGSD. Finally, we computed genetic diversity indices, including allele frequency posterior probability and Tajima's D using the site frequency spectrum as prior information with ANGSD thetaStat (Korneliussen et al. 2014).

Intersexual F_{ST} was calculated using the same procedure and filtering criteria as above except that we filtered out bases where we had data in fewer than half the individuals in males and females separately. We quantified Hudson's F_{ST}, which is less sensitive to small sample sizes (Bhatia et al. 2013; Gammerdinger et al. 2020). Estimates across coding regions of autosomal loci were obtained using weighted averages, where per-gene F_{ST} is the ratio between the sum of the between-populations variance across loci and the sum of the total variance across loci.

Immunity genes can generate patterns of balancing selection via mechanisms such as heterozygote advantage (Stahl et al. 1999; Rockman et al. 2010; Hedrick 2011) and negative-frequency dependent selection (Croze et al. 2016). Therefore, genes with potential immune function were excluded from the population genomic analyses. Specifically, we removed all loci with the terms "immune" or "MHC" in their Gene Ontology annotations from population genomic analyses. Furthermore, we applied a strict minimum expression level threshold of 2 log CPM in at least half of the individuals of both sexes to remove lowly expressed genes that may bias population genomic analyses.

Testing the Overlap between Differentially Spliced and Expressed Genes

We tested whether differentially spliced genes are also differentially expressed. First, we estimated the expected number of genes that are both differentially spliced (DSG) and differentially expressed (DEG) as (total no. DSG $\times$ total no. DEG)/total no. expressed genes. Next, we calculated the representation factor (RF), which is the observed number of overlapping genes divided by the expected number. If RF $<$ 1, there is less overlap between differentially spliced and expressed genes than expected and RF $>$ 1, there is more overlap than expected. We tested whether the overlap was significantly less than expected using the hypergeometric test with the phyper function in R. We calculated adjusted P values using the Benjamini–Hochberg (FDR) correction.

Identifying Orthologous Genes across Species

Coding sequences were downloaded from Ensembl v98 (Zerbino et al. 2018) for the mallard duck (*A. platyrhynchos*; CAU_duck1.0; GCA_002743455.1), wild turkey (*M. gallopavo*; Turkey_2.01; GCA_000146605.1), helmeted guineafowl

(*N. meleagris*; NumMel1.0; GCA_002078875.2), and zebra finch (*Taeniopygia guttata*; taeGut3.2.4). The longest isoform was retained for each species, and reciprocal orthologs across the four taxa were identified using BlastN v2.9.0+ (Altschul et al. 1990) with an e-value cutoff of 1×10^{-10} and minimum percentage identity of 30%. Across the duck, turkey, guineafowl, and zebra finch, 10,622 reciprocal orthologs were identified. We also identified pairwise reciprocal orthologs with the chicken (*Gallus gallus*) for the duck, turkey, and guineafowl using the same approach. This resulted in 13,425, 12,764, and 13,942 orthologs in the duck, turkey, and guineafowl, respectively.

Estimating Isoform Specificity (τ)

Tissue specificity (Yanai et al. 2005) was calculated from the chicken UniGene database, as previously described (Mank et al. 2008), and encompasses expression level patterns from nine tissues. Lower values indicate even expression level distribution across tissues and larger values equate to greater levels of tissue specificity. For each species, we extracted tissue specificity for genes with pairwise reciprocal orthologs in the chicken, resulting in τ values for 4,747, 5,131, and 5,200 genes in the duck, turkey, and guineafowl, respectively.

Estimating Rates of Coding Sequence Evolution

Orthologous sequences were aligned with PRANK v.140603 (Löytynoja and Goldman 2008), using a previously published phylogeny (Harrison et al. 2015). The sequence alignments were then checked for gaps, and for poorly aligned regions using SWAMP v.31-03-14 (Harrison et al. 2014) with a threshold of 4 in a window size of 5 bases and a minimum sequence length of 75 bp. Evolutionary parameters were estimated using the branch model in PAML v.4.8a (Yang 2007). Orthologous genes with $dS>2$ were filtered from subsequent analyses as this represents the point of mutational saturation in avian sequence data (Axelsson et al. 2008; Harrison et al. 2015). We extracted the number of nonsynonymous sites (N), the number of nonsynonymous substitutions (NdN), the number of synonymous sites (S), and the number of synonymous substitutions (SdS) for each taxon in order to calculate dN/dS weighted by alignment length (Mank et al. 2010; Harrison et al. 2015). We then generated 1,000 bootstrap replicates to obtain 95% confidence intervals and tested for significant differences between gene categories using 1,000 permutations. We tested if the pattern of dN/dS was conserved after controlling for gene length and gene expression level using multiple regression and an ANOVA test implemented in R.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

This work was supported by a Natural Environment Research Council (NERC) DTP grant to T.F.R., a NERC Independent Research Fellowship to A.E.W. (NE/N013948/1), a Royal Society Research Grant to A.E.W., and an National Science Foundation Postdoctoral Research Fellowship in Biology to D.H.P. (Award No. 1812164). We thank Judith Mank, Jon Slate and Peter Price for productive discussions, Judith Mank for providing tissue specificity values, Daniel Pitt for input on the figures and two anonymous reviewers for constructive feedback.

Author Contributions

T.F.R. and A.E.W. designed the research. T.F.R. and D.H.P. conducted the analyses. T.F.R. and A.E.W. wrote the article, with input from all authors.

Data Availability

The RNA-seq data in this study are available in the SRA (BioProject ID PRJNA271731).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Mol Biol*. 215(3):403–410.
Andersson M. 1994. Sexual selection. Princeton (NJ): Princeton University Press.
Assis R, Zhou Q, Bachtrog D. 2012. Sex-biased transcriptome evolution in *Drosophila*. *Genome Biol Evol*. 4(11):1189–1200.
Axelsson E, Hultin-Rosenberg L, Brandström M, Zwahlén M, Clayton DF, Ellegren H. 2008. Natural selection in avian protein-coding genes expressed in brain. *Mol Ecol*. 17(12):3008–3017.
Barbosa-Morais NL, Irimia M, Pan Q, Xiong HY, Gueroussov S, Lee LJ, Slobodeniuc V, Kutter C, Watt S, Colak R, et al. 2012. The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338(6114):1587–1593.
Bhatia G, Patterson N, Sankararaman S, Price AL. 2013. Estimating and interpreting FST: the impact of rare variants. *Genome Res*. 23(9):1514–1521.
Blekhman R, Marioni JC, Zumbo P, Stephens M, Gilad Y. 2010. Sex-specific and lineage-specific alternative splicing in primates. *Genome Res*. 20(2):180–189.
Blencowe BJ. 2017. The relationship between alternative splicing and proteomic complexity. *Trends Biochem Sci*. 42(6):407–408.
Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114–2120.
Bonduriansky R, Chenoweth S. 2009. Intralocus sexual conflict. *Trends Ecol Evol*. 23(9):1514–1521.
Brawand D, Soumillon M, Necsulea A, Julien P, Csárdi G, Harrigan P, Weier M, Liechti A, Aximu-Petri A, Kircher M, et al. 2011. The evolution of gene expression levels in mammalian organs. *Nature* 478(7369):343–348.
Brown JB, Boley N, Eisman R, May GE, Stoiber MH, Duff MO, Booth BW, Wen K, Park S, Suzuki AM, et al. 2014. Diversity and dynamics of the *Drosophila* transcriptome. *Nature* 512(7515):393–399.
Buchholz R. 1995. Female choice, parasite load and male ornamentation in wild turkeys. *Anim Behav*. 50(4):929–943.
Buchholz R. 1997. Male dominance and variation in fleshy head ornamentation in wild turkeys. *J Avian Biol*. 28(3):223.
Chen Y, Dokholyan NV. 2006. The coordinated evolution of yeast proteins is constrained by functional modularity. *Trends Genet*. 22(8):416–419.
Cline TW, Meyer BJ. 1996. Vive la difference: males vs females in flies vs worms. *Annu Rev Genet*. 30(1):637–702.
Connallon T, Clark AG. 2010. Sex linkage, sex-specific selection, and the role of recombination in the evolution of sexually dimorphic gene expression. *Evolution* 64(12):3417–3442.
Connallon T, Clark AG. 2011. The resolution of sexual antagonism by gene duplication. *Genetics* 187(3):919–937.
Connallon T, Knowles LL. 2005. Intergenomic conflict revealed by patterns of sex-biased gene expression. *Trends Genet*. 21(9):495–499.

Croze M, Živković D, Stephan W, Hutter S. 2016. Balancing selection on immunity genes: review of the current literature and new analysis in *Drosophila melanogaster*. *Zoology* 119(4):322–329.
Dean R, Harrison PW, Wright AE, Zimmer F, Mank JE. 2015. Positive selection underlies faster-Z evolution of gene expression in birds. *Mol Biol Evol*. 32(10):2646–2656.
Drummond DA, Raval A, Wilke CO. 2006. A single determinant dominates the rate of yeast protein evolution. *Mol Biol Evol*. 23(2):327–337.
Duret L, Mouchiroud D. 1999. Expression pattern and, surprisingly, gene length shape codon usage in *Caenorhabditis, Drosophila, and Arabidopsis*. *Proc Natl Acad Sci U S A*. 96(8):4482–4487.
Ellegren H, Parsch J. 2007. The evolution of sex-biased genes and sex-biased gene expression. *Nat Rev Genet*. 8(9):689–698.
Gallego Romero I, Ruvinsky I, Gilad Y. 2012. Comparative studies of gene expression and the evolution of gene regulation. *Nat Rev Genet*. 13(7):505–516.
Gammerdinger WJ, Toups MA, Vicoso B. 2020. Disagreement in FST estimators: a case study from sex chromosomes. *Mol Ecol Resour*. 00:1–9.
Gershoni M, Pietrokovski S. 2014. Reduced selection and accumulation of deleterious mutations in genes exclusively expressed in men. *Nat Commun*. 5(1):4438.
Gerstein MB, Rozowsky J, Yan KK, Wang D, Cheng C, Brown JB, Davis CA, Hillier L, Sisu C, Li JJ, et al. 2014. Comparative analysis of the transcriptome across distant species. *Nature* 512(7515):445–448.
Gibilisco L, Zhou Q, Mahajan S, Bachtrog D. 2016. Alternative splicing within and between *Drosophila* species, sexes, tissues, and developmental stages. *PLoS Genet*. 12(12):e1006464.
Gout JF, Kahn D, Duret L, Paramecium Post-Genomics Consortium. 2010. The relationship among gene expression, the evolution of gene dosage, and the rate of protein evolution. *PLoS Genet*. 6(6):20.
Grantham ME, Brisson JA. 2018. Extensive differential splicing underlies phenotypically plastic aphid morphs. *Mol Biol Evol*. 35(8):1934–1946.
Harrison PW, Jordan GE, Montgomery SH. 2014. SWAMP: sliding window alignment masker for PAML. *Evol Bioinform Online*. 10:197–204.
Harrison PW, Wright AE, Zimmer F, Dean R, Montgomery SH, Pointer MA, Mank JE. 2015. Sexual selection drives evolution and rapid turnover of male gene expression. *Proc Natl Acad Sci U S A*. 112(14):4393–4398.
Hedrick PW. 2011. Population genetics of malaria resistance in humans. *Heredity* 107(4):283–304.
Hill GE, Doucet SM, Buchholz R. 2005. The effect of coccidial infection on iridescent plumage coloration in wild turkeys. *Anim Behav*. 69(2):387–394.
Innocenti P, Morrow EH. 2010. The sexually antagonistic genes of *Drosophila melanogaster*. *PLoS Biol*. 8(3):e1000335.
Kalsotra A, Cooper TA. 2011. Functional consequences of developmentally regulated alternative splicing. *Nat Rev Genet*. 12(10):715–729.
Kasimatis KR, Abraham A, Ralph PL, Kern AD, Capra JA, Phillips PC. 2020. Sexually antagonistic selection on genetic variation is rare in humans. BioRxiv. 2020.03.26.009670; doi: https://doi.org/10.1101/2020.03.26.009670.
Kasimatis KR, Nelson TC, Phillips PC. 2017. Genomic signatures of sexual conflict. *J Hered*. 108(7):780–790.
Kasimatis KR, Ralph PL, Phillips PC. 2019. Limits to genomic divergence under sexually antagonistic selection. *G3 (Bethesda)* 9(11):3813–3824.
Khaitovich P, Hellmann I, Enard W, Nowick K, Leinweber M, Franz H, Weiss G, Lachmann M, Pääbo S. 2005. Evolution: parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. *Science* 309(5742):1850–1854.
Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 12(4):357–360.
Korneliussen TS, Albrechtsen A, Nielsen R. 2014. ANGSD: analysis of next generation sequencing data. *BMC Bioinformatics* 15(1):356.
Korneliussen TS, Moltke I. 2015. NgsRelate: a software tool for estimating pairwise relatedness from next-generation sequencing data. *Bioinformatics* 31(24):4009–4011.
Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25(16):2078–2079.
Löytynoja A, Goldman N. 2008. Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science* 320(5883):1632–1635.
Mank JE. 2017a. The transcriptional architecture of phenotypic dimorphism. *Nat Ecol Evol*. 1(1):0006.
Mank JE. 2017b. Population genetics of sexual conflict in the genomic era. *Nat Rev Genet*. 18(12):721–730.
Mank JE, Hultin-Rosenberg L, Axelsson E, Ellegren H. 2007. Rapid evolution of female-biased, but not male-biased, genes expressed in the avian brain. *Mol Biol*. 24(12):2698–2706.
Mank JE, Hultin-Rosenberg L, Zwahlen M, Ellegren H. 2008. Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. *Am Nat*. 171(1):35–43.
Mank JE, Nam K, Brunström B, Ellegren H. 2010. Ontogenetic complexity of sexual dimorphism and sex-specific selection. *Mol Biol Evol*. 27(7):1570–1578.
Matlin AJ, Clark F, Smith CWJ. 2005. Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol*. 6(5):386–398.
McIntyre LM, Bono LM, Genissel A, Westerman R, Junk D, Telonis-Scott M, Harshman L, Wayne M, Kopp A, Nuzhdin SV. 2006. Sex-specific expression of alternative transcripts in *Drosophila*. *Genome Biol*. 7(8):1–17.
Meisel RP. 2011. Towards a more nuanced understanding of the relationship between sex-biased gene expression and rates of protein-coding sequence evolution. *Mol Biol Evol*. 28(6):1893–1900.
Melamud E, Moult J. 2009. Stochastic noise in splicing machinery. *Nucleic Acids Res*. 37(14):4873–4886.
Melé M, Ferreira PG, Reverter F, DeLuca DS, Monlong J, Sammeth M, Young TR, Goldmann JM, Pervouchine DD, Sullivan TJ, et al. 2015. The human transcriptome across tissues and individuals. *Science* 348(6235):660–665.
Merkin J, Russell C, Chen P, Burge CB. 2012. Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science* 338(6114):1593–1599.
Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. 2019. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res*. 47(D1):D419–D426.
Moghadam HK, Pointer MA, Wright AE, Berlin S, Mank JE. 2012. W chromosome expression responds to female-specific selection. *Proc Natl Acad Sci U S A*. 109(21):8207–8211.
Nielsen R, Korneliussen T, Albrechtsen A, Li Y, Wang J. 2012. SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data. *PLoS One* 7(7):e37558.
Nilsen TW, Graveley BR. 2010. Expansion of the eukaryotic proteome by alternative splicing. *Nature* 463(7280):457–463.
Pál C, Papp B, Hurst LD. 2001. Highly expressed genes in yeast evolve slowly. *Genetics* 158(2):927–931.
Pan Q, Bakowski MA, Morris Q, Zhang W, Frey BJ, Hughes TR, Blencowe BJ. 2005. Alternative splicing of conserved exons is frequently species-specific in human and mouse. *Trends Genet*. 21(2):73–77.
Papakostas S, Vøllestad LA, Bruneaux M, Aykanat T, Vanoverbeke J, Ning M, Primmer CR, Leder EH. 2014. Gene pleiotropy constrains gene expression in fish adapted to different thermal conditions. *Nat Commun*. 5(1):4071.
Parker GA, Partridge L. 1998. Sexual conflict and speciation. *Philos Trans R Soc Lond B*. 353(1366):261–274.
Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol*. 33(3):290–295.
Pointer MA, Harrison PW, Wright AE, Mank JE. 2013. Masculinization of gene expression is associated with exaggeration of male sexual dimorphism. *PLoS Genet*. 9(8):e1003697.

Ranz JM, Castillo-Davis CI, Meiklejohn CD, Hartl DL. 2003. Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science* 300(5626):1742–1745.

Rice WR. 1984. Sex chromosomes and the evolution of sexual dimorphism. *Evolution* 38(4):735–742.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1):139–140.

Rockman MV, Skrovanek SS, Kruglyak L, Gerke JP, Kruglyak L. 2010. Selection at linked sites shapes heritable phenotypic variation in *C. elegans*. *Science* 330(6002):372–376.

Ruzicka F, Dutoit L, Czuppon P, Jordan CY, Li X, Olito C, Runemark A, Svensson EI, Yazdi HP, Connallon T. 2020. The search for sexually antagonistic genes: practical insights from studies of local adaptation and genomics. *Evol Lett*. 4(5):398–415.

Schütt C, Nöthiger R. 2000. Structure, function and evolution of sex-determining systems in Dipteran insects. *Development* 127(4):667–677.

Shen S, Park JW, Lu ZX, Lin L, Henry MD, Wu YN, Zhou Q, Xing Y. 2014. rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci U S A*. 111(51):e5593–e5601.

Stahl EA, Dwyer G, Mauricio R, Kreitman M, Bergelson J. 1999. Dynamics of disease resistance polymorphism at the Rpm1 locus of Arabidopsis. *Nature* 400(6745):667–671.

Su WL, Modrek B, GuhaThakurta D, Edwards S, Shah JK, Kulkarni AV, Russel A, Schadt EE, Johnson JM, Castle JC. 2008. Exon and junction microarrays detect widespread mouse strain- and sex-bias expression differences. *BMC Genomics* 9(1):273.

Suzuki R, Shimodaira H. 2006. Pvclust: An R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 22(12):1540–1542.

Tress ML, Abascal F, Valencia A. 2017a. Alternative splicing may not be the key to proteome complexity. *Trends Biochem Sci*. 42(2):98–110.

Tress ML, Abascal F, Valencia A. 2017b. Most alternative isoforms are not functionally important. *Trends Biochem Sci*. 42(6):408–410.

Uebbing S, Künstner A, Mäkinen H, Backström N, Bolivar P, Burri R, Dutoit L, Mugal CF, Nater A, Aken B, et al. 2016. Divergence in gene expression within and between two closely related flycatcher species. *Mol Ecol*. 25(9):2015–2028.

Wan Y, Larson DR. 2018. Splicing heterogeneity: separating signal from noise. *Genome Biol*. 19(1):1–10.

Wang M, Zhao Y, Zhang B. 2015. Efficient test and visualization of multi-set intersections. *Sci Rep*. 5(1):1–12.

Weatheritt RJ, Sterne-Weiler T, Blencowe BJ. 2016. The ribosome-engaged landscape of alternative splicing. *Nat Struct Mol Biol*. 23(12):1117–1123.

Wright AE, Fumagalli M, Cooney CR, Bloch NI, Vieira FG, Buechel SD, Kolm N, Mank JE. 2018. Male-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evol Lett*. 2(2):52–61.

Wright AE, Rogers TF, Fumagalli M, Cooney CR, Mank JE. 2019. Phenotypic sexual dimorphism is associated with genomic signatures of resolved sexual conflict. *Mol Ecol*. 28(11):2860–2871.

Yanai I, Benjamin H, Shmoish M, Chalifa-Caspi V, Shklar M, Ophir R, Bar-Even A, Horn-Saban S, Safran M, Domany E, et al. 2005. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. *Bioinformatics* 21(5):650–659.

Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ. 2006. Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Res*. 16(8):995–1004.

Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol*. 24(8):1586–1591.

Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, Billis K, Cummins C, Gall A, Giron CG, et al. 2018. Ensembl 2018. *Nucleic Acids Res*. 46(D1):D754–D761.

Zhang Y, Sturgill D, Parisi M, Kumar S, Oliver B. 2007. Constraint and turnover in sex-biased gene expression in the genus *Drosophila*. *Nature* 450(7167):233–237.

Detecting Signatures Of Selection In Regulatory Variation

Peter D Price[1*], Daniela H Palmer Droguett[1,2], Jessica A Taylor[1,3], Dong W Kim[4], Elsie S Place[5], Thea F Rogers[1], Judith E Mank[6,7,8], Christopher R Cooney[1+] & Alison E Wright[1+*]

[1]*Ecology and Evolutionary Biology, School of Biosciences, University of Sheffield, United Kingdom*

[2]*Ecology, Evolution, and Behavior Program, Michigan State University, USA*

[3]*Department of Biosciences, Durham University, United Kingdom*

[4]*Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, USA*

[5]*Development, Regeneration and Neurophysiology, School of Biosciences, University of Sheffield, United Kingdom*

[6]*Department of Zoology, University of British Columbia*

[7]*Beaty Biodiversity Research Centre, University of British Columbia*

[8]*Centre for Ecology and Conservation, University of Exeter, Penryn, UK*

*Corresponding authors: pprice3@sheffield.ac.uk, a.e.wright@sheffield.ac.uk

+Joint senior author

Keywords: gene regulation, comparative phylogenetic methods, adaptive evolution, transcriptomics, complex phenotypes, stabilizing selection

ABSTRACT

A substantial amount of phenotypic diversity results from changes in gene regulation. Understanding how regulatory diversity evolves is therefore a key priority in identifying mechanisms of adaptive change. However, in contrast to powerful models of sequence evolution, we lack a consensus model of regulatory evolution. Furthermore, recent work has shown that many of the comparative approaches used to study gene regulation are subject to biases that can lead to false signatures of selection. In this review, we first outline the main approaches for describing regulatory evolution and their inherent biases. Next, we bridge the gap between the fields of comparative phylogenetic methods and transcriptomics to reinforce the main pitfalls of inferring regulatory selection and use simulation studies to show that shifts in tissue composition can heavily bias inferences of selection. We close by highlighting the multi-dimensional nature of regulatory variation and identifying major, unanswered questions in disentangling how selection acts on the transcriptome.

INTRODUCTION

A growing body of evidence indicates that changes in gene regulation play a key role in phenotypic divergence[1]. Within species, a single genome can encode multiple distinct phenotypes by varying expression levels of the underlying loci[2]. Similarly, across species, regulatory variation is implicated in major phenotypic differences that underlie adaptive change[1]. Given the importance of gene regulation in shaping phenotypic diversity, transcriptome analyses are widely used as a genomic tool to identify the genes that underlie phenotypic variation and the selective regimes acting on them[3]. However, the dominant mode of evolution acting on gene expression remains controversial. Current evidence supports the notion that global patterns of gene expression evolve predominantly under stabilizing selection, but the extent of neutral evolution is heavily debated[4–7].

Much of this debate is driven by the lack of a consensus neutral model of transcriptome evolution. In contrast to established models of sequence evolution that allow us to predict the phenotypic effects of different types of coding mutations and scan coding sequence data for regions of adaptive evolution, gene regulation can be complex and non-additive in its phenotypic effects. This complexity has resulted in a wide range of approaches to study regulatory evolution[3,8,9]. Importantly, these approaches make direct assumptions about how expression evolves across species, many of which have yet to be robustly validated, and these assumptions vary extensively across models. Over the last decade, statistical frameworks developed in the field of phylogenetic comparative methods have been applied to transcriptome data to infer selection[8,10], and these have provided important insights into patterns of regulatory variation. However, in recent years it has become clear that several of these phylogenetic comparative approaches suffer from biases that often lead to false inferences of stabilizing selection when applied to real phenotypic data[11,12]. Many of the root causes of these biases are even more pronounced in transcriptomic data, but the issues uncovered in the phylogenetic comparative literature[11,12] are only rarely discussed in the genomics field[13,14].

Finally, most studies make the explicit assumption that when differential gene expression is observed, it is the direct result of regulatory change. In reality, this fundamental assumption may often be flawed as most studies measure expression in bulk across heterogeneous tissue samples and so cannot distinguish changes in gene regulation from differences in tissue composition[15,16]. Of course, changes

in tissue composition, which encompass both changes in cell type abundance within tissues and allometric scaling across them, are likely due to regulatory changes in development. However, these developmental regulatory differences will not be detected if transcriptomes are measured after development is completed and instead the resulting differences in gene abundance will be mistaken as causative adaptive changes (Fig. 1). This problem undermines our current understanding of the nature and abundance of regulatory variation, and how it contributes to phenotypic divergence. Although the implications of varying tissue composition across species for measuring regulatory change have been discussed[15,16], the consequences of how it affects the inference of expression evolution have received little attention and so are not widely appreciated.

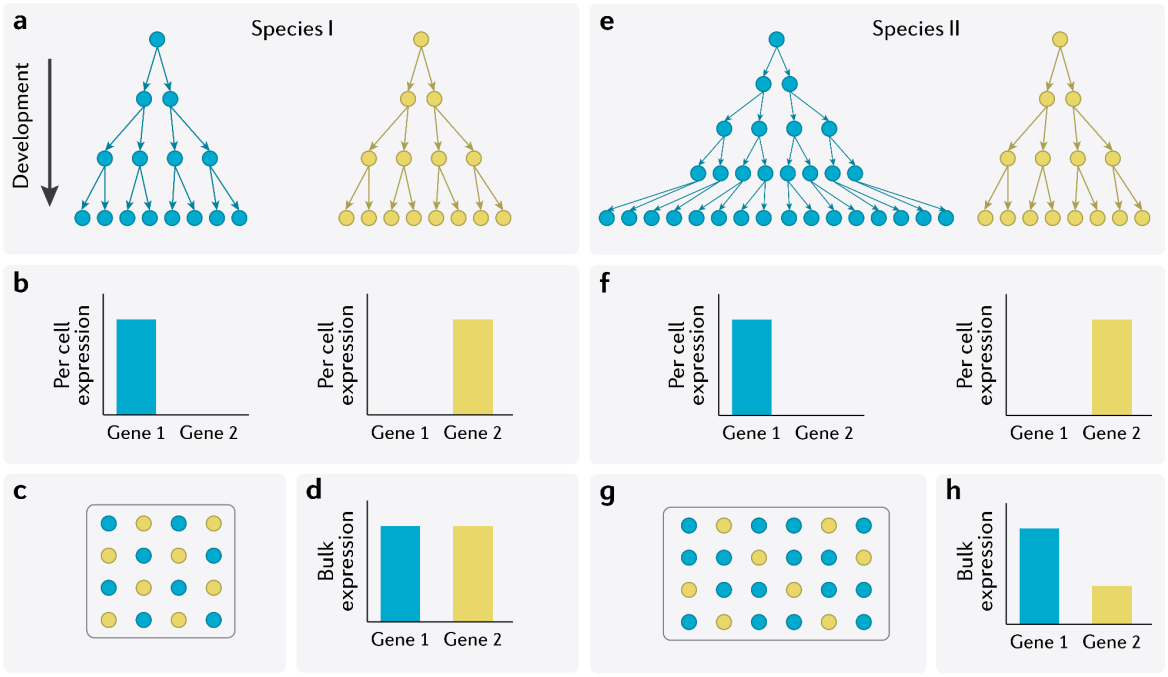

Figure 1. Variation in tissue composition can lead to the perception of differential expression. Schematic illustrating how variation in tissue composition can bias perception of expression measured from bulk RNA-Seq. Here, a single tissue is comprised of two cell types, type 1 (blue) which only expresses gene 1, and type 2 (yellow) which only expresses gene 2. During development in Species I **(a-d)**, cell type 1 and 2 have the same rate of cell proliferation **(a)** and per cell expression is the same for both genes within each cell type **(b)**. The resulting tissue is evenly comprised of each cell type **(c)** and bulk RNA-Seq expression for both gene 1 and 2 is equal **(d)**. In Species II **(e-h)**, a slight increase in the rate of cellular proliferation for cell type 1 **(e)** results in a greater proportion of cells of type 1 in the resulting adult tissue **(g)**. Even though there has been no change in per cell expression of either gene 1 or 2 **(f)**, the relative expression from bulk RNA-Seq of the entire tissue results in the perception of higher expression of gene 1 and lower expression of gene 2 compared to expression in Species 1 **(h)**.

Here, we examine our current understanding of the evolutionary processes generating variation in gene regulation. First, we outline the main approaches for describing regulatory evolution, examine their inherent biases, and synthesize findings to provide new perspectives to the debate over how selection acts on the transcriptome. Second, we attempt to bridge the gap between the fields of comparative phylogenetic methods and transcriptomics to reinforce the main pitfalls of inferring regulatory variation. Importantly, we identify a previously overlooked challenge to the study of expression evolution concerning shifts in tissue composition across taxa, and use simulation studies to show that this issue can heavily bias inferences of selection. We close by highlighting the multi-dimensional nature of regulatory variation and identifying major, unanswered questions in disentangling how selection acts on the transcriptome.

INFERRING THE MODE OF GENE EXPRESSION EVOLUTION

Currently, a number of different approaches for describing regulatory evolution have been proposed in the absence of a single consensus model. These can be divided into three broad categories; (i) contrasts between divergence and variation in expression (Fig 2A), (ii) phylogenetic comparative methods (Fig 2B) and (iii) fitness-based approaches (Fig 2C). Importantly, each makes different assumptions regarding the mode of expression divergence and are subject to distinct biases. With a few exceptions[13,14,17,18], studies rarely interrogate multiple approaches and so it remains unclear whether discrepancies between studies are biologically meaningful or caused by inherent methodological differences. Below we synthesise results from different analytical frameworks to provide an overview on the debate concerning the importance of selection versus genetic drift in shaping regulatory variation.

Contrasting divergence and variation in expression

Many early analyses of regulatory evolution tested for selection by contrasting expression divergence between species and expression diversity within species[19–23]. This method relies on the assumption that neutral changes are based solely on the underlying mutation rate[24,25] and so divergence between species relative to polymorphism within species will be equal at neutral loci[25]. When applied to expression data, mutation leads to polymorphism, which can be inferred through variation in expression level amongst individuals. Thus, a neutral model of evolution can be rejected when there are deviations

from an equal ratio of within to between species regulatory variation (Fig. 2A). Studies employing this approach are dominated by two competing viewpoints. One posits that gene regulation is predominantly neutrally evolving[9,19,20,26] and the other suggests widespread conservation and purifying selection of gene expression levels[21,23,27,28] with evidence of positive selection acting on certain loci[29–34].

Analogous approaches using alternative neutral models of expression divergence have also found broad support for stabilizing selection[3,6]. One such approach uses mutation accumulation studies to estimate neutral expectations of expression divergence and infer selection through contrasts with natural populations[35–37]. Most recently, the distribution of expression levels of F2 offspring from a genetic cross has been used to estimate expected levels of neutral divergence[38]. Here, under neutrality, expression variance of the two parental populations should be equal to the F2 progeny as the F2 phenotypes result from random combinations of segregating alleles. Following this logic, directional selection can be inferred when parental divergence is significantly greater than the neutral expectation and stabilizing selection can be inferred when parents are significantly less diverged than expected. This study found widespread stabilizing selection across a range of species, the magnitude of which was dependent on the species' effective population size, consistent with population genetic theory. Selection has also been inferred through comparisons of additive genetic variance of expression (Q_{ST}) with sequence divergence in neutral molecular markers (F_{ST}) across populations[39]. However, while Q_{ST}:F_{ST} approaches have been successfully applied to gene expression variation in a few instances[40–44], accurately estimating the additive genetic basis of gene regulation can be challenging[45]. There is a tendency for dominance variance to bias Q_{ST} estimates, potentially leading to incorrect inferences of neutrality[39].

Nonetheless, the broad approach of contrasting inter- and intra-specific regulatory variation offers a tractable method to investigate selective forces shaping expression levels. However, one drawback is that these tests assume species or populations are phylogenetically independent and do not account for shared and often complex evolutionary histories. Therefore, in cases where more than one pair of species are compared, these methods can produce evolutionary patterns that are generated by the structure of the underlying phylogeny[46,47]. Furthermore, the neutral expectation that expression divergence equals diversity tends to break down over longer evolutionary time periods. This is because gene expression divergence cannot accumulate indefinitely due to upper limits on the rate of

transcription. With increasing genetic distance, expression divergence among taxa may become nonlinear, leading to instances of genetic drift being mistaken as directional selection[9,14]. To test for selection across multiple species and evolutionary distances, approaches that take a phylogenetic perspective are required.

Phylogenetic comparative methods

Phylogenetic comparative methods have been widely adopted to infer selection acting on phenotypic traits for a number of decades[10,47–49]. By incorporating phylogenetic information, these methods account for shared ancestry and therefore can overcome issues of statistical non-independence. Recently, these approaches have been widely applied to transcriptome data to infer selection acting on gene expression by fitting a number of discrete evolutionary processes to expression data for a given gene[8,50,51] (Fig. 2B). A commonly used model, Brownian Motion (BM), assumes that expression divergence between species will be a function of divergence time and evolutionary rate, and, as such, is often seen as analogous to genetic drift. A second model, the Ornstein-Uhlenbeck (OU) model, adds an 'elastic band' element drawing expression values towards an optimum across the phylogeny, akin to stabilizing selection[8,52]. The OU model can be extended to allow for branch-specific events, such as shifts in optimum trait values[8,53], analogous to directional selection in particular lineages.

Comparative transcriptomic analyses based on the OU model have found overwhelming support for stabilizing selection on expression levels across a wide range of species, including *Drosophila*[8,54], African cichlids[55] and mammals[56]. While this appears consistent with past work[21,23,27,28], using OU models to infer selection has received repeated criticism within the phylogenetic comparative literature (BOX 1). In essence, any factor that leads to a reduction of phylogenetic signal in species' trait values will favour the inference of an OU process over BM, regardless of the underlying evolutionary process Importantly, failing to account for biological intraspecific variance or methodological measurement error by running these models on a mean species expression value has been shown to erode phylogenetic signal and lead to false inferences of stabilizing selection[11–13] or branch-specific selection[14]. These issues are particularly relevant to expression data, which can be noisy (i.e. subject to a high degree of measurement error), particularly when environmental and developmental variance is not strictly controlled for. The OU framework has been adapted to specifically include within-species expression

variability as an error term[13,53,57], and whilst it has been shown to reduce false inferences of stabilizing selection, this approach has only been employed by a handful of studies[18,58].

Recently, Rohlfs *et al.*[14] built on this approach with the Expression Variance and Evolution (EVE) model for testing expression evolution. This approach is grounded in the OU framework but incorporates contrasts of expression variance within versus between species, analogous to divergence-diversity ratio comparisons (Fig. 2A). This is a major advance as it accounts for evolutionary relationships between species as well as incorporating a neutral expectation for expression divergence that is dataset-specific. Interestingly, the few studies that have employed this approach have typically revealed a higher proportion of genes evolving under directional than under stabilizing selection[14,18], and evidence for elevated rates of expression evolution consistent with adaptive evolution[58–61], contrasting with previous findings. However, this method also relies on accurately estimating parameters of the OU process, so it is still likely subject to similar pitfalls identified by the phylogenetic comparative literature (BOX 1).

Fitness-based approaches

Most recently, fitness based approaches have been applied to study contemporary patterns of selection acting on gene regulation[62,63]. One classical approach, which has been used to study a wide range of morphological traits, uses regression-based methods to estimate the strength of selection[64]. In this approach, the covariance between fitness and gene expression is calculated to infer selection differentials at each locus, which signify the mode of selection[62,63] (Fig. 2C). To reduce noise and computation time, as well as increase robustness of model prediction, expression data can be transformed to reduce dimensionality (i.e., by PCA) and selection gradients can then be obtained to estimate direct selection on suites of correlated transcripts. Recent studies have used these principles to measure regulatory selection in experimental contexts (e.g. by quantifying flowering success and fecundity of rice grown in wet versus drought conditions[62]) and in natural settings (e.g. by measuring parasite load and survivorship of wild trout using mark-recapture[63]). In contrast to comparative approaches, neither of these studies found strong support for stabilizing selection, and in one case, the dominant mode of selection was disruptive[63]. Causes of this discrepancy require further investigation, particularly whether or not this reflects methodological biases. However, it is possible that selection

pressures vary over short- versus long-term evolutionary time frames, and these approaches are capturing different snapshots of the evolutionary process.

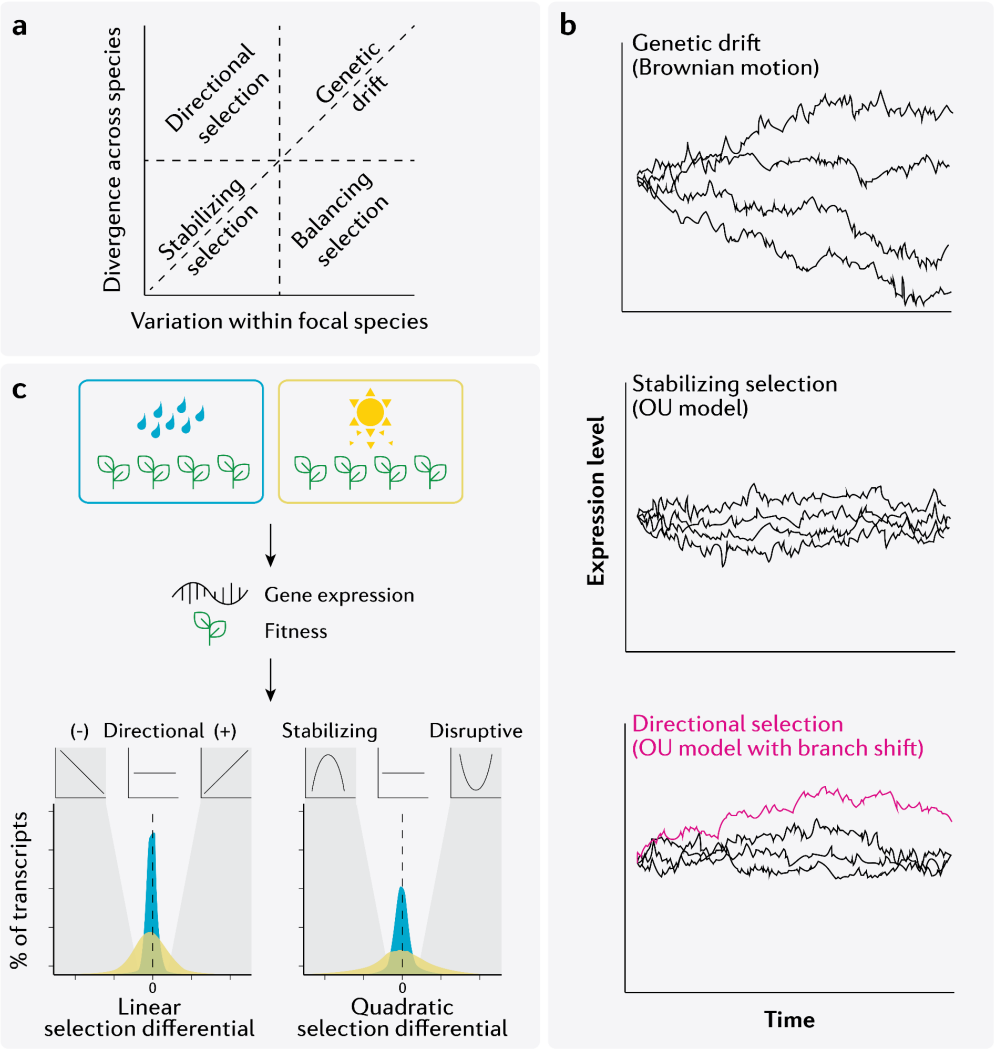

Figure 2. Approaches to detect selection on regulatory variation.

Panel (a) Gene expression evolution has been inferred by contrasting levels of variation within a focal species to divergence across species in a pairwise framework. This principle is analogous to the Hudson Kreitman Aguadé (HKA) test used to detect selection at the DNA level. The neutral expectation is that divergence covaries linearly with intra-specific variance, at least over shorter evolutionary distances. Loci with the highest or lowest levels of regulatory variation relative to neutrality are the best candidates for balancing or directional selection respectively. Loci under stabilizing selection should exhibit limited biological variance and divergence. **Panel (b)** Phylogenetic comparative analyses enable comparisons across species to distinguish between evolutionary processes. Brownian motion models neutral trait evolution via an unconstrained random walk. It assumes that divergence time between species will describe the diversity across the phylogeny with only one parameter σ^2, the drift rate, and that variance at the tips of the phylogeny will equal $T\sigma^2$. The Ornstein-Uhlenbeck (OU) model assumes that gene regulation follows a stochastic process that is attracted towards a single optimum value, consistent with stabilizing selection. The additional parameters are therefore α, the strength of pull, and θ, the evolutionary optima. This framework has been extended to test for branch specific processes by incorporating multiple optima to test for trait

divergence in specific lineages (red line). **Panel (c)** Phenotypic selection analyses have been applied to gene expression data to infer the mode and strength of selection. These employ multiple regression of relative fitness on multiple traits to calculate selection differentials that estimate total selection (direct and indirect) on gene expression. The covariance between fitness and expression is calculated to infer linear (*S*) and quadratic (*C*) selection differentials at each locus, which signify directional, stabilizing, or disruptive selection. The linear selection differential estimates positive versus negative directional selection, while the quadratic selection differential estimates disruptive versus stabilizing selection. This panel is adapted from Groen et al (2020)[62], which used this approach to measure selection on gene expression in rice. Rice was grown under wet (blue) and dry (yellow) environmental conditions, and phenotypes and fitness were measured.

DECOMPOSING TRANSCRIPTIONAL VARIATION

Importantly, approaches designed to test for selection on regulatory variation all make the explicit assumption that when differential gene expression is observed, it is the direct result of regulatory change. However, in most cases, it is unclear whether this assumption is valid as processes other than regulatory evolution can generate apparent gene expression differences among taxa. For example, to date, studies have primarily used bulk sequencing approaches to measure expression across aggregate tissues or even entire body regions, which are often composed of many different cell types with variable expression profiles[1]. In doing so, these 'bulk' expression values represent an average of expression across entire populations of distinct cell types. Importantly, this means that samples that vary in tissue composition can produce patterns of differential expression that are often mistaken as evidence of regulatory change (Fig. 1). Conversely, this approach can also dampen and/or mask genuine regulatory differences[15,16].

Recent advances in single-cell transcriptomics are providing new insights into tissue composition and how this can vary both within and across species. Within species, dramatic changes in tissue composition are well documented throughout development[65–67] and between the sexes[65]. This is exemplified by gonadal tissue, which exhibits sex-specific cell types[65] as well as a mix of haploid and diploid cells at various stages of differentiation[67–70]. Similarly, changes in cell type abundance between homologous tissues are common across species, particularly in the testes[16,71,72], likely as a result of varying levels of sperm competition and sexual selection. For instance, species of New World Blackbirds under more intense sperm competition exhibit a greater proportion of sperm-producing tissue within their testes[71] than species subject to weaker sperm competition. In addition to differences in cell type abundance within a tissue, inter-specific single-cell analyses are starting to show that allometric

shifts might be common in many other tissues, including the brain[73–75]. Therefore, in many instances, differentially expressed genes that are identified from bulk transcriptomic approaches might simply be a product of variation in cellular heterogeneity rather than true regulatory change.

To our knowledge, only a handful of studies have directly addressed the consequences of varying tissue allometry for inferring regulatory variation across species[15,16,76,77]. To provide further insight into this issue, here we use existing single-cell expression data (scRNA-seq) for the developing chicken hypothalamus[66] to investigate this further (Fig. 3). At this stage in development (HH10), the hypothalamus is composed of three major cell types, where the FOXA1 cell type represents the greatest proportion of cells (Fig. 3A). Importantly, each cell type exhibits a distinct gene expression profile (Fig. 3B). We condensed the expression of single cells from these three cell types into a composite expression value for each loci, analogous to a bulk RNA-seq approach for the whole hypothalamus. We find that broad expression patterns across the entire tissue are not reflective of true regulatory variation, although the magnitude of this effect varies across genes (Fig. 3B), consistent with recent work in the mouse gonad[16]. This illustrates that changes in tissue composition can have profound implications for quantifying gene regulation and we urge future studies to carefully consider the composition of samples.

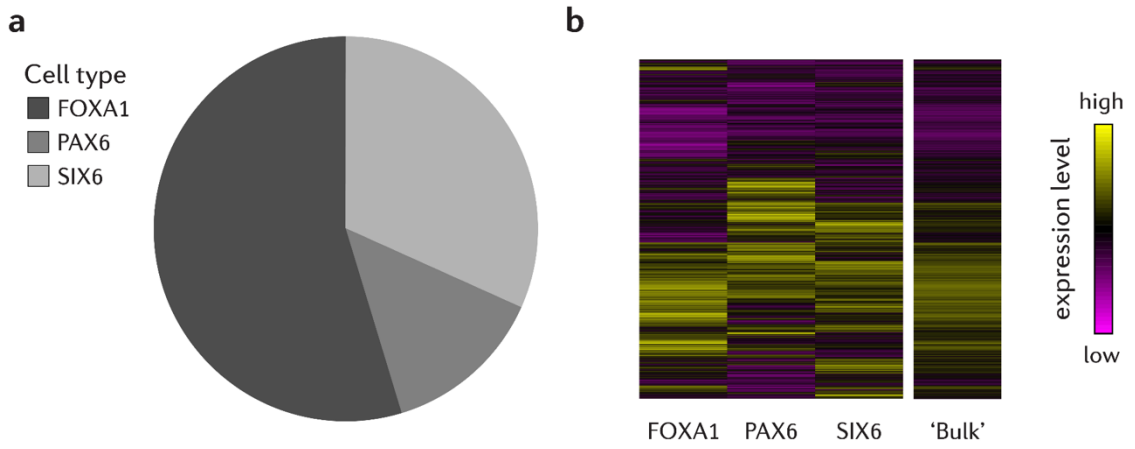

Figure 3. Deconstructing gene expression measured in bulk from heterogeneous tissue.
Tissue composition and gene regulation of the chicken hypothalamus at a single developmental stage (HH10). **Panel (a)** Pie chart shows the proportion of cells in each major cell type in the hypothalamus. **Panel (b)** Heatmap shows gene expression measured across individual cells. Highly expressed genes are shown in yellow and lowly expressed genes are in pink. The first three columns of the heatmap show average expression for each gene across cells in each of the three major cell types. The final column shows average expression estimated across the entire tissue. In this case, we ignore cell identity and convert scRNA-seq data to 'bulk' data, equivalent to generating RNA-seq data from the whole tissue. Data from[66].

CHALLENGES OF INFERRING SELECTION

While the implications of varying tissue allometry for measuring regulatory change across species have been discussed[15,16] (Fig. 1 & 3), the consequences of tissue composition on inferences of expression evolution have received very little attention. Nearly all studies that test for regulatory selection use transcriptomic data generated from heterogeneous tissue, with the exception of recent work that used cell sorting to isolate distinct cell types in the mouse testes[61]. Given that changes in tissue composition across species are likely to be common, this could pose an underappreciated challenge to comparative studies of regulatory evolution. As discussed in BOX 1, there is a tendency for phylogenetic comparative methods to falsely infer stabilizing selection or more complex adaptive processes if non-evolutionary processes (such as measurement error) reduce phylogenetic signal. Changes in expression that are driven by variation in tissue composition across species represent a prominent source of non-evolutionary expression variance and could therefore bias inferences of selection. This possibility has yet to be examined and so, using a series of simulated scenarios, we directly explore how compositional shifts on a phylogeny can bias the inference of evolutionary processes.

We simulated three distinct scenarios to explore how asymmetry in tissue composition across a phylogeny can drive false model inferences of regulatory selection when applying comparative methods (Fig. 4). Specifically, we imagine a simple situation where a tissue is composed of two distinct cell types. We estimate bulk expression values as a function of expression in each cell type and their relative abundances in the tissue, and fit a set of discrete evolutionary models to this bulk expression.

First, we describe a scenario of extreme stabilizing selection on gene regulation of a single locus. This locus is highly expressed in one cell type and lowly expressed in the other, but importantly, expression values are identical (i.e. not evolving) across species. However, the relative abundance of each cell type is evolving under genetic drift and so varies across species (Fig. 4A, scenario i). As predicted, the composite expression value is not reflective of single-cell expression levels nor consistent with extreme stabilizing selection (Fig. 4B, scenario i). Intuitively, a phylogenetic comparative approach consistently rejects a ‘static’ model of expression evolution and finds the greatest support for genetic drift as the dominant mode of regulatory change (Fig. 4C, scenario i). In this instance, the false positive (i.e. Type 1 error rate) rate is around ~85% relative to when these models are run on single-cell expression levels.

This striking result suggests that shifts in tissue composition can lead to false inferences of evolutionary processes acting on gene expression in the complete absence of any regulatory change within each cell type.

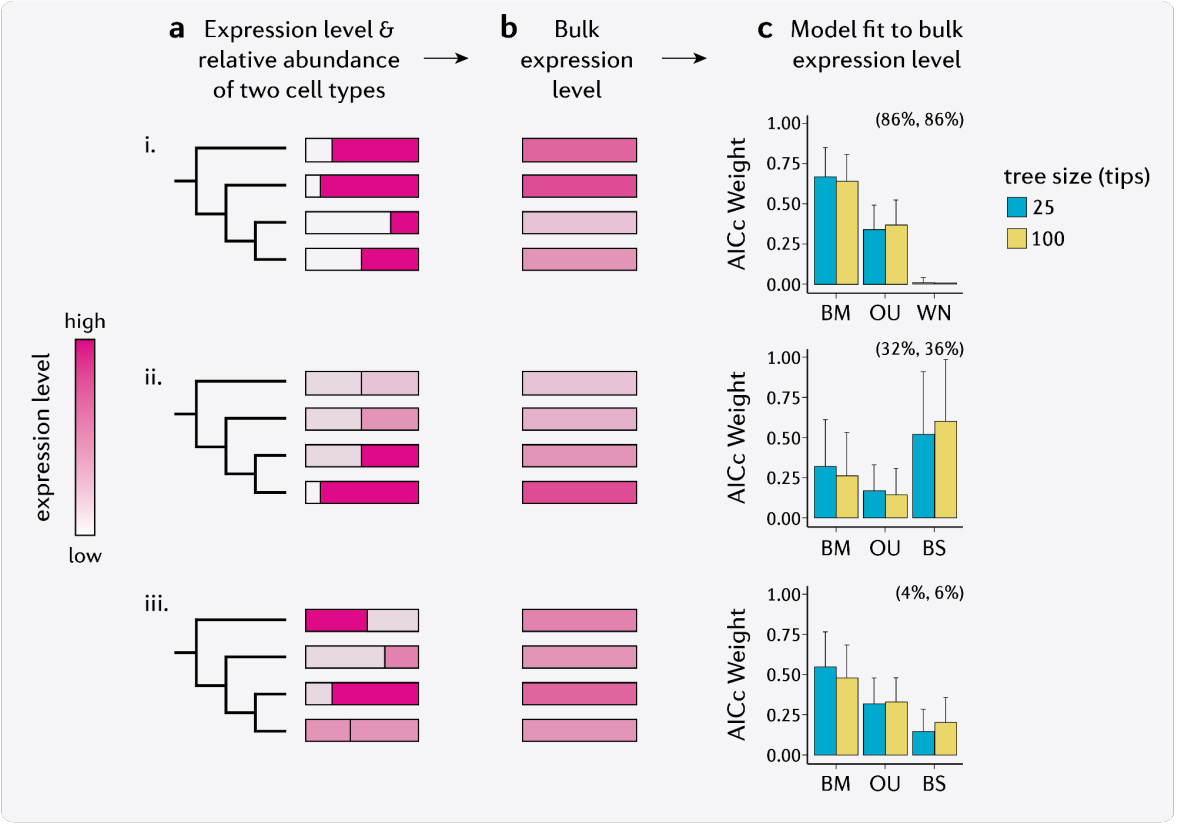

Figure 4. Inferring selection when expression level is measured from a heterogeneous tissue.
Three scenarios illustrating potential pitfalls of inferring regulatory selection at a single locus using phylogenetic approaches when expression is measured from bulk sequencing. **Panel (a)** The first column shows the expression level of a single gene in two different cell types across a phylogeny. High levels of expression are in dark pink and low expression in light pink. The relative proportion of each cell type is indicated by the size of the rectangle where cell type 1 is on the left and cell type 2 is on the right. **Panel (b)** This column shows the composite expression level of the gene as a function of cell type proportion and gene expression in each species. This would be analogous to measuring expression in bulk from a heterogeneous tissue. **Panel (c)** Results of simulated phylogenetic comparative analyses for each scenario with a phylogeny of 25 (blue) or 100 (yellow) tips on 1000 unique trees. Abbreviations of phylogenetic models are BM (Brownian motion), WN (White noise), OU (Ornstein-Uhlenbeck model) and BS (OU model with a branch shift). These models were fitted on the simulated bulk expression values and the relative support for each model is calculated using Akaike weights. Error bars show standard deviation around the mean across simulations. Shown in parenthesis (25 tips, 100 tips) are type 1 error rates for each scenario relative to when these models are fit to expression at the single-cell level. Full details: (https://github.com/Wright-lab-2021-Transcriptome-Evo/Inferring_expression_evolution_review). In scenario (i), expression values are static across the phylogeny for each cell type but cell type abundance is evolving under Brownian Motion (BM). However, phylogenetic approaches falsely infer that expression is evolving under BM. For (ii), expression in both cell types is evolving under BM, whereas tissue composition is stable across the phylogeny with the exception of one tip which has undergone an allometric shift. Here, phylogenetic approaches falsely infer an adaptive shift in expression on a single branch. For (iii), gene expression in both cell types, as well as cell-type abundance, is evolving under BM. However, phylogenetic approaches increasingly falsely infer stabilizing selection on expression evolution.

Second, we assume that gene expression is evolving under genetic drift. The two cell types are of equal abundance in all species with one exception in which a lineage-specific change in cellular composition occurs so that one cell type dominates (Fig. 4A, scenario ii). After model fitting, we find that this type of composition shift in one lineage leads to false inferences of a shift in gene regulation, consistent with adaptive evolution (Fig. 4B & C, scenario ii). The scale of this bias is highly dependent on the size of the allometric shift (Fig. 5A). Where the shift leads to a single cell type dominating, the actual mode of regulatory evolution (i.e. genetic drift), will be rejected in ~35% of instances. While this extreme situation is arguably biologically unrealistic, our simulations show that even marginal shifts in relative proportion result in elevated type 1 error rates. For example, across New World Blackbirds, the proportion of seminiferous tissue in the testes ranges from 87% to 96%[71]. This equates to a shift in the proportion of ~9%. Even though our simulations use different starting conditions, it is clear that shifts of a similar magnitude (e.g. 0.50 to 0.60 in Fig. 5A) can result in increased type 1 errors.

Finally, we simulated a scenario where gene expression and cell type abundance are both evolving under genetic drift (Fig. 4, scenario iii). Here, we are able to recover the true signal of genetic drift more reliably (Fig. 4C, scenario iii). However, in all instances so far, we have assumed that gene expression at a single locus is evolving independently in each cell type. While this is likely a reasonable assumption for some loci that have evolved tissue- or cell-specific regulatory machinery[78,79], expression changes are probably correlated in many instances. Interestingly, we find that this has implications for how regulatory evolution is inferred (Fig. 5B). When tissue composition evolves across the phylogeny, the type 1 error rate is highly dependent on the level of expression covariance between the cellular components of that tissue. In particular, if expression across cell types negatively covaries, where an increase in expression in one cell type is associated with a decrease in expression in another cell type at a single locus, the type 1 error rate can exceed 40% (Fig. 5B). The extent to which gene regulation is decoupled across cell types is, in and of itself, an interesting question. But here we have shown that gene expression covariation across cell types can also have profound implications for how we infer which selective processes are operating.

These scenarios demonstrate the potential challenges of inferring regulatory selection using expression data from heterogeneous tissues. It is also worth noting that our simulations are conservative as we do not model other non-evolutionary sources of variation (such as measurement error and tree topology

error) that are likely to be common in transcriptome studies. We believe this highlights an urgent need to reappraise our current understanding of regulatory evolution in the light of these underlying methodological issues. In particular, establishing (i) how often and by what magnitude changes in tissue composition occur and (ii) the extent to which regulatory variation is correlated across cell types are important prerequisites for studying expression evolution using phylogenetic comparative approaches with bulk RNA-seq. Unfortunately, we are not aware of a simple solution for correcting the biases we have uncovered, beyond recommending the use of single-cell data to study regulatory evolution where possible. However, while single-cell approaches are increasingly available, the technical demands of this approach means that they currently remain unfeasible for many species. In the meantime, we urge caution when using phylogenetic comparative approaches and recommend some steps to minimise other sources of error (BOX 2).

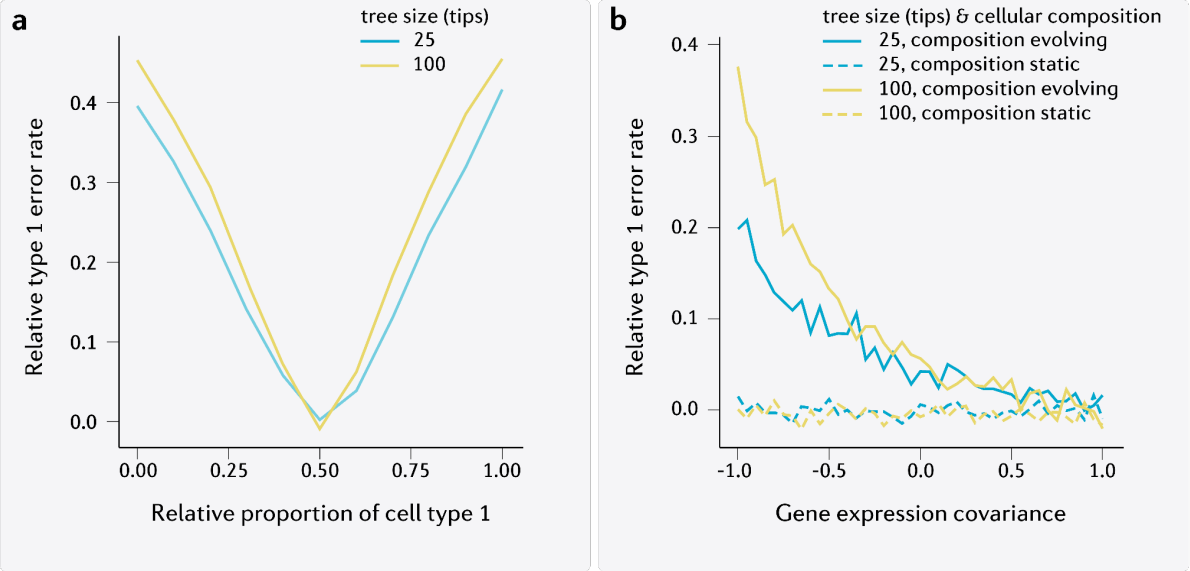

Figure 5. The magnitude of allometric shift and covariance of expression level biases the inference of regulatory evolution. Panel **(a)** The probability that regulatory selection is incorrectly inferred increases substantially with the magnitude of an allometric shift. This plot is a more detailed representation of Fig. 4 (scenario ii), where one species undergoes a shift in tissue composition, ranging from a scenario where a tissue is composed of two cell types at equal proportion to a scenario where only a single cell type is present. All other species have a tissue composition of 50:50 and expression is evolving under Brownian motion in each cell type. Panel **(b)** Covariance of expression between cell types biases inferences of selection. This plot is an extension of Fig. 4 (scenario iii). Expression is evolving under Brownian motion but cell type composition is either static (dotted lines) or also evolving under BM (solid lines). We varied the extent to which gene expression is correlated between cell types, ranging from negative covariance, where expression levels increase in one cell type at the same time as decreasing in the other cell type, to positive covariance, where expression levels decrease or increase in both cell types in a correlated manner. The relative type 1 error rate was calculated as the rate at which a BM model was not best fit to the composite expression value relative to the equivalent error rate when models are fit to single cell simulations.
Full details: (https://github.com/Wright-lab-2021-Transcriptome-Evo/Inferring_expression_evolution_review).

FUTURE DIRECTIONS

To date, studies of regulatory evolution have primarily focused on differences in gene expression level. However, regulatory variation is highly complex and multi-dimensional, and below we identify major, unanswered questions in disentangling how selection acts on the entire transcriptome. As we discuss, our understanding of gene regulatory evolution will make critical advances as we continue to link insights across layers of the genotype-to-phenotype map, developmental contexts, and evolutionary timescales, with organismal ecology as our foundation.

Transcriptional diversity and layers of gene regulation

Variation in splicing, whereby the same gene can express different RNA variants that produce distinct proteins or isoforms, are a common source of regulatory diversity across species[28,80–82] with important phenotypic effects (recently reviewed[83,84]). For genes with constraints on expression levels (e.g. because of pleiotropic effects) alternative splicing may act as another adaptive mechanism of gene regulation[85]. Long-read sequencing methods have the advantage of producing full-length transcript sequences[86], which can be a more reliable way to identify alternatively spliced variants in transcriptomic datasets. Understanding the evolution of gene regulation will ultimately require an integrated understanding of how and when differences in expression level and splicing contribute to phenotypes under selection.

For regulatory variation – whether in terms of expression level or alternative splicing – to be selected upon, it must contribute to variation at the protein layer of the genotype to phenotype map. Due to difficulties in assaying proteins in comparison to RNA, the links between transcription and translation are underexplored, particularly in non-model organisms. Recent methodological advances that measure rates of protein synthesis to assay the translatome, report a higher correlation between the translatome and proteome than between the transcriptome and proteome [87]. However, this effect tends to decrease in instances surrounding functionally relevant loci, such as differentially expressed genes[88]. This indicates that in many cases, mRNA abundance does not fully capture regulatory variation, and more work is needed to understand the complex relationship between transcription and translation (e.g. mechanisms of buffering, feedback, degradation)[5,89].

Regulatory and co-expression networks

The intrinsically correlated nature of gene expression means that identifying selection at a single locus is hard to disentangle from the expression patterns at loci with shared architectures. To account for this, we must either take on network-based approaches and try to account for connectivity or covariance between loci, or we must reduce the dimensionality of our data. Furthermore, recent work identifying key nodes in gene regulatory networks of health and disease phenotypes between sexes also established that genes that appear architecturally central to a phenotype may also not appear differentially expressed[90]. If this is common for evolutionary relevant loci, studying expression on a locus by locus basis and not through inter-locus interactions may limit our ability to understand the architectures underlying adaptive phenotypes.

Developmental context

Phenotypic variation is produced by dynamic developmental changes through space and time. While gene regulation is highly context-dependent in terms of tissue identity and developmental stage, studies primarily test for regulatory selection in a single snapshot, most often in adult tissues[1]. Single-cell transcriptomic methods offer a promising path to better understanding how these sources of variation interface with gene expression through development and inform models of gene expression evolution.

Genotype to phenotype to adaptation

If our goal is to uncover how gene regulation underlies adaptation, we must link regulatory variation with organismal ecology and natural history. This effort is twofold, as it requires understanding when and how selection acts on organisms, and how regulatory variation contributes to phenotypic responses to selection. Methods of surveying regulatory variation offer increasing precision and resolution. However, our ability to identify the evolutionary processes causing this regulatory variation ultimately depends on our understanding of the organisms in question. Model systems like yeast continue to enable high-throughput analyses that have yielded pivotal insights into regulatory evolution[3,91–94], but non-model systems also hold promise for studying regulatory evolution under natural settings which may yield novel and more ecologically relevant findings[63,95]. Furthermore, it remains to be seen how results from microevolutionary studies within or across a single generation integrate with those from

macroevolutionary studies comparing diverged lineages, and the relative roles of stabilizing versus directional selection across these scales.

ACKNOWLEDGEMENTS

This work was funded by a NERC Independent Research Fellowship to AEW (NE/N013948/1), a NERC Independent Research Fellowship to CRC (NE/T01105X/1), a grant from the European Research Council (grant agreement 680951) and a Canada 150 Research Chair to JEM, a NERC ACCE DTP to PDP, and an NSF Postdoctoral Research Fellowship and MSU Presidential Postdoctoral Fellowship to DHPD. We thank Marysia Placzek, Paula Escuer Pifarré, Emily Josephs, Adrian Platts, Miles Roberts, Rebecca Panko, Maya Wilson Brown, and Sophie Buysse for helpful comments and suggestions on the manuscript.

AUTHOR CONTRIBUTIONS

AEW, CRC, DHPD, PDP and JEM designed the review. DWK, ESP, AEW, CRC and PDP analyzed the data. AEW, CRC, DHPD, JAT, JEM and PDP wrote the manuscript with input from all authors.

DATA AND CODE AVAILABILITY STATEMENT

All data has been published previously[66] and all scripts are available at (https://github.com/Wright-lab-2021-Transcriptome-Evo/Inferring_expression_evolution_review).

BOX 1: Common pitfalls of inferring selection using Ornstein Uhlenbeck models

Recent work from the phylogenetic comparative methods field has revealed inherent biases in estimating OU processes, often leading to false inferences of stabilizing selection. As these have already been discussed elsewhere[11,12,96], we summarise the main pitfalls in relation to transcriptome studies.

Small phylogenetic samples

Recent work has shown that the ability to accurately estimate parameters of the OU model is strongly influenced by the number of species. Cooper *et al*[11] simulated a range of phylogenies of varying size under Brownian motion and compared the fit of BM and OU models to test how often stabilizing selection was falsely inferred. They found a high type 1 error rate, especially when the number of sampled taxa was limited. For example, with a phylogeny of 25 species, stabilizing selection was falsely inferred ~10% of the time. This is especially concerning for transcriptomic studies, which are frequently comprised of far fewer species due to sampling and computational costs and employ thousands of model comparisons in order to infer selection at each orthologous locus separately. We do anticipate this concern will lessen as expression data becomes available for more species. However, even with phylogenies of 100 species, Cooper *et al*[11] still estimate a type 1 error rate > 0.05.

Measurement error

Error in measuring traits across lineages can erode phylogenetic signal in the data, falsely biasing model selection away from BM models and towards OU processes and the inference of stabilizing selection[11,12]. Recent work has shown that even small amounts of measurement error can be problematic, particularly when the number of taxa sampled is small. For instance, Cooper *et al*[11] estimate that with a phylogeny of 25 species and a 10% trait measurement error, stabilizing selection will be falsely concluded ~50% of the time. This is a particular concern for gene expression studies, as the environment can strongly influence regulatory variation. Studies should endeavour to control environmental conditions so that regulatory variation across samples reflects the heritable, genetic component of expression, as has been discussed previously[4,97]. Second, it is clear that using a single mean expression value for each species can lead to spurious inferences of selection[13], making multiple replicates essential. Importantly, the OU framework has been extended to parameterise within-species variance as an error term[13,53,57] and appears to be a promising approach.

Complex patterns of trait evolution

Many phenotypic traits exhibit complex patterns of evolution and evolve at different rates across lineages[98]. While few studies have directly tested the tempo of expression change across species[55], it seems likely that gene regulation does not evolve at a constant rate but instead shifts as mutation rate, selective pressures and pleiotropic constraints[42,99,100] vary. However, many evolutionary models, including BM and OU, assume a homogeneous process of trait change across lineages and/or through time. This is analogous to fitting a fixed d_N/d_S across all branches when estimating selection on coding sequences. Recent work has shown that fitting single-process models masks complexity and leads to inaccurate inferences about the underlying evolutionary process[98]. Comparative methods that account for rate heterogeneity are available (discussed in[98]), analogous to allowing d_N/d_S to vary across branches, but to our knowledge have not been widely applied in the context of gene expression evolution.

BOX 2: Best practises for inferring selection in a comparative framework

Best practises for inferring selection on traits using comparative approaches have been discussed in length in the phylogenetic literature. Briefly, to avoid false inferences of stabilizing selection (BOX 1), studies should (i) strive to minimise measurement error, (ii) maximise the number of species sampled and (iii) use comparative approaches that parameterise within-species variance as an error term. Below, we discuss additional recommendations.

Validation of model fit

As discussed, many factors can bias model inference to conclude stabilizing selection over genetic drift. The best fitting model is often chosen by comparing the relative fit of different models. However, studies rarely examine the absolute model fit[98]. This simple step, performed using existing methods such as ARBUTUS[101] or in the probabilistic language RevBayes[102], can be used to assess confidence in model selection. This approach relies on the process of posterior predictive simulations, in which datasets are simulated on the estimated parameters, and then a series of test-statistics are run on the simulated data. Similarly, parametric bootstrapping approaches can be applied, resampling the data to generate a bootstrapped sampling distribution from which test statistics are calculated. These results can then be compared to the empirical data to assess the adequacy of the model. Using such approaches for model estimation has been shown to outperform maximum likelihood approaches in specific cases[103].

Consider tissue composition

By directly comparing regulatory variation across equivalent cell types, comparative single-cell transcriptomics (scRNA-seq) can circumvent problems arising when expression is measured from heterogeneous tissue (Fig. 1 & 3). However, scRNA-seq is not yet feasible for many non-model organisms as it is necessary to isolate and process single cells immediately after harvesting tissue. Although tissue dissociation and storage techniques are being developed, bulk transcriptomic approaches are currently the only feasible option for many species, particularly those sampled from the wild. Accepting these difficulties, we suggest that where possible, studies should quantify cellular composition of the tissue in question and how this varies across species. For instance, if a single cell type dominates or expression level is dominated by one cell type, then our simulations suggest that the potential for bias is reduced. Importantly, if scRNA-seq data is available for the tissue, it is possible to use this to directly test for biases in cellular composition in bulk RNA-seq data[77,104]. Finally, we urge the use of sampling techniques to directly isolate specific regions or cells of interest using microdissection or cell sorting to greatly reduce cell composition complications, as discussed by Hunnicutt *et al*[16].

GLOSSARY

Alternative splicing: a post-transcriptional modification involving the differential removal of introns, resulting in the production of multiple transcripts from a single gene.

Brownian motion (BM) model: a model of neutral evolution via unconstrained, random fluctuations in trait values.

Gene regulation: all pre and post-transcriptional mechanisms involved in controlling the level of gene expression.

Genome: the complete set of genetic elements encoded by the entire DNA sequence of an organism.

Ornstein-Uhlenbeck (OU) model: a modified Brownian Motion model of evolution with random fluctuations in trait values constrained towards a single optimum value.

Proteome: the set of proteins produced in a specific tissue or cell type at a particular time.

Ribo-Seq: a translatome profiling technique that involves sequencing transcripts bound and being actively translated by ribosomes.

Transcriptome: the set of RNA molecules produced by the genome in a specific tissue or cell type at a particular time.

Translatome: the set of mRNA molecules being actively translated in a specific tissue or cell type at a particular time. The regulation of the translatome determines the formation of the proteome.

REFERENCES:

1. Mank, J. E. The transcriptional architecture of phenotypic dimorphism. *Nat Ecol Evol* **1**, 6 (2017).
2. Parsch, J. & Ellegren, H. The evolutionary causes and consequences of sex-biased gene expression. *Nat. Rev. Genet.* **14**, 83–87 (2013).
3. Hill, M. S., Vande Zande, P. & Wittkopp, P. J. Molecular and evolutionary processes generating variation in gene expression. *Nat. Rev. Genet.* **22**, 203–215 (2021).
4. Romero, I. G., Ruvinsky, I. & Gilad, Y. Comparative studies of gene expression and the evolution of gene regulation. *Nat. Rev. Genet.* **13**, 505–516 (2012).
5. Signor, S. A. & Nuzhdin, S. V. The evolution of gene expression in cis and trans. *Trends Genet.* **34**, 532–544 (2018).
6. Fay, J. C. & Wittkopp, P. J. Evaluating the role of natural selection in the evolution of gene regulation. *Heredity* **100**, 191–199 (2008).
7. Khaitovich, P., Enard, W., Lachmann, M. & Pääbo, S. Evolution of primate gene expression. *Nat. Rev. Genet.* **7** 693–702 (2006).
8. Bedford, T. & Hartl, D. L. Optimization of gene expression by natural selection. *Proc. Natl. Acad. Sci. USA* **106**, 1133–1138 (2009).
9. Whitehead, A. & Crawford, D. L. Neutral and adaptive variation in gene expression. *Proc. Natl. Acad. Sci. USA* **103**, 5425–5430 (2006).
10. Hansen, T. F. Stabilizing selection and the comparative analysis of adaptation. *Evolution* **51**, 1341–1351 (1997).
11. Cooper, N., Thomas, G. H., Venditti, C., Meade, A. & Freckleton, R. P. A cautionary note on the use of Ornstein Uhlenbeck models in macroevolutionary studies. *Biol. J. Linn. Soc. Lond.* **118**, 64–77 (2016).
12. Silvestro, D., Kostikova, A., Litsios, G., Pearman, P. B. & Salamin, N. Measurement errors should always be incorporated in phylogenetic comparative analysis. *Methods Ecol. Evol.* **6**, 340–346 (2015).
13. Rohlfs, R. V., Harrigan, P. & Nielsen, R. Modeling gene expression evolution with an extended Ornstein–Uhlenbeck process accounting for within-species variation. *Mol. Biol.* **31**, 201-211 (2014).

14. Rohlfs, R. V. & Nielsen, R. Phylogenetic ANOVA: The Expression Variance and Evolution Model for Quantitative Trait Evolution. *Syst. Biol.* **64**, 695–708 (2015).
15. Montgomery, S. H. & Mank, J. E. Inferring regulatory change from gene expression: the confounding effects of tissue scaling. *Mol. Ecol.* **25**, 5114–5128 (2016).
16. Hunnicutt, K. E., Good, J. M. & Larson, E. L. Unraveling patterns of disrupted gene expression across a complex tissue. Preprint at *bioRxiv* https://doi.org/10.1101/2021.07.08.451646 (2021).
17. Nourmohammad, A. *et al.* Adaptive Evolution of Gene Expression in Drosophila. *Cell Rep.* **20**, 1385–1395 (2017).
18. Catalán, A., Briscoe, A. D. & Höhna, S. Drift and directional selection are the evolutionary forces driving gene expression divergence in eye and brain tissue of heliconius butterflies. *Genetics* **213**, 581–594 (2019).
19. Oleksiak, M. F., Churchill, G. A. & Crawford, D. L. Variation in gene expression within and among natural populations. *Nat. Genet.* **32**, 261–266 (2002).
20. Khaitovich, P. *et al.* A neutral model of transcriptome evolution. *PLoS Biol.* **2**, E132 (2004).
21. Rifkin, S. A., Kim, J. & White, K. P. Evolution of gene expression in the *Drosophila melanogaster* subgroup. *Nat. Genet.* **33**, 138–144 (2003).
22. Gilad, Y., Oshlack, A. & Rifkin, S. A. Natural selection on gene expression. *Trends Genet.* **22**, 456–461 (2006).
23. Lemos, B., Meiklejohn, C. D., Cáceres, M. & Hartl, D. L. Rates of divergence in gene expression profiles of primates, mice, and flies: stabilizing selection and variability among functional categories. *Evolution* **59**, 126–137 (2005).
24. Hudson, R. R., Kreitman, M. & Aguadé, M. A Test of neutral molecular evolution based on nucleotide data. *Genetics* **116**, 153–159 (1987).
25. Kimura, M. Genetic variability maintained in a finite population due to mutational production of neutral and nearly neutral isoalleles. *Genet. Res* **11** 247–270 (1968).
26. Staubach, F., Teschke, M., Voolstra, C. R., Wolf, J. B. W. & Tautz, D. A test of the neutral model of expression change in natural populations of house mouse subspecies. *Evolution* **64**, 549–560 (2010).
27. Somel, M. *et al.* Transcriptional neoteny in the human brain. *Proc. Natl. Acad. Sci. USA* **106**,

5743–5748 (2009).

28. Blekhman, R., Marioni, J. C., Zumbo, P., Stephens, M. & Gilad, Y. Sex-specific and lineage-specific alternative splicing in primates. *Genome Res.* **20**, 180–189 (2010).
29. Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S. & Mank, J. E. W chromosome expression responds to female-specific selection. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 8207–8211 (2012).
30. Gilad, Y., Oshlack, A., Smyth, G. K., Speed, T. P. & White, K. P. Expression profiling in primates reveals a rapid evolution of human transcription factors. *Nature* **440**, 242–245 (2006).
31. Enard, W. Intra- and Interspecific Variation in Primate Gene Expression Patterns. *Science* **296**, 340–343 (2002).
32. Blekhman, R., Oshlack, A., Chabot, A. E., Smyth, G. K. & Gilad, Y. Gene regulation in primates evolves under tissue-specific selection pressures. *PLoS Genet.* **4**, e1000271 (2008).
33. Warnefors, M. & Eyre-Walker, A. A selection index for gene expression evolution and its application to the divergence between humans and chimpanzees. *PLoS One* **7**, e34935 (2012).
34. Ometto, L., Shoemaker, D., Ross, K. G. & Keller, L. Evolution of gene expression in fire ants: the effects of developmental stage, caste, and species. *Mol. Biol. Evol.* **28**, 1381–1392 (2011).
35. Rifkin, S. A., Houle, D., Kim, J. & White, K. P. A mutation accumulation assay reveals a broad capacity for rapid evolution of gene expression. *Nature* **438**, 220–223 (2005).
36. Denver, D. R. *et al.* The transcriptional consequences of mutation and natural selection in Caenorhabditis elegans. *Nat. Genet.* **37**, 544–548 (2005).
37. Huang, W. *et al.* Spontaneous mutations and the origin and maintenance of quantitative genetic variation. *Elife* **5**, (2016).
38. Fraser, H. B. Detecting selection with a genetic cross. *Proc. Natl. Acad. Sci. USA* **117**, 22323–22330 (2020).
39. Leinonen, T., McCairns, R. J. S., O'Hara, R. B. & Merilä, J. Q(ST)-F(ST) comparisons: evolutionary and ecological insights from genomic heterogeneity. *Nat. Rev. Genet.* **14**, 179–190 (2013).
40. Mähler, N. *et al.* Gene co-expression network connectivity is an important determinant of selective constraint. *PLoS Genet.* **13**, e1006402 (2017).

41. Kohn, M. H., Shapiro, J. & Wu, C.-I. Decoupled differentiation of gene expression and coding sequence among *Drosophila* populations. *Genes Genet. Systems* **83**, 265–273 (2008).
42. Papakostas, S. *et al.* Gene pleiotropy constrains gene expression changes in fish adapted to different thermal conditions. *Nat. Commun.* **5**, 4071 (2014).
43. Leder, E. H. *et al.* The evolution and adaptive potential of transcriptional variation in sticklebacks--signatures of selection and widespread heritability. *Mol. Biol. Evol.* **32**, 674–689 (2015).
44. Blanc, J., Kremling, K. A. G., Buckler, E. & Josephs, E. B. Local adaptation contributes to gene expression divergence in maize. *G3* **11**, (2021).
45. Pujol, B., Wilson, A. J., Ross, R. I. C. & Pannell, J. R. Are QST-FST comparisons for natural populations meaningful? *Mol. Ecol.* **17**, 4782–4785 (2008).
46. Dunn, C. W., Zapata, F., Munro, C., Siebert, S. & Hejnol, A. Pairwise comparisons across species are problematic when analyzing functional genomic data. *Proc. Natl. Acad. Sci. USA* **115**, E409–E417 (2018).
47. Felsenstein, J. Phylogenies and the Comparative Method. *Am. Nat.* **125**, 1–15 (1985).
48. Pennell, M. W. & Harmon, L. J. An integrative view of phylogenetic comparative methods: connections to population genetics, community ecology, and paleobiology. *Ann. N. Y. Acad. Sci.* **1289**, 90–105 (2013).
49. Felsenstein, J. Maximum-likelihood estimation of evolutionary trees from continuous characters. *Am. J. Hum. Genet.* **25**, 471–492 (1973).
50. Oakley, T. H., Gu, Z., Abouheif, E., Patel, N. H. & Li, W.-H. Comparative methods for the analysis of gene-expression evolution: an example using yeast functional genomic data. *Mol. Biol. Evol.* **22**, 40–50 (2005).
51. Gu, X. Statistical framework for phylogenomic analysis of gene family expression profiles. *Genetics* **167**, 531–542 (2004).
52. Butler, M. A. & King, A. A. Phylogenetic Comparative Analysis: A Modeling Approach for Adaptive Evolution. *Am. Nat.* **164**, 683–695 (2004).
53. Brawand, D. *et al.* The evolution of gene expression levels in mammalian organs. *Nature* **478**, 343–348 (2011).
54. Kalinka, A. T. *et al.* Gene expression divergence recapitulates the developmental hourglass

model. *Nature* **468**, 811–814 (2010).

55. El Taher, A. *et al.* Gene expression dynamics during rapid organismal diversification in African cichlid fishes. *Nat. Ecol. Evol.* **5**, 243–250 (2021).
56. Chen, J. *et al.* A quantitative framework for characterizing the evolutionary history of mammalian gene expression. *Genome Res.* **29**, 53–63 (2019).
57. Pal, S., Oliver, B. & Przytycka, T. M. Modeling gene expression evolution with EvoGeneX uncovers differences in evolution of species, organs and sexes. Preprint at *bioRxiv* https://doi.org/10.1101/2020.01.06.895615 (2021).
58. Greenway, R. *et al.* Convergent evolution of conserved mitochondrial pathways underlies repeated adaptation to extreme environments. *Proc. Natl. Acad. Sci. USA* **117**, 16424–16430 (2020).
59. Vegesna, R. *et al.* Ampliconic Genes on the Great Ape Y Chromosomes: Rapid Evolution of Copy Number but Conservation of Expression Levels. *Genome Biol. Evol.* **12**, 842–859 (2020).
60. Gillard, G. B. *et al.* Comparative regulomics supports pervasive selection on gene dosage following whole genome duplication. *Genome Biol.* **22**, 103 (2021).
61. Kopania, E. E. K., Larson, E. L., Callahan, C. & Keeble, S. Molecular Evolution across Mouse Spermatogenesis. Preprint at *bioRxiv* https://doi.org/10.1101/2021.08.04.455131 (2021).
62. Groen, S. C. *et al.* The strength and pattern of natural selection on gene expression in rice. *Nature* **578**, 572–576 (2020).
63. Ahmad, F. *et al.* The strength and form of natural selection on transcript abundance in the wild. *Mol. Ecol.* **30**, 2724–2737 (2021).
64. Lande, R. & Arnold, S. J. The measurement of selection on correlated characters. *Evolution* **37**, 1210–1226 (1983).
65. Estermann, M. A. *et al.* Insights into Gonadal Sex Differentiation Provided by Single-Cell Transcriptomics in the Chicken Embryo. *Cell Rep.* **31**, 107491 (2020).
66. Kim, D. W. *et al.* Single-cell analysis of early chick hypothalamic development reveals that hypothalamic cells are induced from prethalamic-like progenitors. Preprint at *bioRxiv* https://doi.org/10.1101/2021.04.09.438683 (2021).
67. Niu, W. & Spradling, A. C. Two distinct pathways of pregranulosa cell differentiation support

follicle formation in the mouse ovary. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 20015–20026 (2020).

68. Witt, E., Benjamin, S., Svetec, N. & Zhao, L. Testis single-cell RNA-seq reveals the dynamics of de novo gene transcription and germline mutational bias in *Drosophila*. eLife **8**, e47138 (2019)
69. Hermann, B. P. *et al.* The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Rep.* **25**, 1650–1667.e8 (2018).
70. Green, C. D. *et al.* A Comprehensive Roadmap of Murine Spermatogenesis Defined by Single-Cell RNA-Seq. *Dev. Cell* **46**, 651–667.e10 (2018).
71. Lüpold, S., Linz, G. M., Rivers, J. W., Westneat, D. F. & Birkhead, T. R. Sperm competition selects beyond relative testes size in birds. *Evolution* **63**, 391–402 (2009).
72. Shami, A. N. *et al.* Single-Cell RNA Sequencing of human, macaque, and mouse testes uncovers conserved and divergent features of mammalian spermatogenesis. *Dev. Cell* **54**, 529–547.e12 (2020).
73. La Manno, G. *et al.* Molecular diversity of midbrain development in mouse, human, and stem cells. *Cell* **167**, 566–580.e19 (2016).
74. Tosches, M. A. *et al.* Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomics in reptiles. *Science* **360**, 881–888 (2018).
75. Bakken, T. E. *et al.* Evolution of cellular diversity in primary motor cortex of human, marmoset monkey, and mouse. Preprint at *bioRxiv* https://doi.org/10.1101/2020.03.31.016972 (2020).
76. Harrison, P. W. *et al.* Sexual selection drives evolution and rapid turnover of male gene expression. *Proc. Natl. Acad. Sci. USA* **112**, 4393–4398 (2015).
77. Bauernfeind, A. L. *et al.* Tempo and mode of gene expression evolution in the brain across Primates. Preprint at *bioRxiv* https://doi.org/10.1101/2021.04.21.440670 (2021).
78. Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A. & Carroll, S. B. Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in *Drosophila*. *Nature* **433**, 481–487 (2005).
79. Prud'homme, B. *et al.* Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. *Nature* **440**, 1050–1053 (2006).
80. Brown, J. B. *et al.* Diversity and dynamics of the *Drosophila* transcriptome. *Nature* **512**, 393–399 (2014).

81. Gibilisco, L., Zhou, Q., Mahajan, S. & Bachtrog, D. Alternative splicing within and between *drosophila* species, sexes, tissues, and developmental stages. *PLoS Genet.* **12**, e1006464 (2016).

82. Mazin, P. V., Khaitovich, P., Cardoso-Moreira, M. & Kaessmann, H. Alternative splicing during mammalian organ development. *Nat. Genet.* **53**, 925–934 (2021).

83. Gómez-Redondo, I., Planells, B., Navarrete, P. & Gutiérrez-Adán, A. Role of Alternative Splicing in Sex Determination in Vertebrates. *Sex Dev.* 1–11 (2021).

84. Singh, P. & Ahi, E. P. The importance of alternative splicing in adaptive evolution. Preprint at EcoEvoRxiv https://doi.org/10.32942/osf.io/wak9g (2021).

85. Rogers, T. F., Palmer, D. H. & Wright, A. E. Sex-Specific Selection Drives the Evolution of Alternative Splicing in Birds. *Mol. Biol. Evol.* **38**, 519–530 (2021).

86. Naftaly, A. S., Pau, S. & White, M. A. Long-read RNA sequencing reveals widespread sex-specific alternative splicing in threespine stickleback fish. *Genome Res.* **31**, 1486–1497 (2021).

87. Wang, Z.-Y. *et al.* Transcriptome and translatome co-evolution in mammals. *Nature* **588**, 642–647 (2020).

88. Koussounadis, A., Langdon, S. P., Um, I. H., Harrison, D. J. & Anne Smith, V. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci. Rep.* **5**, 10775 (2015).

89. Vogel, C. & Marcotte, E. M. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* **13**, 227–232 (2012).

90. Lopes-Ramos, C. M. *et al.* Sex differences in gene expression and regulatory networks across 29 human tissues. *Cell Rep.* **31**, 107795 (2020).

91. Metzger, B. P. H., Yuan, D. C., Gruber, J. D., Duveau, F. & Wittkopp, P. J. Selection on noise constrains variation in a eukaryotic promoter. *Nature* **521**, 344–347 (2015).

92. Metzger, B. P. H. *et al.* Contrasting frequencies and effects of cis- and trans-regulatory mutations affecting gene expression. *Mol. Biol. Evol.* **33**, 1131–1146 (2016).

93. Hodgins-Davis, A., Duveau, F., Walker, E. & Wittkopp, P. J. Empirical measures of mutational effects define neutral models of regulatory evolution in Saccharomyces cerevisiae. *Proc. Nat. Acad. Sci. USA* **116**, 21085-21093 (2019).

94. Vaishnav, E. D. *et al.* A comprehensive fitness landscape model reveals the evolutionary history and future evolvability of eukaryotic cis-regulatory DNA sequences. Preprint at bioRxiv https://doi.org/10.1101/2021.02.17.430503 (2021).

95. Josephson, M. P. & Bull, J. K. Innovative mark–recapture experiment shows patterns of selection on transcript abundance in the wild. *Mol. Ecol.* **30**, 2707–2709 (2021).

96. Ho, L. S. T. & Ané, C. Intrinsic inference difficulties for trait evolution with Ornstein-Uhlenbeck models. *Methods Ecol. Evol.* **5**, 1133–1146 (2014).

97. Harrison, P. W., Wright, A. E. & Mank, J. E. The evolution of gene expression and the transcriptome–phenotype relationship. *Semin. Cell Dev. Biol.* **23**, 222–229 (2012).

98. Chira, A. M. & Thomas, G. H. The impact of rate heterogeneity on inference of phylogenetic models of trait evolution. *J. Evol. Biol.* **29**, 2502–2518 (2016).

99. Allen, S. L., Bonduriansky, R. & Chenoweth, S. F. Genetic constraints on microevolutionary divergence of sex-biased gene expression. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **373**, (2018).

100. Dean, R. & Mank, J. E. Tissue Specificity and Sex-Specific Regulatory Variation Permit the Evolution of Sex-Biased Gene Expression. *Am. Nat.* **188**, E74–84 (2016).

101. Pennell, M. W., FitzJohn, R. G., Cornwell, W. K. & Harmon, L. J. Model Adequacy and the Macroevolution of Angiosperm Functional Traits. *Am. Nat.* **186**, E33–50 (2015).

102. Hohna, S. et al. Probabilistic graphical model representation in phylogenetics. *Syst. Biol.* **63**, 753–771 (2014).

103. Slater, G. J. & Pennell, M. W. Robust regression and posterior predictive simulation increase power to detect early bursts of trait evolution. *Syst. Biol.* **63**, 293–308 (2014).

104. Jew, B. *et al.* Accurate estimation of cell composition in bulk expression through robust integration of single-cell information. *Nat. Commun.* **11**, 1971 (2020).