# Factors influencing fertility in female birds

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*Female Indian peafowl (Pavo cristatus)*

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

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# Declaration

I, Katherine Assersohn, confirm that the work submitted is my own, except where work that has formed part of jointly authored publications has been included. My contribution and the contribution of the other authors of this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others. 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 been previously presented for an award at this, or any other, university.

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This is an additional paper produced during the course of my PhD in collaboration with researchers from my and another institution, which although separate from the main body of my thesis, is relevant to the broad field of research. This published manuscript is included in its full published form here with no alterations. All authors contributed equally to writing the manuscript; K.A. produced figures 1 & 2 and wrote the sections on pollution and fertilisation failure.

# General summary

Fertility is a key component of reproductive success and should be under strong directional selection. However, not all copulations result in successful fertilisation, and at least some degree of infertility is virtually universal across the animal kingdom. How fertility rates remain so consistently variable between individuals and populations is a fundamental question in evolutionary biology, but also has important implications for commercial breeding and conservation. In this thesis, I first perform a systematic review of the avian fertility literature, finding that female fertility is consistently understudied relative to male fertility in birds. I begin to address this gap by summarising the key physiological factors influencing female fertility. I identify several important gaps in knowledge including 1) wild and non-commercial species remain understudied; 2) fertilisation failure is often poorly defined and incorrectly measured in females; 3) there is a broad lack of understanding of the genetic basis of female infertility, and (4) there is little data on the variation in and mechanisms of female sperm storage and selection in birds. The remainder of the thesis focuses on addressing some of these gaps. I first explore how a Z-linked supergene influences female reproductive traits, finding that despite having large effects on male sperm traits, the supergene does not affect female reproductive traits. I then conduct two comparative studies on avian female sperm storage and selection, firstly taking a largely descriptive approach, followed by a phylogenetically controlled analysis exploring the dynamic relationships between post-copulatory sexual selection, vaginal and sperm storage tubule morphology, and sperm traits. I present evidence that not only is there a large amount of morphological variation in female sperm storage structures, but that female reproductive tract morphology, specifically vagina length, is associated with sexual selection intensity across species. These findings suggest that female post-copulatory reproductive traits may be an important (and underappreciated) agent of sexual selection in birds. I then collate the key findings from across this thesis into a unifying discussion, placing them in the context of their wider potential impacts on conservation science and commercial breeding and suggesting several important areas for future research. Overall, this thesis presents an important step in directing the future of this field into a new phase where female factors are taken into greater consideration when exploring the factors influencing fertility and post-copulatory sexual selection across species.

# Chapter 1:

# Introduction

*Female blackbird (Turdus merula)*

# 1 Background

The innate drive to maximise individual fitness is an evolutionary force common to all organisms. Since individuals that produce more offspring transmit their traits into future generations at a higher frequency than their less successful counterparts, we expect there to be strong selection on traits that maximise reproductive success. Despite this, rates of reproductive failure are often remarkably high and/or variable within and between species (Morrow et al., 2002). While some individuals reproduce prolifically, others may fail to ever reproduce in their lifetime, and some degree of reproductive failure is a universal feature across the animal kingdom (Clutton-Brock, 1988). Understanding the forces that shape variation in individual and population reproductive rates is a key challenge in evolutionary and conservation biology alike.

For individuals, even a single failed mating attempt can be costly. Failed reproductive attempts are particularly problematic in species where mating opportunities are likely to be limited, such as threatened populations with a small effective population size, those that experience few reproductive seasons, or those that only reproduce once in a lifetime (i.e. semelparity). Gametic wastage is also generally more costly for females, because egg production requires an energetic investment around three orders of magnitude greater than ejaculate production across species (Hayward and Gillooly, 2011). This is especially true for taxa that produce large yolky ova, like birds.

In some critically threatened bird populations, the loss of a single egg can be devastating for conservation management programmes, and the causes of such failure are often not identified (or identified correctly), making it challenging to appropriately mitigate the issue (Assersohn *et al.*, 2021b - Appendix 1; Savage *et al.*, 2022; Marshall *et al.*, 2023). For conservation management techniques to be as effective as possible, a comprehensive foundational understanding of the factors affecting reproductive success is needed, but this is lacking even in some of the most well-studied birds (Assersohn et al., 2021b; Appendix 1). For example, in domestic turkeys (*Meleagris gallopavo*), a commercially important species usually kept under optimal conditions and with strong selection for reproductive success, on average 15% of fertilised eggs fail to hatch, and the causes of such failure remain largely unexplained (Beaumont et al., 1997).

The IUCN predicts that 12% (one in eight species) of birds are threatened globally (BirdLife International, 2017; IUCN, 2023), and with hatching failure rates averaging 17% across all birds (Marshall et al., 2023), it is essential to improve our knowledge of both the physiological and genetic factors affecting avian fertility and embryo survival. This is especially true for female fertility-related traits, which have been historically understudied relative to males in the fields of reproductive biology and sexual selection (Ah-King, 2022; Assersohn et al., 2021a; Chapter 2).

## 2 Sexual selection and the role of females

Whilst in its infancy, sexual selection theory (Darwin, 1871) hadn't yet acknowledged that competition could occur post-mating (Ah-King, 2022), and the idea that females of any species had an active role in most reproductive processes was widely rejected (Tang-Martínez, 2016). Geoff Parker's theory of sperm competition, developed in 1970, lead to an expansion of sexual selection theory, whereby the concept of post-mating male-male competition was introduced for the first time (Parker, 1970). However, the equivalent female-centric theory – cryptic female choice – was not developed and widely accepted until years later (Eberhard, 1996; Thornhill, 1983). Research into sexual selection in males (including sperm competition) has progressed rapidly in the years since, yet our understanding of sexual selection in females has lagged consistently behind.

In the second chapter of this thesis, I show that the number of male-focused papers published to date on avian fertility far outnumber those in females today, and this gap is widening over time (Assersohn et al., 2021a; Chapter 2). More recently, a similar pattern of male-bias was found across the entire field of sexual selection research (Ah-King, 2022). While we cannot attribute with certainty the causes of this imbalance, male reproductive function is often more conspicuous (e.g., male external sexual characteristics), and more easily studied (e.g., sperm being often more readily obtained and manipulated than eggs) (Ah-King, 2022). Birds make a useful system for studying female reproductive function owing to the fact their large eggs are laid external to the mother's body, making them more amenable to experimental examination and manipulation than viviparous taxa like mammals. However, exploring the function of the female reproductive tract (particularly when

attempting to disentangle male and female effects) is still challenging owing to its cryptic nature within the female body (Firman et al., 2017).

Above and beyond the practical advantages of studying male relative to female reproduction, it is widely acknowledged that social context and the beliefs of wider society often influences current scientific thinking (Ah-King, 2022; Tang-Martínez, 2016). Darwin published *The Descent of Man and Selection in Relation to Sex* at a time where females were widely viewed as passive and coy participants in reproduction. These biases likely translated into the belief that sperm were the active participants in fertilisation, with eggs (and the female reproductive tract) remaining passive (Martin, 1991). These ideas were further supported by Bateman in 1948, following his seminal experiment in fruit flies and ensuing arguments that later became known as Bateman's principles (Bateman, 1948).

Bateman argued that because male reproductive success is limited mainly by the number of inseminations they can achieve, whereas females are primarily limited by the number of eggs they can produce, males have the potential to produce a greater number of offspring than females and thus have more variable reproductive success. He suggested that males should be indiscriminately promiscuous, while females should be passive and choosy, thereby cementing Darwin's intuition that sexual selection should act much more strongly in males (Bateman, 1948; Tang-Martínez, 2016). Bateman's ideas were initially largely overlooked until their revival by Trivers in 1972 (Trivers, 1972), after which point they became a paradigm in sexual selection theory. Despite receiving widespread criticism for both his original experimental setup and the analysis and conclusions made thereof (which have also been found to not be replicable) (Gowaty et al., 2013, 2012; Snyder and Gowaty, 2007; Tang-Martínez, 2016), Bateman's conclusions often remain at the forefront of attempts to explain differences in male and female reproductive behaviour (Tang-Martínez, 2016).

Current thinking is transitioning towards a greater appreciation for the role of female agency in reproductive processes: females are now known to exhibit often highly promiscuous and competitive behaviours, and be actively selective both pre- and post- copulation. The rise of molecular paternity analyses in the 1990s also led to the revelation that extra pair paternity, even among apparently monogamous species, is common (albeit variable within populations), and that sexual selection in females may be a ubiquitous phenomenon (Burke, 1989; Fromonteil et al., 2023; Pizzari and Wedell, 2013; Taylor et al., 2014). Furthermore, in a recent

meta-analysis of a broad range of taxa, female reproductive success was found to be positively correlated with mating success, indicating that females gain individual fitness benefits from multiple mating (Fromonteil et al., 2023). This suggests that polyandry is not just a consequence of high pressure from males to mate, but a female-driven sexually selected trait, with important consequences for individual, population, and species level fitness (Fromonteil et al., 2023; Taylor et al., 2014).

The increasing evidence that polyandrous females may bias fertilisation towards sperm from some males over others (i.e. cryptic female choice) further emphasises the degree of control females may have over reproductive outcomes, the processes of sexual selection and the coevolution of male and female sexual traits (Eberhard, 1996; Firman et al., 2017; Kustra and Alonzo, 2023). Despite these advances, the deficit of research focusing on females in the fields of sexual selection and reproductive science is a lingering issue. In Chapter 2 of this thesis, I identify that within avian reproductive science, we particularly lack knowledge of the genetic factors affecting female reproductive function, as well as the variation in and mechanisms of female sperm storage and selection and its relevance to female fertility (Figure 1) (Assersohn et al., 2021a).

## 3 The genetic basis of variation in reproductive success in female birds

The field of avian genomics is dominated by studies in the domestic chicken (*Gallus gallus*). Not only is the chicken one of the most valuable agricultural species globally but is a model for the study of embryology, and disease and immunology (Kraus, 2019). The chicken was the first avian species (and one of the first vertebrates) to have its genome sequenced (International Chicken Genome Sequencing Consortium, 2004), swiftly becoming a leading model for genomics research. Since then, with the advent of new, faster, and more cost-effective sequencing technologies, there has been a rapid expansion in the number of completed avian genomes, including at least one representative species from almost all bird families (Bravo et al., 2021; Feng et al., 2020; Kraus, 2019). Although it currently covers only 5% of total avian diversity (Bravo et al., 2021), the Bird 10,000 Genomes Project (B10K) aims

to expand coverage to include sequences from every extant bird species (China National GeneBank, 2016). This will be essential for improving our understanding of the phylogenetic relationships between modern birds and building the field of avian comparative genomics (Bravo et al., 2021; Feng et al., 2020).

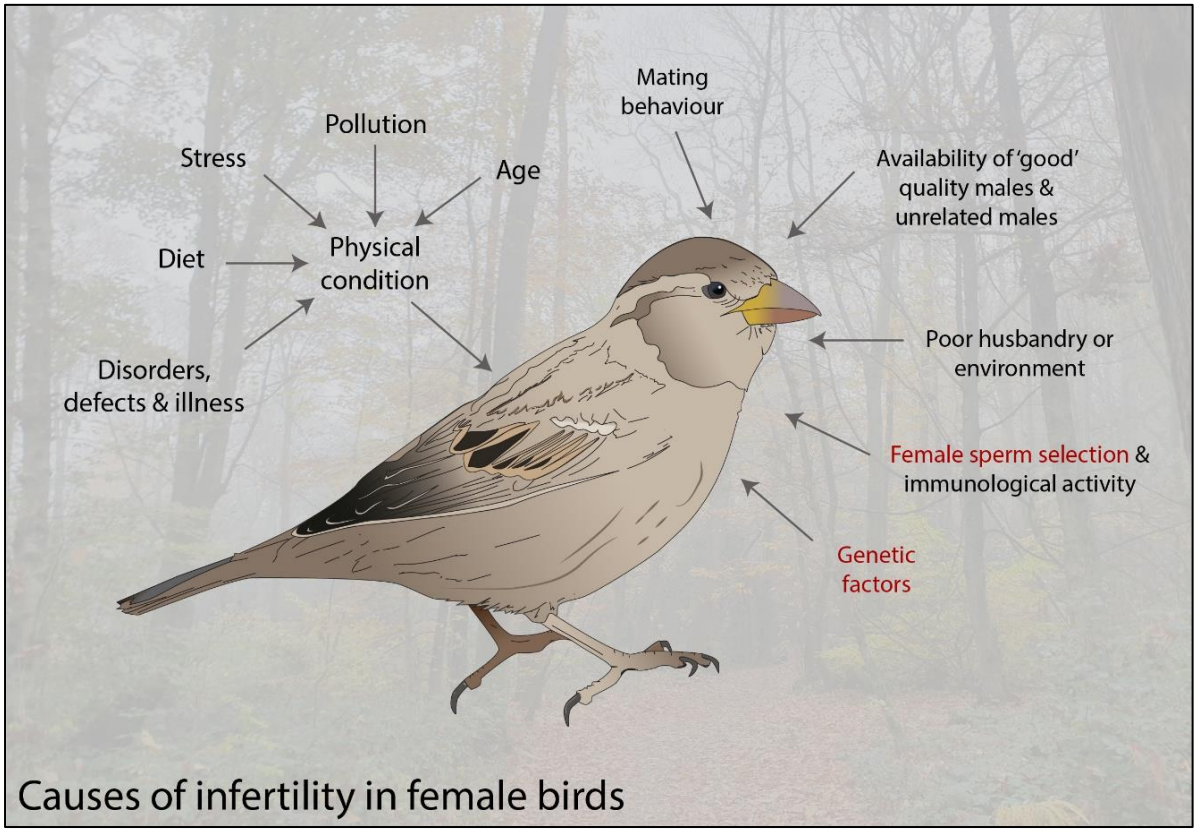


***Figure 1*** *Schematic summarising the primary causes of infertility in female birds identified in* Assersohn et al., (2021a) *(**Chapter 2**). Highlighted in red are fields thought to be particularly poorly understood (the genetic factors, and female sperm storage and selection), on which **Chapters 3, 4 and 5** of this thesis will focus. Drawn in Adobe Illustrator (version 28.3).*

Unsurprisingly, the bulk of our knowledge of the genetic architecture of reproductive traits in birds currently comes from studies in poultry, which have focused primarily on identifying and improving traits that act as indicators of laying performance (e.g., egg production, age at first egg, bodyweight at first egg, laying rate, etc.) (reviewed in Du et al., 2020). Despite these advances, research remains relatively diffuse, with a lack of a unifying and clear overarching understanding of reproductive genetics in birds. Furthermore, most research to date has focused on identifying genes important for reproduction with the practical goal of improving laying performance in poultry, while little attention has been paid to the ways in which these

genes might influence infertility and hatching failure in other species, especially those that suffer from low rates of reproductive success. Even among chickens, a more complete understanding of the factors affecting egg laying performance first requires the identification of a greater number of candidate genes (Du et al., 2020).

Reproductive failure is likely a complex polygenic trait, influenced by many genes, each of small effect, and likely with many linked and interacting genetic components (Du et al., 2020; Pei et al., 2020; Santure et al., 2013). For example, at least 31 genes have been identified as having significant associations with egg-laying performance in chickens, with several further candidate genes having already been identified through GWAS (Genome Wide Association Studies) (Du et al., 2020). In another GWAS in collared flycatchers, only a few significant QTLs for clutch size were identified (Husby et al., 2015), and in great tits (*Parus major)*, studies have found a positive association between chromosome size and genetic variation in clutch size and egg mass but detected no significant genome-wide quantitative trait loci (QTL), suggesting these traits are polygenic (Santure et al., 2015, 2013). In addition to individual/candidate genes (of which there are likely to be many, each of small effect) and their interactions, variation in reproductive outcomes may also be influenced by other non-mutually exclusive genetic factors such as selfish genetic elements (Lindholm et al., 2016; Price and Wedell, 2008), intra-locus sexual antagonism (Pei et al., 2020; Pennell and Morrow, 2013), frequency-dependent selection (Morrow et al., 2002), genetic incompatibilities (Tregenza and Wedell, 2000; Zeh et al., 1996), inbreeding (Charlesworth and Charlesworth, 1987), chromosomal mutations (such as inversions) (Giraldo-Deck et al., 2022), and gene x environment interactions (Armbruster and Reed, 2005).

It is currently difficult to draw broad conclusions about what is known or important for other avian species in terms of the genetic basis of traits involved in reproductive failure. This will become clearer as the number of species with their genomes sequenced continues to increase in the coming years, no doubt spurring further research including the identification of new candidate genes involved in reproductive processes. As is highlighted in Chapter 2, a more collaborative approach between poultry science and the wider field of evolutionary biology would aid progress.

In this thesis, I take a targeted approach to exploring the genetic basis of variation in reproductive success in birds, by building upon an existing body of work in a model songbird,

the zebra finch (*Taeniopygia guttata*). In this system, it is already known that a suite of linked genes (known as an inversion polymorphism, or supergene, see Chapter 3) have strong effects on male fertility, but the effects on females are not well understood. This system therefore presents an ideal opportunity to expand our knowledge of the genetic basis of variation in reproductive traits in a non-poultry species, whilst simultaneously addressing the disparity in knowledge between males and females in this field (Chapter 3).

## 4 Sperm storage and selection in female birds

In internally fertilising animals, successful fertilisation depends on the precise timing of the release of the ova with the arrival of sperm at the site of fertilisation. In birds, there is only a very short window between ovulation and the laying down of the outer perivitelline layer (approximately 15 minutes in chickens; Wishart and Staines, 1999) – which is impenetrable by sperm (Bakst et al., 1994; Hemmings and Birkhead, 2015; Wishart, 1997). To guard against the wastage of gametes, females must ensure sperm are available for fertilisation within that window at every ovulation. This can be achieved either by precise timing of insemination, or via the storage of sperm within the reproductive tract (Sasanami, 2017). Sperm storage allows for the decoupling of mating from fertilisation: sperm are stored and maintained within the reproductive tract long after insemination, and then released at the precise timing of ovulation (Bakst et al., 1994; Hemmings et al., 2015; Sasanami, 2017). The reproductive tract is a highly hostile environment for sperm, and so storage must occur in specialised sperm storage tubules (SSTs), without which sperm would not survive long enough to participate in fertilisation (Bakst, 2011; Das et al., 2008). Even with the presence of SSTs, only 1% of inseminated sperm make it into storage and reach the site of fertilisation (Bakst et al., 1994). Sperm storage not only guards against a lack of viable sperm for fertilisation, but provides females with the opportunity to selectively store/release a subset of sperm from preferred males, thereby providing a mechanism of cryptic female choice (Eberhard, 1996; Firman et al., 2017).

In birds, SSTs are often defined as ‘simple tubular invaginations’ (Sasanami, 2017), and have been most well studied in poultry, but rarely in non-commercial or wild species (Shankar et al., 2022). Even among commercially important species like chickens (*Gallus gallus domesticus*) and turkeys *(Meleagris gallopavo*), we still lack a comprehensive understanding

of the precise molecular and biochemical events occurring within SSTs during the acceptance, storage, and release of sperm (Khillare et al., 2018). Chickens and turkeys are also comparatively quite abnormal in their reproductive physiology (e.g., non-seasonal breeders, having undergone strong artificial selection for constant high productivity), making them a non-representative model for reproductive function for many other species. Despite this, there has been a general assumption that all birds have SSTs that look and function in the same way as those observed in poultry. There is already evidence that even among commercial birds, SSTs have large interspecific variation in storage capabilities. For example, sperm are stored for an average of 5-10 days in Japanese quail (*Coturnix* japonica), 2-3 weeks in domestic chickens, and 10-15 weeks in turkeys (Birkhead and Moller, 1990; Sasanami, 2017). The cause of this variation in sperm storage duration is unknown and rarely discussed in the context of SST diversity in birds.

Given that sperm are the most diverse cell types known (Pitnick et al., 2009), it stands to reason that the structures that receive and store them may also be diverse (Cramer et al., 2023). In a comparative study of 20 passerines, Briskie & Montgomerie (1993) found that sperm length was correlated positively with SST length and negatively with SST number, suggesting that the large variation in sperm size observed in these species is likely explained by the co-evolutionary dynamics of post-copulatory sexual selection. Correlations between sperm morphology and sperm storage organ morphology have been shown in other taxa as well (primarily invertebrates), with growing evidence that the female reproductive tract can act as a driver of evolution in sperm form and function (Higginson et al., 2012; Miller and Pitnick, 2002). We ultimately know little about the degree to which sperm storage tubules vary or how such variation might influence – or be influenced by – the trajectory of sperm/ejaculate and/or mating system evolution in birds. An important step in assessing these relationships more thoroughly – and for understanding the factors affecting female reproductive function across birds in general – will be to first identify and quantify the diversity of morphology and function in SSTs across a greater range of avian species, a goal I aim to begin addressing within this thesis (Chapters 4 & 5).

# 5 Thesis outline and aims

During my PhD, I aimed to improve our understanding of female reproductive function in birds, and the factors that shape variation in reproductive success across avian species. This thesis begins by summarising the primary physiological factors that can affect female fertility in birds. Chapter 2 (published) provides a systematic review that collates the extensive and often under-cited poultry science literature and combines it with our current knowledge across non-poultry species. I provide evidence that females and non-commercial bird species are understudied relative to males and commercially important birds, respectively, and identify several knowledge gaps that would benefit from future research. I identify that the genetic factors underpinning variation in female fertility, as well as female sperm storage physiology, remain particularly understudied in the field of avian fertility (Figure 1). The successive chapters are focused specifically on addressing these gaps, with Chapter 3 (published) focusing on a key knowledge gap in female reproductive genetics, exploring the effects of a sex-linked supergene on female fertility in a system already well studied in males. Chapters 4 (*in press*) and 5 then adopt a physiological perspective, describing and quantifying the degree of variation in sperm storage tubule structure and number across the Order Galliformes, and testing functional hypotheses to explain this variation. The findings of all four data chapters are then unified in a final discussion. In Appendix 1, I also present a review paper (Assersohn et al., 2021b) that I wrote and published during my PhD candidature, on which I am joint first author. While the work does not form part of my core PhD research, its focus on the causes of hatching failure in birds is relevant to the rest of the work presented in my thesis and has influenced my thinking across all chapters.

# 6 Study systems

## *6.1 The zebra finch*

The zebra finch (*Taeniopygia guttata*) is a small estrildid finch with an extensive distribution in Australia and Indonesia (Zann, 1996). They became known to science in the early 1800s, with the Australian zebra finch being first formally described in 1837 by the ornithologist John Gould (Zann, 1996). They quickly became popular cage birds as they took well to captivity and captive breeding. In addition to their hardy nature, and inexpensive and simple husbandry requirements, zebra finches have a quick generational turnover and breed opportunistically,

making them ideal for the study of reproductive physiology and behaviour in the lab (Zann, 1996). Furthermore, the zebra finch was the second avian species to have its genome sequenced (after domestic fowl) (Warren et al., 2010), cementing their place as one of the most popular animal models for an array of scientific disciplines (Hauber et al., 2021), with 5,831 published papers to date (between 1941 and 2024: from a Web of Knowledge search with "*Taeniopygia guttata"* or "*zebra finch*" in the topic or title).

Zebra finches make particularly good species for exploring the factors affecting reproductive performance in birds. This is in part due to the wealth of relevant knowledge and resources available, and the ease with which they are kept and bred in captivity, but also because their reproductive success is highly variable – and this is true of both captive and wild populations. In the wild, zebra finch hatching failure rate is reported to be >15%, whilst in the lab estimates range from 17-75% (Pei et al., 2020). Even in a species as well studied as the zebra finch, the causes of such high and often variable levels of hatching failure remain largely unexplained (Griffith et al., 2017).

### *6.2 Galliformes*

The order Galliformes are heavy-bodied ground feeding birds with wide ecological, economic, and cultural importance (see Chapter 5, Figure 1, for a phylogeny) (Wang et al., 2013). They consist of around 290 species, many of which are widely domesticated and form an important commercial food source (e.g., chicken, turkey and quail spp.) (Wang et al., 2013). Many species have also become important model systems across diverse disciplines, with poultry science forming an expansive and discrete discipline in and of itself. The red jungle fowl (*Gallus gallus*) was also the first avian species to have its genome sequenced (International Chicken Genome Sequencing Consortium, 2004), making them a model system with a wealth of well-developed genomics resources. Galliformes are extremely varied in physiology and breeding ecology, with a wide variety of mating systems (Wang et al., 2013). As such, they are an ideal system in which to explore the causes of interspecific variation in reproductive ability. In poultry, hatching rate varies considerably depending on breed, but on average, 15% of eggs fail to hatch in both chickens and turkeys (Beaumont et al., 1997; "Hatchability," 2017; Islam et al., 2002), despite considerable efforts to maximise egg production and fertilisation rates through intense artificial selection.

# 7 References

Ah-King, M., 2022. The history of sexual selection research provides insights as to why females are still understudied. Nat. Commun. 13, 6976. https://doi.org/10.1038/s41467-022-34770-z

Armbruster, P., Reed, D.H., 2005. Inbreeding depression in benign and stressful environments. Heredity 95, 235–242.

Assersohn, K., Brekke, P., Hemmings, N., 2021a. Physiological factors influencing female fertility in birds. R. Soc. Open Sci. 8, 202274. https://doi.org/10.1098/rsos.202274

Assersohn, K., Marshall, A.F., Morland, F., Brekke, P., Hemmings, N., 2021b. Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. Anim. Conserv. 1–12. https://doi.org/10.1111/acv.12674

Bakst, M.R., 2011. Role of the oviduct in maintaining sustained fertility in hens. J. Anim. Sci. 89, 1323–1329. https://doi.org/10.2527/jas.2010-3663

Bakst, M.R., Wishart, G., Brillard, J.-P., 1994. Oviducal sperm selection, transport, and storage in poultry. Poult. Sci. Rev. 5, 117–143.

Bateman, A.J., 1948. Intra-sexual selection in Drosophila. Heredity 2, 349–368. https://doi.org/10.1038/hdy.1948.21

Beaumont, C., Millet, N., Le Bihan-Duval, E., Kipi, A., Dupuy, V., 1997. Genetic parameters of survival to the different stages of embryonic death in laying hens. Poult. Sci. 76, 1193–1196. https://doi.org/10.1093/ps/76.9.1193

BirdLife International, 2017. More than one in eight of all bird species is threatened with global extinction.

Birkhead, T.R., Moller, A.P., 1990. Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study 45, 363–372. https://doi.org/doi.org/10.1111/j.1095-8312.1992.tb00649.x

Bravo, G.A., Schmitt, C.J., Edwards, S.V., 2021. What Have We Learned from the First 500 Avian Genomes? Annu. Rev. Ecol. Evol. Syst. 52, 611–639. https://doi.org/10.1146/annurev-ecolsys-012121-085928

Briskie, J.V., Montgomerie, R., 1993. Patterns of Sperm Storage in Relation to Sperm Competition in Passerine Birds. The Condor 95, 442–454. https://doi.org/10.2307/1369366

Burke, T., 1989. DNA fingerprinting and other methods for the study of mating success. Trends Ecol. Evol. 4, 139–144.

Charlesworth, D., Charlesworth, B., 1987. Inbreeding depression and its evolutionary consequences. Annu. Rev. Ecol. Syst. 18, 237–268.

China National GeneBank, 2016. B10K [WWW Document]. b10k.genomics. URL https://b10k.genomics.cn/index.html (accessed 1.31.24).

Clutton-Brock, T., 1988. Reproductive Success: Studies of Individual Variation in Contrasting Breeding Systems. University of Chicago Press.

Cramer, E.R.A., Yilma, Z.B., Lifjeld, J.T., 2023. Selection on sperm size in response to promiscuity and variation in female sperm storage organs. J. Evol. Biol. 36, 131–143. https://doi.org/10.1111/jeb.14120

Darwin, C., 1871. The descent of man, and selection in relation to sex. John Murray, London, UK.

Das, S.C., Isobe, N., Yoshimura, Y., 2008. Mechanism of prolonged sperm storage and sperm survivability in hen oviduct: A review. Am. J. Reprod. Immunol. 60, 477–481. https://doi.org/10.1111/j.1600-0897.2008.00651.x

Du, Y., Liu, L., He, Y., Dou, T., Jia, J., Ge, C., 2020. Endocrine and genetic factors affecting egg laying performance in chickens: a review. Br. Poult. Sci. 61, 538–549. https://doi.org/10.1080/00071668.2020.1758299

Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Feng, S., Stiller, J., Deng, Y., Armstrong, J., Fang, Q., Reeve, A.H., Xie, D., Chen, G., Guo, C., Faircloth, B.C., Petersen, B., Wang, Z., Zhou, Q., Diekhans, M., Chen, W., Andreu-Sánchez, S., Margaryan, A., Howard, J.T., Parent, C., Pacheco, G., Sinding, M.-H.S., Puetz, L., Cavill, E., Ribeiro, Â.M., Eckhart, L., Fjeldså, J., Hosner, P.A., Brumfield, R.T., Christidis, L., Bertelsen, M.F., Sicheritz-Ponten, T., Tietze, D.T., Robertson, B.C., Song, G., Borgia, G., Claramunt, S., Lovette, I.J., Cowen, S.J., Njoroge, P., Dumbacher, J.P., Ryder, O.A., Fuchs, J., Bunce, M., Burt, D.W., Cracraft, J., Meng, G., Hackett, S.J., Ryan, P.G., Jønsson, K.A., Jamieson, I.G., da Fonseca, R.R., Braun, E.L., Houde, P., Mirarab, S., Suh, A., Hansson, B., Ponnikas, S., Sigeman, H., Stervander, M., Frandsen, P.B., van der Zwan, H., van der Sluis, R., Visser, C., Balakrishnan, C.N., Clark, A.G., Fitzpatrick, J.W., Bowman, R., Chen, N., Cloutier, A., Sackton, T.B., Edwards, S.V., Foote, D.J., Shakya,

S.B., Sheldon, F.H., Vignal, A., Soares, A.E.R., Shapiro, B., González-Solís, J., Ferrer-Obiol, J., Rozas, J., Riutort, M., Tigano, A., Friesen, V., Dalén, L., Urrutia, A.O., Székely, T., Liu, Y., Campana, M.G., Corvelo, A., Fleischer, R.C., Rutherford, K.M., Gemmell, N.J., Dussex, N., Mouritsen, H., Thiele, N., Delmore, K., Liedvogel, M., Franke, A., Hoeppner, M.P., Krone, O., Fudickar, A.M., Milá, B., Ketterson, E.D., Fidler, A.E., Friis, G., Parody-Merino, Á.M., Battley, P.F., Cox, M.P., Lima, N.C.B., Prosdocimi, F., Parchman, T.L., Schlinger, B.A., Loiselle, B.A., Blake, J.G., Lim, H.C., Day, L.B., Fuxjager, M.J., Baldwin, M.W., Braun, M.J., Wirthlin, M., Dikow, R.B., Ryder, T.B., Camenisch, G., Keller, L.F., DaCosta, J.M., Hauber, M.E., Louder, M.I.M., Witt, C.C., McGuire, J.A., Mudge, J., Megna, L.C., Carling, M.D., Wang, B., Taylor, S.A., Del-Rio, G., Aleixo, A., Vasconcelos, A.T.R., Mello, C.V., Weir, J.T., Haussler, D., Li, Q., Yang, H., Wang, J., Lei, F., Rahbek, C., Gilbert, M.T.P., Graves, G.R., Jarvis, E.D., Paten, B., Zhang, G., 2020. Dense sampling of bird diversity increases power of comparative genomics. Nature 587, 252–257. https://doi.org/10.1038/s41586-020-2873-9

Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating Female Control: 20 Years of Cryptic Female Choice. Trends Ecol. Evol. 32, 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Fromonteil, S., Marie-Orleach, L., Winkler, L., Janicke, T., 2023. Sexual selection in females and the evolution of polyandry. PLOS Biol. 21, e3001916. https://doi.org/10.1371/journal.pbio.3001916

Giraldo-Deck, L.M., Loveland, J.L., Goymann, W., Tschirren, B., Burke, T., Kempenaers, B., Lank, D.B., Küpper, C., 2022. Intralocus conflicts associated with a supergene. Nat. Commun. 13, 1384. https://doi.org/10.1038/s41467-022-29033-w

Gowaty, P.A., Kim, Y.-K., Anderson, W.W., 2013. Mendel's law reveals fatal flaws in Bateman's 1948 study of mating and fitness. Fly (Austin) 7, 28–38. https://doi.org/10.4161/fly.23505

Gowaty, P.A., Kim, Y.-K., Anderson, W.W., 2012. No evidence of sexual selection in a repetition of Bateman's classic study of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 109, 11740–11745. https://doi.org/10.1073/pnas.1207851109

Griffith, S.C., Crino, O.L., Andrew, S.C., Nomano, F.Y., Adkins-Regan, E., Alonso-Alvarez, C., Bailey, I.E., Bittner, S.S., Bolton, P.E., Boner, W., Boogert, N., Boucaud, I.C.A., Briga, M., Buchanan, K.L., Caspers, B.A., Cichoń, M., Clayton, D.F., Derégnaucourt, S., Forstmeier,

W., Guillette, L.M., Hartley, I.R., Healy, S.D., Hill, D.L., Holveck, M.-J., Hurley, L.L., Ihle, M., Tobias Krause, E., Mainwaring, M.C., Marasco, V., Mariette, M.M., Martin-Wintle, M.S., McCowan, L.S.C., McMahon, M., Monaghan, P., Nager, R.G., Naguib, M., Nord, A., Potvin, D.A., Prior, N.H., Riebel, K., Romero-Haro, A.A., Royle, N.J., Rutkowska, J., Schuett, W., Swaddle, J.P., Tobler, M., Trompf, L., Varian-Ramos, C.W., Vignal, C., Villain, A.S., Williams, T.D., 2017. Variation in Reproductive Success Across Captive Populations: Methodological Differences, Potential Biases and Opportunities. Ethology 123, 1–29. https://doi.org/10.1111/eth.12576

Hatchability [WWW Document], 2017. . Hatchability.com. URL (accessed 1.30.24).

Hauber, M.E., Louder, M.I., Griffith, S.C., 2021. Neurogenomic insights into the behavioral and vocal development of the zebra finch. eLife 10, e61849. https://doi.org/10.7554/eLife.61849

Hayward, A., Gillooly, J.F., 2011. The cost of sex: Quantifying energetic investment in gamete production by males and females. PLOS One 6, 16557. https://doi.org/10.1371/journal.pone.0016557

Hemmings, N., Birkhead, T.R., 2015. Polyspermy in birds: sperm numbers and embryo survival. Proc. R. Soc. B Biol. Sci. 282, 20151682. https://doi.org/10.1098/rspb.2015.1682

Hemmings, N., Birkhead, T.R., Brillard, J.P., Froment, P., Briere, S., 2015. Timing associated with oviductal sperm storage and release after artificial insemination in domestic hens. Theriogenology 83, 1174-1178.e1. https://doi.org/10.1016/j.theriogenology.2014.12.022

Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. 109, 4538–4543. https://doi.org/10.1073/pnas.1111474109

Husby, A., Kawakami, T., Rönnegård, L., Smeds, L., Ellegren, H., Qvarnström, A., 2015. Genome-wide association mapping in a wild avian population identifies a link between genetic and phenotypic variation in a life-history trait. Proc. R. Soc. B Biol. Sci. 282, 20150156. https://doi.org/10.1098/rspb.2015.0156

International Chicken Genome Sequencing Consortium, 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432, 695–716. https://doi.org/10.1038/nature03154

Islam, M.S., Howlider, M.A.R., Kabir, F., Alam, J., 2002. Comparative assessment of fertility and hatchability of Barred Plymouth Rock, White Leghorn, Rhode Island Red and White Rock Hen. Int. J. Poult. Sci. 1, 85–90. https://doi.org/10.3923/ijps.2002.85.90

IUCN, 2023. IUCN Redlist Summary Statistics.

Khillare, G.S., Sastry, K.V.H., Agrawal, R., Prakash, A., Mohan, J., Singh, R.P., 2018. Molecular and biochemical events in sperm storage tubules of oviduct in birds. Worlds Poult. Sci. J. 74, 35–44. https://doi.org/10.1017/S004393391700085X

Kraus, R.H.S., 2019. Avian Genomics in Ecology and Evolution: From the Lab into the Wild, 1st ed. Springer International Publishing AG.

Kustra, M.C., Alonzo, S.H., 2023. The coevolutionary dynamics of cryptic female choice. Evol. Lett. 7, 191–202. https://doi.org/10.1093/evlett/qrad025

Lindholm, A.K., Dyer, K.A., Firman, R.C., Fishman, L., Forstmeier, W., Holman, L., Johannesson, H., Knief, U., Kokko, H., Larracuente, A.M., Manser, A., Montchamp-Moreau, C., Petrosyan, V.G., Pomiankowski, A., Presgraves, D.C., Safronova, L.D., Sutter, A., Unckless, R.L., Verspoor, R.L., Wedell, N., Wilkinson, G.S., Price, T.A.R., 2016. The Ecology and Evolutionary Dynamics of Meiotic Drive. Trends Ecol. Evol. 31, 315–326. https://doi.org/10.1016/j.tree.2016.02.001

Marshall, A.F., Balloux, F., Hemmings, N., Brekke, P., 2023. Systematic review of avian hatching failure and implications for conservation. Biol. Rev. 807–832. https://doi.org/10.1111/brv.12931

Martin, E., 1991. The egg and the sperm : How science has constructed a romance based on stereotypical male-female roles. Univ. Chic. Press J. 16, 485–501. https://doi.org/10.1086/494680

Miller, G.T., Pitnick, S., 2002. Sperm-Female Coevolution in *Drosophila*. Science 298, 1230–1233. https://doi.org/10.1126/science.1076968

Morrow, E.H., Arnqvist, G., Pitcher, T.E., 2002. The evolution of infertility: Does hatching rate in birds coevolve with female polyandry? J. Evol. Biol. 15, 702–709. https://doi.org/10.1046/j.1420-9101.2002.00445.x

Parker, G., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. Camb. Philos. Soc. 45, 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Pei, Y., Forstmeier, W., Wang, D., Martin, K., Rutkowska, J., Kempenaers, B., 2020. Proximate causes of infertility and embryo mortality in captive zebra finches. Am. Nat. 196, 577–596. https://doi.org/10.1086/710956

Pennell, T.M., Morrow, E.H., 2013. Two sexes, one genome: The evolutionary dynamics of intralocus sexual conflict. Ecol. Evol. 3, 1819–1834. https://doi.org/10.1002/ece3.540

Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm Biology: an evolutionary perspective. Academic Press, pp. 69–149.

Pizzari, T., Wedell, N., 2013. The polyandry revolution. Philos. Trans. R. Soc. B Biol. Sci. 368, 20120041. https://doi.org/10.1098/rstb.2012.0041

Price, T.A.R., Wedell, N., 2008. Selfish genetic elements and sexual selection: their impact on male fertility.

Santure, A.W., De Cauwer, I., Robinson, M.R., Poissant, J., Sheldon, B.C., Slate, J., 2013. Genomic dissection of variation in clutch size and egg mass in a wild great tit (*Parus major*) population. Mol. Ecol. 22, 3949–3962. https://doi.org/10.1111/mec.12376

Santure, A.W., Poissant, J., De Cauwer, I., Van Oers, K., Robinson, M.R., Quinn, J.L., Groenen, M.A.M., Visser, M.E., Sheldon, B.C., Slate, J., 2015. Replicated analysis of the genetic architecture of quantitative traits in two wild great tit populations. Mol. Ecol. 24, 6148–6162. https://doi.org/10.1111/mec.13452

Sasanami, T., 2017. Avian Reproduction: From Behaviour to Molecules. Springer Singapore. https://doi.org/10.1007/978-981-10-3975-1

Savage, J.L., Crane, J.M.S., Kākāpō Recovery Team, Hemmings, N., 2022. Low hatching success in the critically endangered kākāpō is driven by early embryo mortality not infertility. Anim. Conserv. 25, 352–360. https://doi.org/10.1111/acv.12746

Shankar, G., Gagan, T.A., Kumari, T.R.S., Marathe, G.K., 2022. Sperm storage by females across the animal phyla: A survey on the occurrence and biomolecules involved in sperm storage. J. Exp. Zool. PART B-Mol. Dev. Evol. https://doi.org/10.1002/jez.b.23189

Snyder, B.F., Gowaty, P.A., 2007. A reappraisal of Bateman's classic study of intrasexual selection. Evolution 61, 2457–2468. https://doi.org/10.1111/j.1558-5646.2007.00212.x

Tang-Martínez, Z., 2016. Rethinking Bateman's Principles: Challenging Persistent Myths of Sexually Reluctant Females and Promiscuous Males. J. Sex Res. 53, 532–559. https://doi.org/10.1080/00224499.2016.1150938

Taylor, M.L., Price, T.A.R., Wedell, N., 2014. Polyandry in nature: a global analysis. Trends Ecol. Evol. 29, 376–383. https://doi.org/10.1016/j.tree.2014.04.005

Thornhill, R., 1983. Cryptic Female Choice and Its Implications in the Scorpionfly Harpobittacus nigriceps. Am. Nat. 122, 765–788. https://doi.org/10.1086/284170

Tregenza, T., Wedell, N., 2000. Genetic compatibility, mate choice and patterns of parentage Invited Review. Mol. Ecol. 9, 1013–1027.

Trivers, R., 1972. Parental investment and sexual selection, in: Sexual Selection and the Descent of Man. Aldine, Chicago.

Wang, N., Kimball, R.T., Braun, E.L., Liang, B., Zhang, Z., 2013. Assessing Phylogenetic Relationships among Galliformes: A Multigene Phylogeny with Expanded Taxon Sampling in Phasianidae. PLoS ONE 8, e64312. https://doi.org/10.1371/journal.pone.0064312

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Künstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., Heger, A., Kong, L., Ponting, C.P., Jarvis, E.D., Mello, C.V., Minx, P., Lovell, P., Velho, T.A.F., Ferris, M., Balakrishnan, C.N., Sinha, S., Blatti, C., London, S.E., Li, Y., Lin, Y.-C., George, J., Sweedler, J., Southey, B., Gunaratne, P., Watson, M., Nam, K., Backström, N., Smeds, L., Nabholz, B., Itoh, Y., Whitney, O., Pfenning, A.R., Howard, J., Völker, M., Skinner, B.M., Griffin, D.K., Ye, L., McLaren, W.M., Flicek, P., Quesada, V., Velasco, G., Lopez-Otin, C., Puente, X.S., Olender, T., Lancet, D., Smit, A.F.A., Hubley, R., Konkel, M.K., Walker, J.A., Batzer, M.A., Gu, W., Pollock, D.D., Chen, L., Cheng, Z., Eichler, E.E., Stapley, J., Slate, J., Ekblom, R., Birkhead, T., Burke, T., Burt, D., Scharff, C., Adam, I., Richard, H., Sultan, M., Soldatov, A., Lehrach, H., Edwards, S.V., Yang, S.-P., Li, X., Graves, T., Fulton, L., Nelson, J., Chinwalla, A., Hou, S., Mardis, E.R., Wilson, R.K., 2010. The genome of a songbird. Nature 464, 757–762. https://doi.org/10.1038/nature08819

Wishart, G., 1997. Quantitative aspects of sperm:egg interaction in chickens and turkeys. Anim. Reprod. Sci. https://doi.org/10.1016/S0378-4320(97)00042-0

Wishart, G., Staines, H., 1999. Measuring sperm:egg interaction to assess breeding efficiency in chickens and turkeys. Poult. Sci. 78, 428–436. https://doi.org/10.1093/ps/78.3.428

Zann, R.A., 1996. The Zebra Finch: A Synthesis of Field and Laboratory Studies, C.M. Perrins. ed, Oxford Ornithology Series. Oxford University Press.

Zeh, J.A., Zeh, D.W., Zeh, W., 1996. The evolution of polyandry I : intragenomic conflict and genetic incompatibility. Proceeding B R. Soc. 263, 1711–1717.

# Chapter 2:

## Physiological factors influencing female fertility in birds

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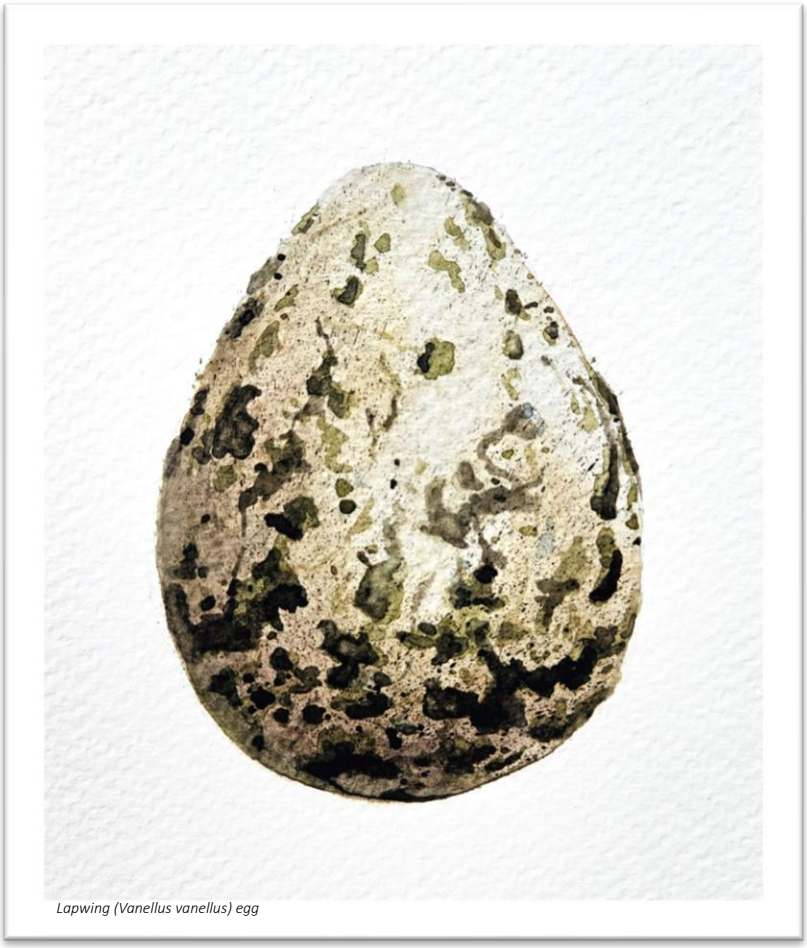
*Supplementary material associated with this publication is provided in* ***Appendix 2****. This can also be found here: https://royalsocietypublishing.org/doi/suppl/10.1098/rsos.202274*



*Lapwing (Vanellus vanellus) egg*

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Review

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# Physiological factors influencing female fertility in birds

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Fertility is fundamental to reproductive success, but not all copulation attempts result in a fertilized embryo. Fertilization failure is especially costly for females, but we still lack a clear understanding of the causes of variation in female fertility across taxa. Birds make a useful model system for fertility research, partly because their large eggs are easily studied outside of the female's body, but also because of the wealth of data available on the reproductive productivity of commercial birds. Here, we review the factors contributing to female infertility in birds, providing evidence that female fertility traits are understudied relative to male fertility traits, and that avian fertility research has been dominated by studies focused on Galliformes and captive (relative to wild) populations. We then discuss the key stages of the female reproductive cycle where fertility may be compromised, and make recommendations for future research. We particularly emphasize that studies must differentiate between infertility and embryo mortality as causes of hatching failure, and that non-breeding individuals should be monitored more routinely where possible. This review lays the groundwork for developing a clearer understanding of the causes of female infertility, with important consequences for multiple fields including reproductive science, conservation and commercial breeding.

## 1. Introduction

Fertility is fundamental to reproductive success, and so we should expect fertility traits to be under strong selection to maximize reproductive output and minimize the wastage of gamete investment [1,2]. Despite this, fertility varies remarkably across individuals, species and populations [3,4], and some degree of infertility is ubiquitous across taxa. Gametic wastage is likely to be more costly for females than males [4], since they typically invest

considerably more in gamete production [5]. These costs are also likely to be greater in taxa that produce large yolky ova, such as birds. Despite this, females are thought to have received comparatively less attention than males within avian fertility research [6], especially in poultry research, where attempts to increase fertility have historically been focused on male performance with far less attention given to females [7,8].

Successful fertilization occurs when a male pronucleus and a female pronucleus fuse to form a zygote (i.e. syngamy) [9]. We therefore define infertility as the failure of syngamy, and any male or female process contributing to failed syngamy is a cause of infertility. Confusingly, the term infertility has been used interchangeably in the literature to describe both fertilization failure and embryo mortality across taxa, though these two processes often have a very different mechanistic basis [10]. Differentiating between infertility and embryo mortality is often difficult, particularly if the embryo dies during the very early stages of development [11], but the failure to distinguish between them presents an important barrier to addressing the underlying causes of reproductive failure.

Birds are well suited to the study of reproductive failure, primarily because—unlike mammals—they produce large, well-protected eggs which make them easy to examine externally before they degrade [11]. It is also possible to precisely determine whether a bird's egg failed because it was unfertilized or because the embryo died very early, using microscopic methods, but many studies still fail to make the distinction [10,12,13]. Avian reproduction science also benefits from a wealth of knowledge from commercial poultry research. Despite intensive, long-term selection for consistent and efficient egg production in certain lines of commercially important species such as the domestic fowl (*Gallus gallus domesticus)* and turkey (*Meleagris gallopavo)*, hatching failure is still a pervasive issue in many commercial breeds, and the reasons for this are not fully understood [11,14]. In the wild, hatching failure is also ubiquitous; on average 10% of eggs never hatch [2], and in some threatened and bottlenecked species more than 60–70% of eggs fail [15,16]. While there has been some attention paid to embryo mortality in birds, we still lack a clear understanding of the incidence of true infertility and the factors that contribute towards it [17]. The incidence of infertility relative to embryo mortality in wild populations has most likely been overestimated by many studies [11], while in captive birds, infertility may be more likely [10]. Understanding the mechanisms that cause infertility could therefore be particularly important for captive breeding programmes.

Here, we provide a thorough review of female-specific physiological factors that lead to infertility in birds. We have consolidated the most valuable insights from across a broad range of literature, including behavioural ecology, evolutionary biology and reproductive physiology, integrating these with key findings from the vast but often under-cited poultry science literature. We reveal that there has been a deficit of fertility research in wild and non-commercial species, and that female fertility traits are consistently understudied relative to those of males. We then identify and explore key phases in the female reproductive cycle where fertility may be compromised, and broadly categorize the physiological mechanisms of infertility into five key processes: (i) failure to produce fertilizable eggs, (ii) failure during ovulation, (iii) failure to obtain sufficient sperm, (iv) failure to store and transport sperm, and (v) failure of fertilization. We draw particular attention to the relationships between senescence, environmental factors and female reproductive function. Our aim is to develop a clearer understanding of the proximate causes of variation in female fertility and highlight key directions for future research.

## 2. How much do we know about female fertility traits in birds?

We conducted a systematic search of the avian fertility literature (see electronic supplementary material for methods), identifying 718 relevant papers on avian fertility traits, of which 42% considered both male and female fertility, 37% focused on male fertility only and 20% focused on female fertility only. As expected, the number of avian fertility papers published each year is increasing, but since 1985, the number of published papers that focus on male fertility have increased at a faster rate than the number of papers focused on female fertility (figure 1*a*) ($x^2 = 15$, d.f. = 2, $p < 0.001$). By April 2020, published papers focusing on male avian fertility outnumbered those on female avian fertility by a factor of 1.84, indicating that there is a deficit of papers focusing on females (compared with males) within avian fertility research, a gap that appears to be widening over time. However, studies that considered fertility traits in both males and females were almost as numerous as those that considered males only, perhaps indicating that many researchers are taking a more holistic approach. This may reflect the inherent difficulties involved in disentangling the effects of male and female factors on fertilization success, since they are likely to be non-independent processes exhibiting complex interactions [18]. Across all years (from 1921 to 2020), 79% of articles exclusively investigated captive populations, with only 16% investigating wild populations and 5% investigating both captive and

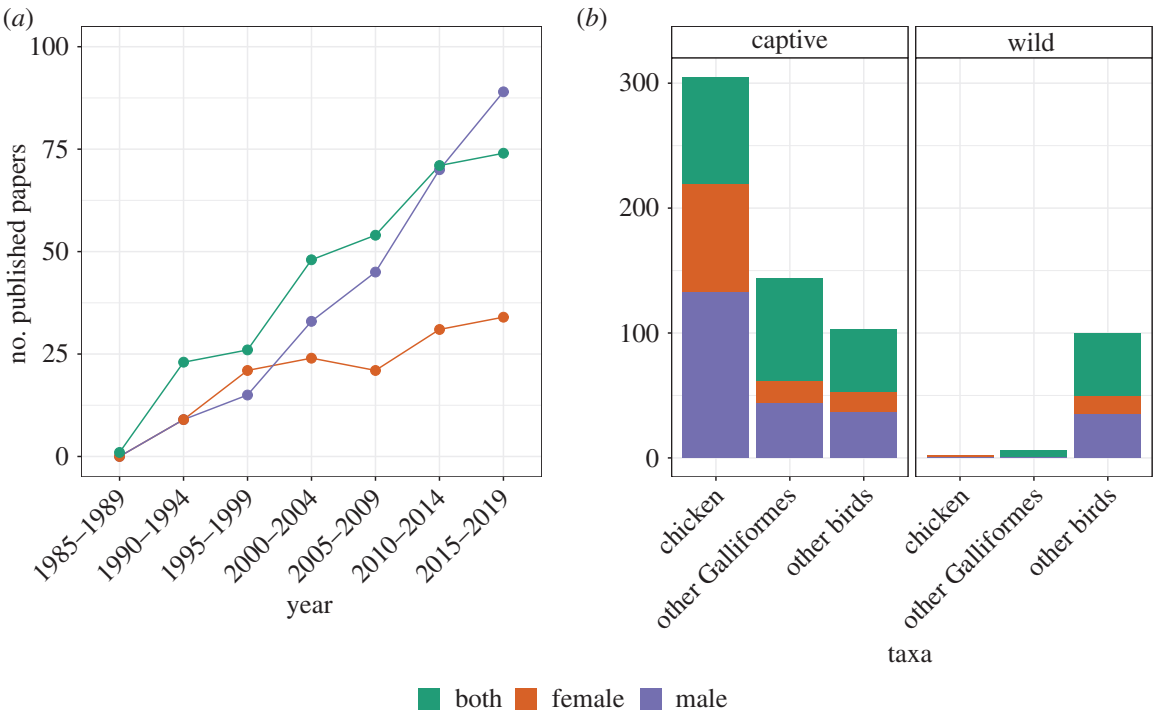


**Figure 1.** (*a*) The number of papers on avian fertility published between 1985 and 2019 on males only (purple), females only (orange) and both males and females (green). (*b*) The number of papers on avian fertility published between 1921 and 2020 (all years) either focusing exclusively on the domestic chicken (*Gallus gallus domesticus*), other Galliformes, or other (non-Galliform) bird orders, for both captive (left) and wild (right) populations.

wild populations. Furthermore, of the captive species studied, 80% focused on the order Galliformes, with 54% focused exclusively on a single species: the domestic chicken (*Gallus gallus domesticus*). This indicates that the avian fertility literature has also been heavily dominated by studies on gallinaceous birds of commercial importance, with far fewer studies investigating non-commercial and wild species. The greater focus towards male fertility appears to be a consistent pattern across both captive and wild populations (figure 1*b*). Within wild populations, only 15% of papers focus on females relative to 32% that focus on males. Within captive populations, the greater focus on male fertility is not solely driven by studies in poultry, since it is consistent even when domestic fowl are excluded, in which case only 14% of studies focus on females relative to 33% that focus on males.

The deficit of female-only avian fertility papers may reflect that, relative to ova, it is more practical to study sperm traits, partly because it is easier to collect sperm in a way that is non-invasive and repeatable. In birds, sperm biotechnology has also been developed considerably in poultry over the last century [19], which has advanced the ways in which sperm can be examined, preserved and manipulated. There may also be a degree of positive feedback, with ease of collection and study of sperm yielding greater advances in methodology, which in turn yields further research. Beyond the practical advantages to studying sperm relative to ova, the over-representation of studies on male fertility may also be a consequence of the historical view that sperm are the 'active' participants in fertilization: seeking, binding to and penetrating the somewhat 'passive' egg [20]. Cultural biases have also been suggested to drive a male-orientated research focus across other taxa including mammals [21]. While this view has been challenged in recent years (especially with regard to post-copulatory processes such as cryptic female choice [22]), the gap between the number of male and female fertility papers (both for wild and captive populations) suggests that female fertility traits in birds are still understudied, and the role of the female in determining reproductive success is therefore underappreciated. The following sections explore the physiological mechanisms that may contribute to variation in female fertility in birds, and suggest new hypotheses and future directions that may help fill the gaps in our current understanding of avian female fertility.

# 3. What causes infertility in female birds?

## 3.1 Failure during egg formation

In birds, infertility is typically measured as the number of unfertilized eggs, but this makes the assumption that a female is already able to produce an ovum that can be fertilized. Female fertility is

the product of not only fertilization rate, but also the number of eggs produced that are capable of being fertilized. The process of egg formation—from follicular development through to release of the ovum during ovulation—is a metabolically demanding process [23,24] and problems occurring during these early stages of reproduction are a costly and important cause of infertility. The incidence of infertility resulting from egg production problems is unknown for most wild bird populations, making it difficult to determine how important it is as a driver of individual variation in fitness. Regardless, determining the incidence of egg production dysfunction is a logical step towards establishing the causes of reproductive failure. Future studies should (where possible) attempt to collect data on failed breeding attempts (i.e. where copulation was successful, but no eggs were produced), particularly when the fertility status of the male breeding partner is known. This is likely to be somewhat easier for captive populations relative to long-term wild study populations, where data are not routinely collected on non-breeding individuals. Much of the avian fertility literature has a heavy focus on seasonally breeding species, where photoperiod provides a reliable cue eliciting an annual reproductive response. Generally, less is known about tropical/a-seasonal species and opportunistic breeders, where an individual's ability to respond rapidly to more unpredictable environmental cues is likely to significantly influence their fertility.

Reproductively active females of most bird species have only one functional oviduct in which there is usually just one ovary (figure 2). The right oviduct regresses during development, and this is thought to occur via hormonally controlled apoptosis (caused by the release of anti-Müllerian hormone), while the left oviduct is protected from regression by elevated concentrations of oestrogen (which inhibits the anti-Müllerian hormone receptor) [25], although the molecular mechanisms underpinning this process are not yet fully defined. The mature avian ovary contains multiple maturing follicles each at a different stage of development [26] (labelled $F_1$–$F_5$ in figure 2). Mature follicles consist of a large, protein rich yolky oocyte and a small germinal disc (which contains the genetic material), surrounded by a granulosa cell layer, multicellular theca layer and an epithelial layer (figure 3) [27,28]. At the vegetal pole of the follicle, the epithelial layer becomes thin, forming a region known as the stigma that acts as the point of follicle rupture during ovulation [28]. During the later stages of follicular growth, a glycoprotein structure known as the perivitelline layer forms between the granulosa cells and the oocyte (figure 3). The perivitelline layer functions to bind with sperm during fertilization and initiate the acrosome reaction [29].

In birds, the mechanisms underpinning follicular development, maintenance and selection have yet to be well defined, although the implications of these processes are likely to be significant for fertility [30]. Follicular selection (i.e. the selection of one white follicle to rapidly uptake yolk, undergo further differentiation and eventually ovulate as a mature yellow follicle) is thought to be mediated by cyclic adenosine monophosphate (cAMP) signalling, which acts through G protein-coupled receptors to upregulate the expression of multiple genetic factors important for follicular development [25,30–32]. The unselected white follicles are maintained in an undifferentiated/arrested yet viable state within the ovary until the next follicular selection [28]. This is thought to be regulated in part by the β-arrestin protein, which desensitizes G protein-coupled receptors (and thus inhibits cAMP signalling), and depresses granulosa cell differentiation [30]. Understanding the mechanisms governing follicular recruitment and maintenance is considered a primary challenge in avian reproductive research [28,33].

### 3.1.1 Hormonal factors

The proper functioning of the avian endocrine system is vital for egg production. In seasonally breeding species, photoperiodic cues are received by deep brain photoreceptors that stimulate activity of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis is a tightly regulated system that, among other things, regulates physiological processes associated with reproduction [28]. Specifically, following an increase in photoperiod, the mediobasal hypothalamus is stimulated to produce local thyroid hormone which regulates the release of gonadotrophin releasing hormone. This in turn stimulates the pituitary to produce gonadotropins that initiate seasonal gonadal growth and activity [28,34]. Following breeding, the HPG axis is promptly 'switched off', resulting in a significant regression of the gonads [28]. In male Japanese quail (*Coturnix japonica*), lesions of the mediobasal hypothalamus can inhibit the photoperiodic response and gonadal growth [35], but whether such lesions affect seasonal gonad development in females is unclear.

The degree to which endocrine disorders naturally affect wild birds is largely unknown, but disorders such as cystic hyperplasia, cystic ovaries and hypothyroidism are a clinical issue in captive birds [36–40]. There is also extensive experimental evidence showing that hormonal disruption can significantly

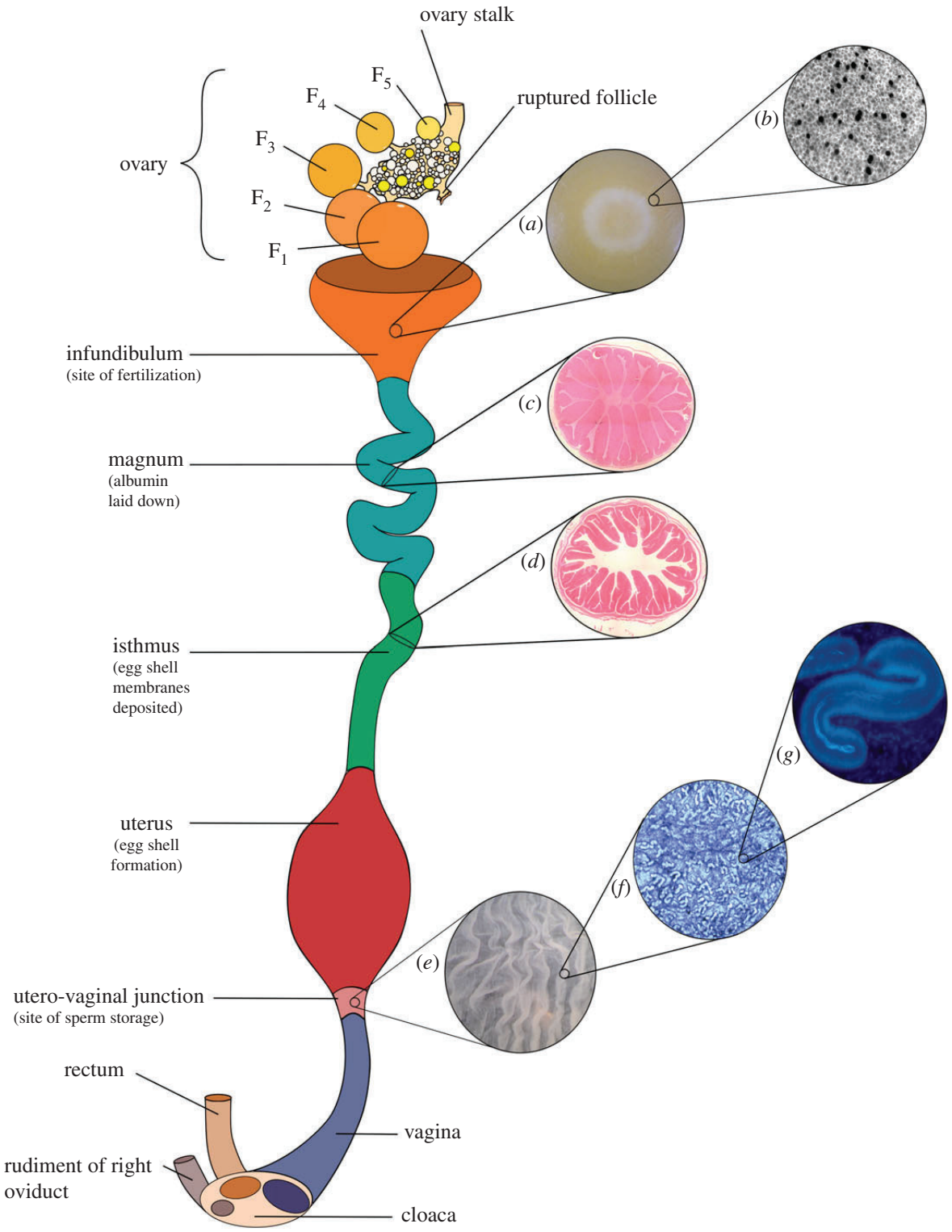


**Figure 2.** Schematic of the avian oviduct and ovary (not to scale). Note that the avian ovary consists of multiple follicles at different stages of development. The largest yellow follicles are labelled $F_{1–5}$ where $F_1$ is the largest follicle and will be the next to rupture. (*a*) The germinal disc of a fertilized ovum (from a zebra finch (*Taeniopygia guttata*)). Note the clear outer ring and paler center of the germinal disc which indicates embryonic development. (*b*) Sperm penetration holes visible on the inner perivitelline layer (IPVL) of an ovum after fertilization (from a bullfinch (*Pyrrhula pyrrhula*)). (*c*) A cross section of the magnum (from a helmeted guineafowl (*Numida meleagris*)). Sperm is transported through the magnum prior to fertilization, but this region functions mainly to produce the albumin which is laid down during egg development. (*d*) A cross section of the isthmus (from Reeves pheasant (*Syrmaticus reevesii*)). Sperm is also transported through the isthmus prior to fertilization, but this region functions mainly to produce and deposit shell membranes during egg development. (*e*) The internal tissue lining and folds of the vagina and utero-vaginal junction region (from a bobwhite quail (*Colinus virginianus*)). The vagina is considered the primary site of sperm selection in the oviduct, and the utero-vaginal junction functions as the primary site of sperm storage, containing numerous sperm storage tubules. (*f*) A single fold of the utero-vaginal junction (from a zebra finch (*Taeniopygia guttata*)) stained with Hoechst 33342 dye under a fluorescence microscope. The many small tubular structures are sperm storage tubules. (*g*) A single sperm storage tubule (and visible trapped sperm) from a single fold of the utero-vaginal junction (from a Japanese quail (*Coturnix japonica*)), stained with Hoechst 33342 dye and viewed under a fluorescence microscope. Image credits: (*a–d*) Nicola Hemmings; (*e–g*) Paul Richards.

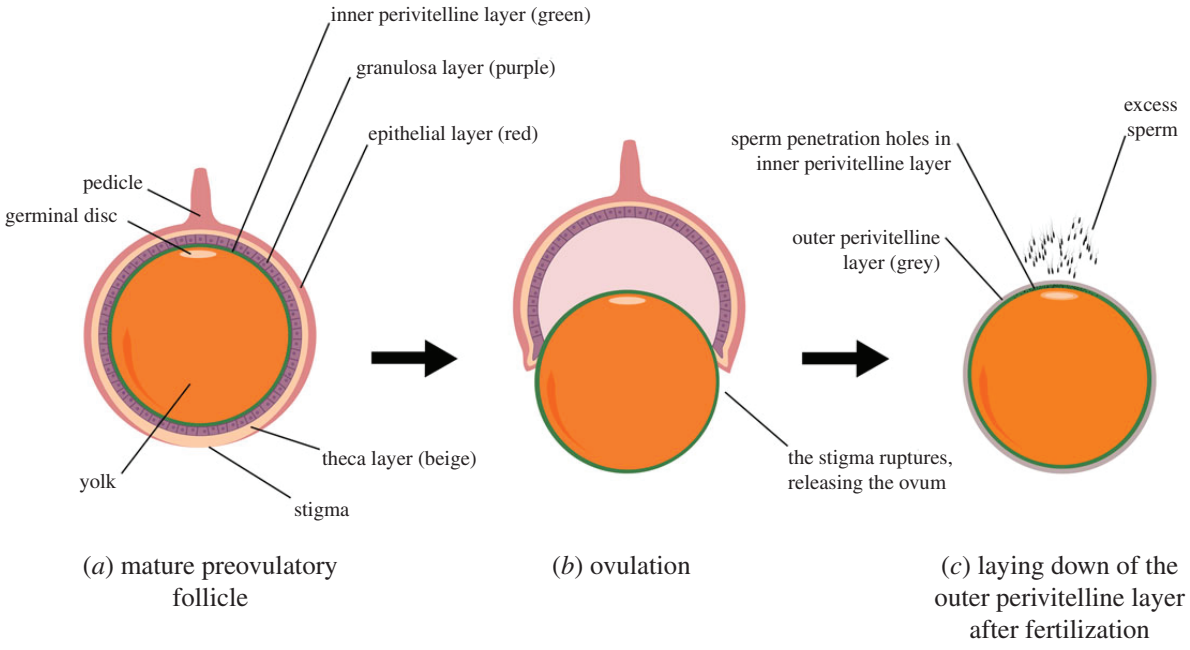


**Figure 3.** Schematic of a mature avian follicle. (*a*) An avian follicle prior to ovulation. (*b*) The ovum and follicle during ovulation, whereby a mature follicle ruptures at the stigma region, releasing the ovum. Sperm present in the infundibulum will begin to move towards the ovum in preparation for fertilization, where they will penetrate the IPVL (green). (*c*) The ovum after fertilization, the outer perivitelline layer (OPVL) (grey) has been laid down (which blocks further sperm entry). The IPVL (green) has an abundance of sperm penetration holes around the germinal disc region where sperm have penetrated during fertilization (figure 2*b*).

influence egg production in captive species. For example, in domestic fowl, administering luteinizing hormone 8.5 h after ovulation causes follicular degeneration (atresia) of the next follicle within the follicular hierarchy [41], and in pigeons (*Columba livia)*, administering synthetic gonadotropin-releasing hormone can reduce luteinizing hormone concentrations, depressing egg production [42]. Treatment with the inhibin A protein can increase the proliferation of granulosa cells and increase the secretion of granulosa steroid production, while decreased expression of the inhibin $\alpha$ subunit (which has been observed in cystic follicles in pigs [43]) is associated with follicle atresia in chickens [32]. Counterintuitively, injections of follicle stimulating hormone can decrease egg production in zebra finches (*Taeniopygia guttata*), possibly because of a negative feedback effect with endogenous secretion of follicle stimulating hormone [44], although timing of treatment is also likely to be important since the function of follicle stimulating hormone is known to vary with follicle size under normal conditions [28]. Inappropriate levels of the anti-Müllerian hormone have been shown to disrupt normal reproductive development in some species [45,46], and experimentally inhibiting oestrogen synthesis in chicken embryos increases the expression of the anti-Müllerian hormone receptor, resulting in masculinization of the reproductive tract [47]. The anti-Müllerian hormone is thought to play a vital role in follicular development within the ovary, and elevated levels have been associated with periods of restricted fertility in hens [48]. Elucidating the full significance of the anti-Müllerian hormone for avian female fertility is an active area of research [33,48].

### 3.1.2 Disease and immune factors

The proper functioning of the immune system is of great importance in the defence against bacterial, fungal and viral pathogens within the ovary. Toll-like receptors (TLRs) produced in the follicular tissue of domestic fowl are known to be involved in the recognition of pathogens, and play a key role in inducing an innate immune response in the ovary [28]. In particular, TLRs respond to pathogenic stimuli by producing avian β-defensins (antimicrobial peptides), and proinflammatory cytokines and chemokines [28,49]. Other cellular members involved in the innate immune response include macrophages, natural killer cells and antimicrobial peptides [28]. TLR signalling may also cause disruption to steroidogenesis, and result in apoptosis of undifferentiated granulosa cells, thereby providing a mechanism to prevent the selection of infected follicles into the preovulatory hierarchy [50]. The adaptive immune response then involves the migration of certain immunocompetent cells

into the follicles, including major histocompatibility complex (MHC) antigen-presenting cells, T cells, B cells and macrophages [28]. The distribution of immunocompetent cells in the oviduct increases during sexual maturation, but then decreases significantly thereafter with age [51]. In humans, ovarian autoimmunity has been associated with premature ovarian failure and infertility [52]. Less is known about the incidence and mechanisms of ovarian autoimmunity in birds (particularly wild populations), but antibodies that target ovarian tissue have been identified and been associated with a decline in egg production with age in laying hens [51,53], and autoimmune thyroiditis is a clinical issue associated with obesity and fertility declines in chickens [40]. The frequency of immunocompetent cells present in the follicles of the hen ovary also decreases with age, suggesting a reduction in infection resistance in older hens that could have an associated impact on egg production [54]. The follicular reserve also depletes as birds age [55], inevitably resulting in changes to the HPG axis. This may occur via reduced secretion of gonadal steroids and peptides and/or reduced sensitivity of the hypothalamus to ovarian steroids, either because of diminished steroid stimulation or a general pattern of neural senescence [55]. Oestrogen has been associated with the upregulation of immunocompetent cells into maturing follicles, and so may be involved in the age-related decline of the immune response in the ovary [28,51,54].

In humans, ovarian disorders such as hormonal dysfunction, ovarian abnormalities (such as polycystic ovarian disease), premature menopause and genetic defects [56], explain 30% of female infertility cases. Although less well studied in birds, ovarian disorders are known to reduce or stop egg production in poultry [57], and cystic ovarian disease is common in other bird species (e.g. cockatiels (*Nymphicus hollandicus*), budgerigars (*Melopsittacus undulatus*) and pheasants (*Phasianus colchicus*) [37]) [38]. Microbes present in the intestines and cloaca may be transported to—and colonize—the ovaries [28]. Inflammation of the oviduct (such as salpingitis or metritis) caused by bacterial or viral infection can result in oviductal impaction, egg abnormalities and infertility [38]. Little is known about the incidence of such ovarian disorders, or the degree to which they explain variation in female fertility in wild populations.

### 3.1.3. Environmental factors

#### 3.1.3.1 Diet

A wealth of experimental evidence in birds (mostly poultry) shows that diet strongly influences egg production and fertility. Striking the balance between optimum nutrient uptake (to maximize production) and nutrient toxicity and/or obesity is of primary concern to the poultry industry [58]. Modern broiler breeder hens are particularly sensitive to overfeeding during the weeks prior to laying, and even minor overfeeding can result in oviducal inflammation, prolapse and a reduction in egg production [59]. When broiler breeder hens are fed ad libitum, this can result in obesity and the onset of erratic oviposition and defective egg syndrome (EODES), which is thought to be caused by excessive follicle development and the occurrence of multiple follicular hierarchies which disrupts ovulation [59]. Yolk formation is energetically demanding and requires substantial changes in the body's metabolism of lipids [60]. Lipogenesis is responsive to both hormonal control as well as dietary changes, and overfed hens exhibit symptoms of lypotoxicity including ovarian abnormalities and follicular atresia [60,61]. Feed restriction is a commonly used method for controlling obesity and the onset of EODES [59,60]. Over the last few decades, intense selection for greater body mass has resulted in an increase in food consumption during ad libitum feeding. Consequently, there has been an increase in the use and intensity of feed restriction regimes in broilers [58,60,61]. It is becoming increasingly difficult for poultry breeders to achieve a diet sufficient for growth and reproductive maintenance without overfeeding. Restricted feeding protocols also come with additional welfare issues, namely an increase in stress and social aggressiveness related to hunger [62].

Nutrients thought to be important for egg production and fertility in birds include: manganese, selenium, iodine, fluoride, sodium, zinc, copper, vitamin A, vitamin E, vitamin $B_{12}$, protein and linoleic acid [58,63–68]. An excess or deficiency in any of these can be disruptive. Nutritional deficiency and toxicity are common in captive birds but thought to be more rare in wild populations [63], although the dietary requirements and nutrient availability for wild populations are less well studied and may be impacted by environmental change and/or supplemental feeding. In particular, the nutritional needs of endangered populations with reduced natural habitat may be restricted, especially if they have been translocated to habitats with different food sources to those in their native range (but see Jamieson [63]).

#### 3.1.3.2 Stress

It has long been known that stress also plays a vital role in the productivity of laying hens, and stressors may include fear (either of humans [69] or of novel social or physical environments [70]), insufficient space [71] and heat stress [72]. Heat stress reduces egg production by decreasing feed intake and causing nutritional deficiencies, but also by causing widespread disruption to the hormones important for ovulation [72]. In birds, temperatures above 30°C can trigger heat stress [73], and Deng *et al*. [74] found that when laying hens were exposed to 34°C heat for two weeks, egg production decreased by 28.8%. Increasing environmental temperatures predicted under climate change is expected to have important repercussions for commercial egg production [73,75]. Evidence for thermally induced female fertility loss in non-commercial species is lacking [76], although in male zebra finches exposed to 30°C and 40°C heat there was an increase in the production of abnormal sperm [77]. Regarding wild populations, it is likely that small, isolated or endemic species are particularly vulnerable to heat stress-induced reductions in fertility, because lack of gene flow and genetic variation impose limitations on their ability to adapt to novel environmental stress. Species with limited ranges may also be at risk if they are unable to shift to cooler climates [76].

Early-life/developmental conditions and stress may also heavily influence individual patterns of reproductive ageing. For example, rates of reproductive senescence are higher in guillemots that invest more heavily in early-life reproduction [78]; the effects of early-life stress (in the form of predation pressure) increases the rate of reproductive senescence in barn swallows (*Hirundo rustica*) [79]; and female collared flycatchers (*Ficedula ablbicollis*) from a low-competition natal environment experience higher reproductive rates early in life, but with a cost of earlier reproductive senescence [80]. In seasonally reproducing wild birds, older females with previous breeding experience often (initially) lay earlier in the season, and lay larger clutches than inexperienced females [81–83]. This has been attributed to females having prior experience responding to photostimulation, resulting in more robust photo-induced reproductive development, including having higher circulating levels of reproductive hormones and greater seasonal increases in follicle size than in photo-naive birds [81,82]. Initial increases in reproductive output with age are often followed by gradual declines in fertility with age across species [83]. In the endangered and bottlenecked whooping crane (*Grus Americana*)—known for suffering from fertility problems—female age is a key predictor of egg fertility, where younger females have a greater probability of producing fertile eggs [84]. Fertility senescence can be very variable across species, however, and while some short-lived species (such as poultry) experience rapid declines in female fertility with age, many long-lived birds apparently experience little or even no reproductive decline throughout their lives [83,85]. We do not fully understand the mechanisms of fertility senescence in birds [79]; more long-term longitudinal studies on age-related changes in fertility traits are necessary, especially in wild (non-poultry) species and where environmental/developmental effects are incorporated [78,80,83,86].

#### 3.1.3.3 Pollution

Exposure of wild birds to environmental pollutants has been shown to have a significant impact on fertility [87]. Over 90 000 anthropogenic chemicals are estimated to have been released into the environment, and several hundred of these pollutants are confirmed to act as endocrine-disrupting compounds (EDCs; although the majority remain untested for their effects on wildlife) [88,89]. If passed on to developing embryos, EDCs can disrupt reproductive development and cause sterility [90]. Negative effects on reproductive success in birds have been observed even when they are exposed to very low and environmentally relevant concentrations of certain EDCs. For example, small amounts of crude oil are sufficient to depress egg yolk formation in seabirds [91,92]; exposure to flame-retardant additives at concentrations typically seen in the environment can depress reproductive success (including fertility) in American kestrels (*Falco sparverius*) [93], and in areas polluted with environmental oestrogens, severe reproductive tract abnormalities have been found in exposed females [92]. Toxic heavy metals are also known to act as EDCs and disrupt reproduction in exposed birds: a single dose of cadmium was enough to significantly reduce egg production in Japanese quail [94]; lead is known to accumulate in the ovaries of pheasants [95], Japanese quail [96] and chickens [97] following exposure (which can reduce egg production and cause histopathological damage and developmental delays in the ovaries); and exposure to mercury (even at very low concentrations) can significantly reduce reproductive success in zebra finches [98]. EDCs have also been shown to disrupt mating behaviour in several seasonally breeding birds [99] and are linked with population declines [100]. Understanding how EDCs influence reproductive physiology in wild birds is crucial,

particularly for endangered birds where even small reductions in fertility can jeopardize species survival [101]. Relatively few long-term studies of wild birds have monitored the effects of EDCs on avian fertility, and in particular there is a lack of knowledge on the population-level effects of EDCs on fertility in wild birds [102,103]. Detecting the varied and often sublethal effects of EDCs is difficult: wild birds are probably exposed to many different types of EDC at one time [104,105], each with potentially complex and different effects [103]. The risks of EDC exposure to fertility is also likely to differ between species and across individual lifetimes [106]. Identifying the mechanisms by which EDCs affect fertility in wild birds therefore requires a combination of laboratory studies, long-term monitoring, and continued development of analytical methods [103].

## 3.2 Failure during ovulation

Ovulation occurs when the largest mature yellow follicle (labelled $F_1$ in figure 2) ruptures at the stigma region (figure 3) [28], releasing the ovum which is then captured by the infundibulum—the site of fertilization. Unlike mammals, the granulosa layer provides the main source of gonadal steroids [32], and ovulation is initiated by the production of testosterone in the granulosa cells, which stimulates the release of granulosa cell progesterone. Progesterone then creates a positive feedback response in the hypothalamus which stimulates an increase in the secretion of gonadotropin-releasing hormone, and consequently causes a surge of pituitary luteinizing hormone [31,107,108]. Clock genes expressed within granulosa cells after follicle selection are thought to provide a degree of circadian control over the timing of ovulation [31,109]. Proper regression of the post-ovulatory follicle is also thought to be required for managing the timing of ovulation and egg-laying [25].

### 3.2.1 Hormonal factors

As with egg formation, hormones play a critical role in regulating the process of ovulation. [25]. Exposure to environmental EDCs is likely to disrupt normal ovulation in exposed birds [110], and experimental hormonal manipulation can significantly influence ovulation (and therefore fertility) in captive species. For example, an increase in progesterone at the wrong time may induce a spike in luteinizing hormone, which triggers premature ovulation [38,111]. Inhibition of luteinizing hormone (e.g. via serotonin injections) [111] can lead to anovulation and disruption of thyroid hormone function [65], and ovulation can be prevented in domestic fowl when treated with the testosterone antagonist flutamide, which blocks the production of preovulatory hormones [112].

### 3.2.2 Disease and immune factors

In captive pet birds, excessive ovulations are one of the most common forms of reproductive abnormalities [113], the causes of which are not fully understood, but may be improved with husbandry and dietary measures [114]. Coelomitis is another common clinical problem in domestic birds, causing inflammation of the ovaries (oophoritis) and ectopic ovulation [37]. Viral infections such as avian influenza, infectious bronchitus and avian hepatitis can cause the formation of chronic lesions within the oviduct, that may prevent the successful capture of ova following ovulation [115,116]. This can result in extensive damage to the oviduct [117], often leading to further bacterial infection due to the presence of yolk in the coelomic cavity (egg yolk peritonitis) [118]. The spontaneous development of ovarian cancers is extremely common in the laying hen [28], the incidence of which increases with age, occurring in 24% of hens aged over 2 years [119], and 30–35% of hens by 3.5 years [25]. The increase in the incidence of ovarian cancers with age is thought to be, at least in part, a consequence of the accumulation of ovarian surface and DNA damage caused by ovulatory events over time [120]. Laying hens may therefore be at particular risk from ovarian cancers because of the selection for frequent ovulations in commercial breeds [28]. Progesterone can be effective at reducing the incidence of ovarian cancers, possibly because it limits the number of ovulations experienced [121]. Increased levels of progesterone have also been implicated in an increase in the number of apoptotic events in the ovary, which may act to remove damaged cells [122].

In broiler breeder hens which have been selected for rapid growth at the expense of fertility, double-yolk eggs are fairly common, and occur more frequently during the onset of egg production [25,123]. Double-yolk eggs are associated with a greater incidence of embryo mortality (at all stages of development) and are also more likely to be infertile [123], possibly because ova are ovulated early and in an immature state. Ovulation order of double-yolk eggs also affects the likelihood of

fertilization: in duck (*Anas platyrhynchos domesticus*) eggs, the first yolk captured by the infundibulum has a higher probability of being fertilized [124]. This may explain why double-yolk eggs commonly contain only one fertilized ovum [123]. Age, nutrition (e.g. feed restriction) and changes in photostimulation are all thought to play a role in the production of double-yolk eggs, the occurrence of which can also be increased via selection [124], indicating a genetic component.

## 3.3 Failure to obtain sperm

Even if ova are produced and ovulated normally, sperm must have been obtained and be present in the infundibulum at the precise time of ovulation. In the majority of bird species (greater than 97%), sperm are obtained by the female through cloacal contact during copulation, although there are a few examples of extant bird species that copulate with the use of a true intromittent organ (e.g. in the ratites, many Tinamiformes, Anseriformes and Cracidae), or a pseudo-phallus (such as in some Galliformes) [125–127].

### 3.3.1 Copulation

Sperm may be prevented from reaching the site of fertilization in several ways. Mechanical difficulties during mating resulting from physical injury (e.g. impaired vision or balance [38]) may prevent sperm from entering the reproductive tract. Access to the cloaca (figure 2) may be physically blocked due to obesity or clogged feathers (e.g. due to fecal build-up, or heavy cloacal feathering) [39,128,129], though this may be more likely to occur in captive populations. In captive birds, failed copulation may also occur due to inappropriate husbandry, for example a lack of proper perching or nesting sites, aviary disturbances, a lack of flock stimulation or illness [39]. Immaturity and sexual inexperience may also result in failed mating in young birds [39]. If mating proceeds normally, then females could theoretically ensure sufficient sperm are available for fertilization by copulating more frequently. Using an experimental approach that restricted inseminations by males, Török *et al*. [130] showed that multiple copulations were necessary to achieve a normal (unmanipulated) level of egg fertilization success in wild collared flycatchers, implying copulation frequency is important for fertility assurance [131]. However, multiple copulations could also reduce fertility if they damage the female reproductive tract. Copulation provides an opportunity for bacterial transfer [132,133], which can cause local inflammation in the vaginal wall, impairing sperm transport and reducing fertility [134]. When artificial insemination is performed with poor technique, the risk of vaginal infections is increased [135,136]. In domestic turkey hens, inflammatory effects of repeated artificial insemination appear to be transient, with quick recovery [137], but the long-term consequences of repeated infections are unknown. The main defence against microbial infection in the oviduct is provided by the vaginal mucosa and associated mucin substances, although cilial action within the oviduct may also play a role in the removal of microbes [28]. Similarly to the ovary, TLRs and avian β-defensins are also expressed within the rest of the oviduct, as well as macrophages, natural killer cells, cathelicidin and other antimicrobial defensins such as gallin [28]. There is a high density of T cells and B cells in the vagina [138], and the expression of proinflammatory cytokines are also increased in response to oviductal infection [139].

### 3.3.2 Timing of insemination

If ovulation proceeds normally, the ovum progresses into the infundibulum and a second glycoprotein layer is formed around it within approximately 15 min, preventing additional sperm from penetrating (figure 3). This short fertilization window requires precise timing of insemination and/or the release of sperm from female storage to ensure sufficient sperm are in the infundibulum at ovulation [140]. In turkeys, sperm storage is more efficient when artificial insemination is performed just prior to (rather than just after) the onset of egg production [141]. Similarly, in chickens, more sperm reach the infundibulum when they are inseminated within 1–6 days of ovulation (relative to 6–8 days before ovulation) [142]. The greater the time delay between insemination and ovulation, the fewer sperm will be available for fertilization, since sperm are lost passively from storage at a constant rate [143]. The time interval between ovulation and egg-laying takes approximately 24–28 h in chickens, turkeys and quail [144]. Inseminations performed immediately before or after egg-laying reduce fertility, possibly because egg-laying contractions impede the ability of sperm to move through the oviduct and/or the passage of sperm is blocked by the egg [26,28]. In chickens, sperm storage is up to 40 times more

efficient when insemination occurs more than 4 h after egg-laying [134]. Similar evidence of low sperm uptake during and just after egg-laying has also been observed for natural copulations [145].

### 3.3.3 Vaginal sperm selection

Ensuring sufficient sperm are available for fertilization has clear benefits for the female, but the mechanisms that might facilitate this are at odds with those facilitating female sperm selection. The vagina is considered the main sperm selection site in the avian oviduct [146,147], and only 1% of inseminated sperm make it through to the sperm storage tubules (labelled (*f*) and (*g*) in figure 2). Domestic fowl, for example, eject the sperm of undesirable males following forced copulations [148], thereby reducing the number of sperm available for fertilization. The vaginal fluid of female barn swallows has also been shown to reduce sperm performance by varying degrees depending on female quality [149]. Huang *et al*. [150] found that vagina mucosal tissue of chickens produces exosomes (membrane vesicles enriched in transmembrane proteins) that significantly reduce sperm viability (possibly because they contain cytotoxic factors) and therefore may play a role in sperm selection. During egg production, vaginal pH and immunological activity also varies [26,138], and immune-competent cells are expressed in the vagina [151]. Van Krey *et al*. [152] found that infertile females express antibody-producing plasma cells in their reproductive tract, and Higaki *et al*. [153] showed that the number of leukocytes present in the vagina increases following copulation. Localized immune responses are predicted to participate in non-random sperm selection [154], and the likelihood of an anti-sperm response may depend on male genotype [155]. For example, Løvlie *et al*. [156] show that post-copulatory sperm selection in female red junglefowl (*Gallus gallus*) is biased towards males dissimilar at the MHC. Sperm are rapidly coated with immunoglobulin cells produced by the vaginal mucosa, and immunoglobulin IgA and IgG are thought to be at least partially responsible for the massive reduction in sperm viability during transport through the vagina [26]. Determining how female anti-sperm responses vary across individuals is a crucial step towards understanding the importance of the immune response for avian female fertility. Immune response strength probably depends on complex phenotypic trade-offs between fertility and infection resistance [157], similar to the trade-off females face between fertility and sperm quality during sperm selection. If sperm selection and/or immune response mechanisms are too effective, insufficient sperm may reach the site of fertilization. Sperm selection and transport in the vagina may therefore be considered a balance between selecting high-quality sperm, avoiding infection, and ensuring sufficient sperm remain available for fertilization.

## 3.4. Failure to maintain and transport sperm

### 3.4.1 Sperm storage tubule function

Within the female reproductive tract, sperm are stored in blind-ended tubular invaginations known as sperm storage tubules (SSTs) [147] (labelled (*f*) and (*g*) in figure 2) found in the utero-vaginal junction (UVJ). While SSTs are considered the primary sperm storage site, it has been suggested that sperm may also be stored in the infundibulum. However, evidence for this is equivocal [154]. Once sperm are in storage, the proper functioning of the SSTs is likely to be crucial to fertility, and a decline in sperm storage ability has been associated with fertility senescence in birds [158]. The number of sperm stored is strongly associated with the number that reach the ovum [141], and chickens selected for high fertility have significantly greater numbers of SSTs [159]. The mechanisms controlling sperm acceptance, storage and release are assumed to be under fine temporal control based on hormonal changes [142]. Fertilization failure is therefore likely if hormonal imbalances result in a mismatch between the timing of ovulation and arrival of sperm in the infundibulum [142,154]. The significant variation in fertile periods across species has been attributed to differences in SST number and therefore sperm storage capacity [154], but intra- and inter-specific variation in the structure and function sperm storage tubules has not yet been quantified.

The mechanisms by which sperm are maintained in a viable state in the SSTs are not fully understood, but it is thought that numerous compounds are produced to maintain a suitable environment for long-term sperm survival [154,160]. Recent work provides some experimental evidence to support this. For example, lactic acid—now known to be produced in SST cells in response to hypoxic conditions—can induce a reduction in sperm flagellar movement and so may contribute to the quiescence of Japanese quail sperm [28,161]. Additionally, Huang *et al*. [162]

identified a number of fatty acids in the UVJ mucosa of domestic fowl, which have also been shown to depress rooster sperm motility, and *in vitro* sperm survival was found to be higher in the presence of oleic and linoleic acid. They also found that SST cells express lipid receptors, which may enable lipid droplets to accumulate and be used by resident sperm to maintain structural integrity. SST cells in turkeys are also known to shed microvillus vesicles that interact with sperm, transferring metabolic substrates that may be capable of temporarily inhibiting fertilizing ability, protecting from oxidative stress and transporting fluid from the SST cells into the SST lumen [163]. While evidence suggests that these compounds play a key part in sperm maintenance, there are probably a number of other important compounds expressed in SSTs that have yet to be discovered [28]. The degree to which individuals and species vary in their ability to maintain sperm in storage is not clear, but this may play an important role in determining female fertility.

One factor that is essential for sperm survival in storage is the suppression of the female immune response, which if triggered can have highly detrimental effects on sperm. In domestic fowl, repeated artificial inseminations were associated with a complete lack of stored sperm and a 57% decrease in fertility [164,165]. This has been attributed to an influx of lymphocytes and antigen-presenting cells into SSTs that probably impair sperm survivability, but also prevent sperm from entering storage [164]. A significant decrease in the expression of oestrogen receptors in the sperm storage tubules was also observed following infection, probably impairing the hormonal control of sperm storage tubule function [165]. Das *et al*. [166] demonstrated enhanced local expression of transforming growth factor $\beta$(TGFb) within sperm storage tubules, which suppresses the anti-sperm immune response by depressing the activity of lymphocytes.

### 3.4.2 Sperm release and transport

As the rate of sperm release from storage increases, the duration of fertility will decline unless more sperm are inseminated [26]. Older hens tend to release sperm faster, possibly due to a decline in hormone production needed to regulate ovulation and sperm release [167]. This may partially explain why older hens have shorter fertile periods [26]. The mechanisms of sperm release are not fully understood, but SSTs have been shown to possess a constricted 'gate-like' entrance, that may act as a physical (and/or selective) barrier preventing sperm from leaving [168]. Constriction is likely to be hormonally triggered, since progesterone has been shown to induce contractions of the SSTs, and images taken using electron microscopy show sperm leaving the SSTs after intravenous injection with progesterone [169]. Furthermore, the specific membrane progestin receptor mPR$\alpha$ has been shown to be expressed within SSTs of Japanese quail [169]. Additionally, in a comprehensive study, Hiyama *et al*. [170] demonstrated that heat shock protein 70 (HSP70)—a widespread and highly conserved molecular chaperone—is expressed in the utero-vaginal junction and its expression increases prior to ovulation. They also found that HSP70 binds to sperm and stimulates flagellar movement *in vitro*, and injection of an HSP70 antibody significantly reduces fertilization success *in vivo*. Hiyama *et al*. speculate that HSP70 expression in the UVJ may be stimulated by progesterone; the progesterone surge experienced prior to ovulation may therefore function in part to allow sperm to be released from storage at the right time while also ensuring sperm regain function. Imbalances in circulating progesterone levels can be triggered by conditions such as nutrient toxicity (e.g. excess fluoride [68]) and heat stress [171], and future work should explore if such imbalances reduce fertility by disrupting sperm release from storage.

Once released from storage, sperm are thought to travel passively through the uterus, isthmus (labelled (*d*) in figure 2) and magnum (labelled (*c*) in figure 2) [154], as evidenced by the fact that dead sperm inseminated beyond the utero-vaginal junction reach the infundibulum in as great numbers as live sperm [172]. Past the utero-vaginal junction the reproductive tract is apparently free of immunoglobulins, and anti-peristaltic activity is thought to aid in the passive and rapid transport of sperm to the infundibulum, [173] where they remain until fertilization [154].

## 3.5. Failure of sperm–egg fusion

Following successful ovulation, the avian ovum is captured by the infundibulum where it encounters sperm (figures 2 and 3). Successful fertilization involves the initiation of multiple events in sequence: sperm–egg binding, acrosomal exocytosis, sperm penetration through the perivitelline layer and fusion of the male and female pronuclei in the germinal disc. The mechanisms of sperm–egg interactions in birds are not well understood, but the roles of several important molecules have been discovered.

### 3.5.1. Sperm–egg interactions

The inner perivitelline layer (IPVL) (figure 3), which is homologous to the zona pellucida (ZP) in mammals, is composed of a mesh of fibre that forms a three-dimensional extracellular matrix. Unlike in mammals, the IPVL of birds does not inhibit polyspermy [29], and in fact a degree of physiological polyspermy is required for normal development in birds [140]. There are at least six known avian ZP glycoproteins [6,17] (there has been significant confusion in the literature regarding the nomenclature of ZP proteins [174], here we provide the common aliases in parentheses), most notably ZP1 (ZPB1), ZP3 (ZPC) and ZPD which are major components of the IPVL and play a key role in the binding of sperm and initiation of the acrosome reaction [17,29]. Other minor constituents of the IPVL include ZP2 (ZPA), ZP4 (ZPB or ZPB2) and ZPAX (ZPX1) [28,175], where ZP2 accumulates primarily in the germinal disc region in chickens [176]. Interestingly, ZP4 (but not ZP2) mRNA was found to be expressed in the germinal disc region in the turkey [6], suggesting differences in sperm binding mechanisms may occur across species. Acrosin, located in the sperm plasma membrane, was discovered to be a complementary molecule that supports the binding of sperm to the ZP proteins in quail PVL [17].

Diagrammatic representations of fertilization often depict the ovum oriented such that the germinal disc faces towards the reproductive tract (and oncoming sperm). However, we suggest that the animal pole—where the germinal disc is located—faces towards the ovary during ovulation and fertilization (figure 3), because the ovum is in that orientation while inside the follicle [28], and to our knowledge there is no known mechanism by which it would turn to face the opposite direction after ovulation. If true, the consequence is that sperm would have to travel around the ovum to reach the tiny germinal disc target. Sperm are known to bind to the germinal disc region in higher concentrations than elsewhere on the ovum [176], suggesting there must be an underlying mechanism by which sperm locate and/or preferentially bind to this region. Such a mechanism has yet to be discovered, but the egg plasma membrane is reported to differ in morphology around the germinal disc region compared with other regions of the ovum [177]. Specifically, numerous microvilli and cytoplasmic processes of the plasma membrane have been observed to protrude through the IPVL exclusively at the germinal disc region [177–179]. In other (non-germinal disc) areas of the ovum, the discontinuous nature of the ovum plasma membrane has been hypothesized to inhibit sperm from hydrolysis over these regions [180]. It has also been suggested that sperm may locate the germinal disc region via egg chemo-attractants [181], and/or by using site-specific egg coat receptors [176]. PVL glycoproteins ZP2 and ZP4 are promising egg coat receptor candidates, since ZP2 and ZP4 are concentrated primarily in the germinal disc region of chicken and turkey PVL respectively, but their sperm binding properties have yet to be investigated [6,17,176]. Recently, a number of new PVL proteins have been identified which appear to vary across species [182], but their function in fertilization has not yet been determined.

In mammals, a variety of protein coding genes associated with gamete cell surfaces have been discovered [183–185]. This includes Juno and Izumo—the only known interacting pair of sperm–egg adhesion proteins. Izumo is a sperm protein [186], and Juno is the more recently discovered egg Izumo receptor [187]. In mice, Juno and Izumo knockouts result in sterility, and Juno is vital in preventing polyspermy; its rapid loss from the egg surface membrane following fertilization causes the blocking of the zona pellucida to further sperm entry [187]. Reproductive proteins are known to evolve rapidly compared with many other gene classes, and both Juno and Izumo have been found to be under positive selection in mammals [188]. No such interacting proteins have been discovered in birds: comparisons of the genomic regions containing Juno (and surrounding loci) in mice and humans with that of the chicken shows that they are generally syntenic (the gene order is conserved); however, Juno loci are absent in the chicken [28]. A key step for avian fertility research will be to identify avian Juno and Izumo equivalents. Also essential for sperm–egg fusion is the ubiquitously expressed membrane protein CD9: female (but not male) CD9 knockout mice are infertile [185]—CD9 was the first identified gene with female-specific fertility effects [189]. There is one known homologue of CD9 in the chicken (ID: AB032767) though to our knowledge no studies have explored the involvement of this (or any other gene) on sperm–egg fusion in birds [28].

In addition to proteins, the consistency and structure of the IPVL differs markedly between species. For example, Damaziak *et al*. [182] observed that cockatiels have a more densely and irregularly arranged IPVL than that of three other species studied (pigeons, grey partridges (*Perdix perdix*) and pheasants). They also found that the pigeon PVL is markedly different in structure; its numerous sublayers are more homogeneous, less porous and unusually loose in arrangement compared with the other species. Pigeon PVL is also composed of flat sheets rather than the cylindrical fibres which are observed in the PVL of all the other species. It is unknown how this variation in structure affects the

integrity of the PVL, its interaction with sperm, or whether this variation corresponds to post-copulatory sexual selection intensity and/or sperm traits. Damaziak *et al*. [182] suggest that interspecific variation in PVL structure may be related to differences in the function of the PVL during embryo development, which may vary depending on whether the species is precocial or superaltricial. The germinal disc region is also known to show subtle intra- and inter-specific variation in terms of morphology, and also in terms of the location, size and number of sperm penetration holes [190]. It is currently unknown how variation in PVL structure affects fertility, but fertilization rates are positively correlated with the number of sperm that penetrate the PVL [191]. There is known to be variation in how readily sperm can bind to the PVL [146,192], and it seems logical that intra- and inter-specific differences in PVL structure may affect how easy it is for sperm to bind to and penetrate the PVL. In turkey lines where females are selected for increased body weight at the expense of fertility, fewer sperm penetration holes are visible on the IPVL compared with hens selected for high fertility [193]. While fewer IPVL sperm holes could indicate that fewer sperm are reaching the site of fertilization, it has also been associated with reduced mRNA expression of ZP1 and ZP3 sperm binding proteins on the IPVL [8]. This not only suggests that individuals may vary in terms of the number of sperm that are able to penetrate the IPVL, but also that selection for higher expression of sperm binding proteins may improve fertility in some populations [8,193]. In taxa where polyspermy is lethal to the egg (such as in mammals), egg 'fertilizability' is known to vary according to the risk of polyspermy [194]. Consequently, females and males will be locked in an apparent cycle of coevolutionary conflict where females are selected for greater 'egg defensiveness' (resistance to sperm) and males selected to counter this with greater fertilizing ability and competitiveness [21]. Currently, egg defensiveness has been most largely explored in sea urchins and in mice [22,194], with virtually nothing known in birds. Since polyspermy is a normal and important part of fertilization in birds, this suggests that mechanisms of polyspermy avoidance are unlikely to be important other than to prevent excessive sperm penetration that might damage the integrity of the ovum. Theoretically, females might be expected to evolve mechanisms of resistance to sperm for other reasons, for example, to alleviate the costs of hybridization, avoid incompatible sperm, or as a mechanism of selection for high-quality sperm [22,175]. Indeed, evidence suggests that the strength of positive selection on gamete-recognition genes is similar between birds and mammals, suggesting that in the absence of polyspermy avoidance, there must be some other adaptive mechanism to explain the rapid evolution of avian gamete-recognition genes [175]. Recently, Hurley *et al*. [195] found significant variation in PVL sperm numbers between breeding pairs of estrildid finches, as well as variation in PVL sperm numbers across the laying order. It was unclear, however, if this variation was male or female mediated. Exploring the degree of variation in egg quality, egg defensiveness and sperm selection at the gametic level is challenging but important for elucidating the full role of the avian ovum for fertility.

Chromosomal abnormalities, such as whole genome triploidy, can significantly or completely impair fertility in some affected individuals, and some triploid embryos are non-viable and die after a few days of incubation [196]. Triploidy is usually (but not always) maternally derived, and is thought to arise from diploid gametes produced as a result of chromosomal non-disjunction (where homologous chromosomes fail to separate during meiosis) [197,198]. Reports of triploid birds in the wild are rare (but see [199]), possibly because of the reduced survival of triploid embryos, although the true rate of incidence in wild populations is unknown. In addition to chromosomal abnormalities, the presence of multiple germinal discs on a single yolk has been reported [200], although the cause and incidence of this is unknown, as well as the implications it might have for fertility and embryo development.

Whether other aspects of ovum quality, such as physiological abnormalities, or the integrity of DNA in the female pronucleus, influences the likelihood of successful sperm–egg fusion in birds remains unclear. It seems likely, however, given that in mammals, the accumulation of DNA damage in oocytes is known to impact fertility [201]. Oocytes may be especially vulnerable to the accumulation of DNA damage (relative to sperm) given that they remain in an arrested state for an extended period of time [201]. While oxidative DNA damage is expected to be minimized by various innate protective mechanisms, the efficacy of these mechanisms is known to decline with age [202]. If a similar process occurs in birds, this could be a factor that contributes to fertility senescence in captive species. Increased DNA damage due to oxidative stress has also been associated with exposure to endocrine disrupting compounds in mammals [203], as well as certain reproductive diseases in humans [204]. In mammals, heritable mutations of ZP2 and ZP3 are also known to cause infertility [205], and antibodies raised against ZP proteins can depress ovarian function [6]. Investigating the incidence of similar ovum abnormalities and immunological activity, and the degree to which they might affect avian fertility, may be a fruitful avenue for future research.

### 3.5.2. Syngamy

Once bound with the PVL, the sperm acrosomal contents (including proteases and endopeptidases) are released during the acrosomal reaction and locally degrade the PVL, forming a hole via which sperm can penetrate the ovum (figure 3) [17,28]. Sperm penetration holes are visible on the PVL *in vitro* (labelled (*b*) in figure 2) and can be used as a reliable proxy for the number of sperm that reach and penetrate the ovum [140,195]. Following the acrosomal reaction, the inner acrosomal membrane of sperm becomes exposed, binds to the ovum and the male pronucleus is released. While multiple sperm can penetrate the PVL in birds, only one male pronucleus typically fuses with the female pronucleus in the germinal disc during syngamy. This provides additional potential for the female pronucleus itself to be selective [206], although the exact mechanisms of avian syngamy remain unknown [28]. In the comb jelly (*Beroe ovata*), where fertilization is also polyspermic, the female pronucleus migrates within the egg cell prior to syngamy, moving between different immobile male pronuclei before finally fusing with just one [206,207]. Whether sexual selection occurs at the point of syngamy in birds remains to be seen, but could present an additional opportunity for females to influence the outcome of fertilization by discriminating between males, for example, based on male genotype, or on the integrity of the male's DNA [155,206].

Supernumerary male pronuclei are probably degraded by DNAses in the germinal disc and PVL of mature oocytes [208], though the precise molecular mechanisms involved have not yet been fully elucidated [28]. During or immediately after fertilization, a granular continuous layer is laid down around the ovum, followed by the outer perivitelline layer (OPVL), which blocks further sperm entry (figure 3) [28]. The OPVL is multi-layered and composed of proteins secreted by the infundibular mucosa [209]. Macrophages present within the infundibulum are thought to function in the phagocytosis of superfluous sperm (i.e. those that did not participate in fertilization) [210]. Elucidating the full molecular mechanisms involved in avian syngamy is challenging [28], but will be an important step in understanding how fertility can be compromised at the gametic level.

## 4. Conclusion and future directions

It is clear that females can exert far more control over fertilization than has historically been assumed, but if and how females influence whether their ova are successfully fertilized is often ignored in favour of male processes (such as sperm quality and quantity) [6]. Here we have quantitatively demonstrated that avian fertility research has been dominated by studies on males, with a deficit in research effort on female fertility. We also show that the vast majority of avian fertility research has concentrated on captive populations, with a significant taxonomic focus on gallinaceous birds and the domestic chicken in particular. We have also highlighted key advances and gaps in knowledge on the role of female physiological processes in determining fertilization success. In particular, we have identified five key stages in the reproductive cycle during which fertility can be compromised: (i) failure to produce fertilizable eggs, (ii) failure during ovulation, (iii) failure to obtain sufficient sperm, (iv) failure to store and transport sperm, and (v) failure of fertilization. We highlight that the field of avian fertility would benefit from more studies investigating variation in fertility in non-poultry species (i.e. that have not undergone intense artificial selection for high productivity) and wild populations. Within wild birds, more attention to a-seasonal/tropical species and opportunistic breeders would also be valuable. Although we acknowledge that detailed study of variation in female fertility may be difficult in wild populations, because information about non-breeders is not always easy to collect, we nonetheless urge that such efforts are made, particularly in non-poultry species and managed and/or experimental populations where male processes can be controlled for. If studies are unable to monitor failed breeders (i.e. those that did not produce any eggs following a successful copulation), it would be useful to acknowledge that infertility may be underestimated. Obtaining a more accurate estimate of fertility rates across wild populations, and improving our understanding of the mechanisms that influence fertility, will aid in the management of threatened populations that suffer high levels of hatching failure, and improve predictions for how fertility will be influenced by a changing climate.

When investigating reproductive failure, the fact that infertility and embryo mortality are fundamentally distinct processes needs to be explicitly acknowledged. Specifically, that infertility is used to describe failed fertilization, and any process contributing to failed fertilization is a mechanism of infertility (rather than embryo mortality). Similarly, if an egg fails to hatch but was fertilized, then the cause of hatching failure must be referred to as embryo mortality, even if development arrested

after only a few cell divisions. If fertility status cannot be unequivocally determined using the appropriate techniques [13], then the mechanisms of hatching failure cannot be conclusively known. Very early embryo mortality is likely to be mistaken for infertility when using traditional methods (e.g. candling or macroscopic examination), which may result in an overestimation of infertility [11]. Moving forward, a clearer estimation of the incidence of infertility in a given population will require a combination of both careful monitoring (to identify failed breeders) as well as an accurately determined fertilization status for unhatched eggs.

The female reproductive tract typically offers a hostile environment for sperm, providing considerable potential for female processes to influence sperm survival and transport to the ovum. While the processes of sperm selection, storage, release and transport within the reproductive tract have received increasing research attention over the past few decades, we still lack fundamental understanding of the underlying mechanisms, and the degree of intra- and inter-specific variation in these processes, with the vast majority of work having focused on a very limited number of domestic species. Many of the female-mediated processes required for high fertility also deteriorate to some degree with age, making fertility problems more likely in older birds. This may have particularly important consequences for captive and managed threatened populations, where individuals may reproduce to an older age than their wild counterparts, due to reduced predation and competition pressure, and high accessibility of food and other resources. The field of avian reproductive science will also benefit from better understanding the impact of other factors on female fertility, such as stress, hormonal and physiological disorders (particularly in wild birds where less is known), environmental pollutants, intra- and inter-individual variation in egg production, egg quality, sperm selection and the female immune response within the oviduct (including the ovaries).

The causes and maintenance of variation in fertility is a key question in evolutionary biology, and one in which the role of the female is often sidelined. Our hope is that this review challenges the field of avian reproductive science and evolutionary biology to consider female processes to a greater degree when investigating the causes of depressed fertility in birds. Using birds as a model system for the study of female fertility across taxa presents several advantages and will provide insights not only in the field of reproductive biology, but also for fields such as conservation and commercial animal breeding as well.

Data accessibility. The dataset supporting this article has been uploaded as part of the electronic supplementary material [211].

Authors' contributions. K.A. carried out the data collection, data analysis, created the figures and drafted the manuscript; P.B. critically revised the manuscript; N.H. participated in data collection, assisted in data analysis and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

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# References

1. Greenway GEV, Balfour VL, Shuker DM. 2017 Can females choose to avoid mating failure in the seed bug *Lygaeus simulans*? *Anim. Behav.* **129**, 61–69. (doi:10.1016/j.anbehav.2017.05.004)
2. Koenig WD. 1982 Ecological and social factors affecting hatchability of eggs. *Auk* **99**, 526–536.
3. Lifjeld J, Laskemoen T, Fossøy F, Johnsen A, Kleven O. 2007 Functional infertility among territorial males in two passerine species, the willow warbler *Phylloscopus trochilus* and the bluethroat *Luscinia svecica*. *J. Avian Biol.* **38**, 267–272. (doi:10.1111/j.2007.0908-8857.04126.x)
4. Morrow EH, Arnqvist G, Pitcher TE. 2002 The evolution of infertility: does hatching rate in birds coevolve with female polyandry? *J. Evol. Biol.* **15**, 702–709. (doi:10.1046/j.1420-9101.2002.00445.x)
5. Hayward A, Gillooly JF. 2011 The cost of sex: quantifying energetic investment in gamete production by males and females. *PLoS ONE* **6**, 16557. (doi:10.1371/journal.pone.0016557)
6. Benson AP, Malloy MN, Steed JR, Christensen VL, Fairchild BD, Davis AJ. 2017 Zona pellucida protein B2 messenger ribonucleic acid expression varies with follicular development and granulosa cell location. *Poult. Sci.* **96**, 3414–3421. (doi:10.3382/ps/pex126)
7. Donoghue AM. 1999 Prospective approaches to avoid flock fertility problems: predictive assessment of sperm function traits in poultry. *Poult. Sci.* **78**, 437–443. (doi:10.1093/ps/78.3.437)
8. Benson AP, Christensen VL, Fairchild BD, Davis AJ. 2009 The mRNA for zona pellucida proteins B1, C and D in two genetic lines of turkey hens that differ in fertility. *Anim. Reprod. Sci.* **111**, 149–159. (doi:10.1016/j.anireprosci.2008.02.013)
9. Johnson MH. 2018 *Essential reproduction*, 8th edn. New York, NY: Wiley-Blackwell.
10. Hemmings N, West M, Birkhead TR. 2012 Causes of hatching failure in endangered birds. *Biol. Lett.* **8**, 964–967. (doi:10.1098/rsbl.2012.0655)
11. Hemmings N, Evans S. 2020 Unhatched eggs represent the invisible fraction in two wild bird

populations. *Biol. Lett.* **16**, 20190763. (doi:10.1098/rsbl.2019.0763)
12. Birkhead TR, Hall J, Schut E, Hemmings N. 2008 Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis (Lond. 1859)*. **150**, 508–517. (doi:10.1111/j.1474-919X.2008.00813.x)
13. Assersohn K, Marshall AF, Morland F, Brekke P, Hemmings N. 2021 Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. *Anim. Conserv.* 1–12. (doi:10.1111/acv.12674)
14. Beaumont C, Millet N, Le Bihan-Duval E, Kipi A, Dupuy V. 1997 Genetic parameters of survival to the different stages of embryonic death in laying hens. *Poult. Sci.* **76**, 1193–1196. (doi:10.1093/ps/76.9.1193)
15. Heber S, Briskie JV. 2010 Population bottlenecks and increased hatching failure in endangered birds. *Conserv. Biol.* **24**, 1674–1678. (doi:10.1111/j.1523-1739.2010.01553.x)
16. Jamieson IG, Ryan CJ. 2000 Increased egg infertility associated with translocating inbred takahe (*Porphyrio hochstetteri*) to island refuges in New Zealand. *Biol. Conserv.* **94**, 107–114. (doi:10.1016/S0006-3207(99)00158-5)
17. Ichikawa Y, Matsuzaki M, Hiyama G, Mizushima S, Sasanami T. 2016 Sperm-egg interaction during fertilization in birds. *J. Poult. Sci.* **53**, 173–180. (doi:10.2141/jpsa.0150183)
18. Lüpold S, Reil JB, Manier M, Zeender V, Belote J, Pitnick S. 2020 Female × male and male × male interactions have limited influence on competitive fertilization in *Drosophila melanogaster*. *Evol. Lett.* **4**, 416–429. (doi:10.1002/evl3.193)
19. Blesbois E. 2007 Current status in avian semen cryopreservation. *Worlds Poult. Sci. J.* **63**, 213–222. (doi:10.1017/S0043933907001419)
20. Martin E. 1991 The egg and the sperm: How science has constructed a romance based on stereotypical male-female roles. *Univ. Chicago Press J.* **16**, 485–501. (doi:10.1086/494680)
21. Firman RC. 2020 Of mice and women: advances in mammalian sperm competition with a focus on the female perspective. *Phil. Trans. R. Soc. B* **375**, 20200082. (doi:10.1098/rstb.2020.0082)
22. Firman RC, Gasparini C, Manier MK, Pizzari T. 2017 Postmating female control: 20 years of cryptic female choice. *Trends Ecol. Evol.* **32**, 368–382. (doi:10.1016/j.tree.2017.02.010)
23. Vézina F, Williams TD. 2002 Metabolic costs of egg production in the European starling (*Sturnus vulgaris*). *Physiol. Biochem. Zool.* **75**, 377–385. (doi:10.1086/343137)
24. Houston DC, Donnan D, Jones PJ. 1995 The source of the nutrients required for egg production in zebra finches *Poephila guttata*. *J. Zool.* **235**, 469–483. (doi:10.1111/j.1469-7998.1995.tb01763.x)
25. Johnson AL. 2014 The avian ovary and follicle development: some comparative and practical insights. *Turkish J. Vet. Anim. Sci.* **38**, 660–669. (doi:10.3906/vet-1405-6)
26. Bakst MR, Wishart G, Brillard J-P. 1994 Oviducal sperm selection, transport, and storage in poultry. *Poult. Sci. Rev.* **5**, 117–143.
27. Hung-Chang Yao H, Volentine KK, Bahr JM. 1998 Destruction of the germinal disc region of an immature preovulatory chicken follicle induces atresia and apoptosis. *Biol. Reprod.* **59**, 516–521. (doi:10.1095/biolreprod59.3.516)
28. Sasanami T. 2017 *Avian reproduction: from behaviour to molecules*, 1st edn. Singapore: Springer.
29. Rodler D, Sasanami T, Sinowatz F. 2012 Assembly of the inner perivitelline layer, a homolog of the mammalian zona pellucida: an immunohistochemical and ultrastructural study. *Cells Tissues Organs* **195**, 330–339. (doi:10.1159/000327013)
30. Johnson AL. 2015 Ovarian follicle selection and granulosa cell differentiation. *Poult. Sci.* **94**, 781–785. (doi:10.3382/ps/peu008)
31. Kim D, Johnson AL. 2016 Vasoactive intestinal peptide promotes differentiation and clock gene expression in granulosa cells from prehierarchal follicles. *Mol. Reprod. Dev.* **83**, 455–463. (doi:10.1002/mrd.22641)
32. Cui Z, Liu L, Zhu Q, Wang Y, Yin H, Li D, Tian Y, Shu G, Zhao X. 2020 Inhibin A regulates follicular development via hormone secretion and granulosa cell behaviors in laying hens. *Cell Tissue Res.* **381**, 337–350. (doi:10.1007/s00441-020-03207-8)
33. Scanes CG. 2017 Grand and less grand challenges in avian physiology. *Front. Physiol.* **8**, 222. (doi:10.3389/fphys.2017.00222)
34. Shinomiya A, Shimmura T, Nishiwaki-Ohkawa T, Yoshimura T. 2014 Regulation of seasonal reproduction by hypothalamic activation of thyroid hormone. *Front. Endocrinol. (Lausanne).* **5**, 1–7. (doi:10.3389/fendo.2014.00012)
35. Sharp PJ, Follet BK. 1969 The effect of hypothalamic lesions on gonadotrophin release in Japanese quail (*Coturnix coturnix japonica*). *Neuroendocrinology* **5**, 205–218. (doi:10.1159/000121861)
36. Rae M. 1995 Endocrine disease in pet birds. *Semin. Avian Exot. Pet Med.* **4**, 32–38. (doi:10.1016/S1055-937X(05)80007-6)
37. Rosen LB. 2012 Avian reproductive disorders. *J. Exot. Pet Med.* **21**, 124–131. (doi:10.1053/j.jepm.2012.02.013)
38. Crosta L, Gerlach H, Bürkle M, Timossi L. 2003 Physiology, diagnosis, and diseases of the avian reproductive tract. *Vet. Clin. North Am. Exot. Anim. Pract.* **6**, 57–83. (doi:10.1016/S1094-9194(02)00026-9)
39. Crosta L, Gerlach H, Burkle M, Timossi L. 2002 Endoscopic testicular biopsy technique in Psittaciformes. *J. Avian Med. Surg.* **16**, 106–110. (doi:10.1647/1082-6742(2002)016[0106:ETBTIP]2.0.CO;2)
40. Dietrich HM, Cole RK, Wick G. 1999 The natural history of the obese strain of chickens—an animal model for spontaneous autoimmune thyroiditis. *Poult. Sci.* **78**, 1359–1371. (doi:10.1093/ps/78.10.1359)
41. Gilbert AB, Davidson MF, Hardie MA, Wells JW. 1981 The induction of atresia in the domestic fowl (*Gallus domesticus*) by ovine LH. *Gen. Comp. Endocrinol.* **44**, 344–349. (doi:10.1016/0016-6480(81)90011-3)
42. Cowan ML, Martin GB, Monks DJ, Johnston SD, Doneley RJT, Blackberry MA. 2014 Inhibition of the reproductive system by deslorelin in male and female pigeons (*Columba livia*). *J. Avian Med. Surg.* **28**, 102–108. (doi:10.1647/2013-027)
43. Wang C, Li C, Li H, Ma W, Chen S, Zhao Y, Rao J, Zhou X. 2015 Downregulation of the expression of inhibin $\alpha$ subunit and betaglycan in porcine cystic follicles. *J. Vet. Med. Sci.* **77**, 1419–1425. (doi:10.1292/jvms.14-0617)
44. Christians JK, Williams TD. 2002 Effects of porcine follicle-stimulating hormone on the reproductive performance of female zebra finches (*Taeniopygia guttata*). *Gen. Comp. Endocrinol.* **125**, 121–131. (doi:10.1006/gcen.2001.7737)
45. Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL. 1990 Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. *Nature* **345**, 167–170. (doi:10.1038/345167a0)
46. Lambeth LS *et al.* 2016 Overexpression of anti-Müllerian hormone disrupts gonadal sex differentiation, blocks sex hormone synthesis, and supports cell autonomous sex development in the chicken. *Endocrinology* **157**, 1258–1275. (doi:10.1210/en.2015-1571)
47. Cutting AD, Ayers K, Davidson N, Oshlack A, Doran T, Sinclair AH, Tizard M, Smith CA. 2014 Identification, expression, and regulation of anti-Müllerian hormone type-II receptor in the embryonic chicken gonad. *Biol. Reprod.* **90**, 1–12. (doi:10.1095/biolreprod.113.116491)
48. Lemcke RA, Stephens CS, Hildebrandt KA, Johnson PA. 2018 Anti-Müllerian hormone type II receptor in avian follicle development. *Biol. Reprod.* **99**, 1227–1234. (doi:10.1093/biolre/ioy140)
49. Yoshimura Y. 2015 Avian β-defensins expression for the innate immune system in hen reproductive organs. *Poult. Sci.* **94**, 804–809. (doi:10.3382/ps/peu021)
50. Woods DC, Schorey JS, Johnson AL. 2009 Toll-like receptor signaling in hen ovarian granulosa cells is dependent on stage of follicle maturation. *Reproduction* **137**, 987–996. (doi:10.1530/REP-08-0320)
51. Yoshimura Y. 2004 Significance of local immunity in hen reproductive organs. *Anim. Sci.* **75**, 183–191. (doi:10.1111/j.1740-0929.2004.00174.x)
52. Mande PV, Parikh FR, Hinduja I, Zaveri K, Vaidya R, Gajbhiye R, Khole VV. 2011 Identification and validation of candidate biomarkers involved in human ovarian autoimmunity. *Reprod. Biomed. Online* **23**, 471–483. (doi:10.1016/j.rbmo.2011.06.013)
53. Barua A, Yoshimura Y. 2001 Ovarian autoimmunity in relation to egg production in laying hens. *Reproduction* **1**, 117–122. (doi:10.1530/rep.0.1210117)
54. Barua A, Yoshimura Y, Tamura T. 1998 Effects of ageing and oestrogen on the localization of immunoglobulin-containing cells in the chicken ovary. *J. Reprod. Fertil.* **114**, 11–16. (doi:10.1530/jrf.0.1140011)
55. Ellison PT, Ottinger MA. 2014 A comparative perspective on reproductive aging, reproductive cessation, post reproductive life, and social behaviour. In *Sociality, hierarchy, health: comparative biodemography: a collection of papers* (eds M Weinstein, MA Lane), pp. 315–338. Washington, DC: The National Academic Press.

56. Fairley DH, Taylor A. 2003 Anovulation. *Br. Med. J.* **327**, 546–549. (doi:10.1136/bmj.327.7414.546)
57. Srinivasan P, Balasubramaniam GA, Gopala Krishna Murthy TR, Balachandran P. 2014 Prevalence and pathology of oviduct impaction in commercial white leghorn layer chicken in Namakkal region of India. *Vet. World* **7**, 553–558. (doi:10.14202/vetworld.2014.553-558)
58. Briere S, Brillard JP, Panheleux M, Froment P. 2011 Diet, welfare and male and female bird fertility: the complex relationship. *Prod. Anim.* **24**, 171–180. (doi:10.20870/productions-animales.2011.24.2.3251)
59. Eitan Y, Soller M. 2009 Problems associated with broiler breeder entry into lay: a review and hypothesis. *Worlds Poult. Sci. J.* **65**, 641–648. (doi:10.1017/S0043933909000452)
60. Richards MP, Poch SM, Coon CN, Rosebrough RW, Ashwell CM, McMurtry JP. 2003 Feed restriction significantly alters lipogenic gene expression in broiler breeder chickens. *J. Nutr.* **133**, 707–715. (doi:10.1093/jn/133.3.707)
61. Chen SE, McMurtry JP, Walzem RL. 2006 Overfeeding-induced ovarian dysfunction in broiler breeder hens is associated with lipotoxicity. *Poult. Sci.* **85**, 70–81. (doi:10.1093/ps/85.1.70)
62. De Jong IC, Guémené D. 2011 Major welfare issues in broiler breeders. *Worlds Poult. Sci. J.* **67**, 73–82. (doi:10.1017/S0043933911000067)
63. Jamieson IG. 2004 No evidence that dietary nutrient deficiency is related to poor reproductive success of translocated takahe. *Biol. Conserv.* **115**, 165–170. (doi:10.1016/S0006-3207(03)00216-7)
64. Attia YA, Abdalah AA, Zeweil HS, Bovera F, El-Din AA, Araft MA. 2010 Effect of inorganic or organic selenium supplementation on productive performance, egg quality and some physiological traits of dual-purpose breeding hens. *Czech J. Anim. Sci.* **55**, 505–519. (doi:10.17221/1702-CJAS)
65. Lewis PD. 2004 Responses of domestic fowl to excess iodine: a review. *Br. J. Nutr.* **91**, 29–39. (doi:10.1079/BJN20031017)
66. Whitehead CC, Shannon DW. 1974 The control of egg production using a low-sodium diet. *Br. Poult. Sci.* **15**, 429–434. (doi:10.1080/00071667408416130)
67. Yaripour M, Seidavi A, Dadashbeiki M, Laudadio V, Tufarelli V, Ragni M, Payan-Carreira R. 2018 Impact of dietary supra-nutritional levels of vitamins A and E on fertility traits of broiler breeder hens in late production phase. *Agriculture* **8**, 149. (doi:10.3390/agriculture8100149)
68. Miao LP, Li LL, Zhu MK, Dong XY, Elwan HAM, Zou XT. 2019 Excess dietary fluoride affects laying performance, egg quality, tissue retention, serum biochemical indices, and reproductive hormones of laying hens. *Poult. Sci.* **98**, 6873–6879. (doi:10.3382/ps/pez443)
69. Barnett J, Hemsworth P, Newman E. 1992 Fear of humans and its relationship with productivity in laying hens at commercial farms. *Br. Poult. Sci.* **33**, 699–710. (doi:10.1080/00071669208417510)
70. Jones B. 1996 Fear and adaptability in poultry: insights, implications and imperatives. *Worlds Poult. Sci. J.* **52**, 131–174. (doi:10.1079/WPS19960013)
71. Adams A, Craig J. 1985 Effect of crowding and cage shape on productivity and profitability of caged layers: A survey. *Poult. Sci.* **64**, 238–242. (doi:10.3382/ps.0640238)
72. Lara L, Rostagno M. 2013 Impact of heat stress on poultry production. *Animals* **3**, 356–369. (doi:10.3390/ani3020356)
73. Nardone A, Ronchi B, Lacetera N, Ranieri MS, Bernabucci U. 2010 Effects of climate changes on animal production and sustainability of livestock systems. *Livest. Sci.* **130**, 57–69. (doi:10.1016/j.livsci.2010.02.011)
74. Deng W, Dong XF, Tong JM, Zhang Q. 2012 The probiotic *Bacillus licheniformis* ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult. Sci.* **91**, 575–582. (doi:10.3382/ps.2010-01293)
75. Mack LA, Felver-Gant JN, Dennis RL, Cheng HW. 2013 Genetic variations alter production and behavioral responses following heat stress in 2 strains of laying hens. *Poult. Sci.* **92**, 285–294. (doi:10.3382/ps.2012-02589)
76. Walsh BS, Parratt SR, Hoffmann AA, Atkinson D, Snook RR, Bretman A, Price TAR. 2019 The impact of climate change on fertility. *Trends Ecol. Evol.* **34**, 249–259. (doi:10.1016/j.tree.2018.12.002)
77. Hurley LL, McDiarmid CS, Friesen CR, Griffith SC, Rowe M. 2018 Experimental heatwaves negatively impact sperm quality in the zebra finch. *Proc. R. Soc. B* **285**, 20172547. (doi:10.1098/rspb.2017.2547)
78. Reed TE, Kruuk LEB, Wanless S, Frederiksen M, Cunningham EJA, Harris MP. 2008 Reproductive senescence in a long-lived seabird: rates of decline in late-life performance are associated with varying costs of early reproduction. *Am. Nat.* **171**, E89–E101. (doi:10.1086/524957)
79. Balbontin J, Møller AP. 2015 Environmental conditions during early life accelerate the rate of senescence in a short-lived passerine bird. *Ecology* **96**, 948–959. (doi:10.1890/14-1274.1)
80. Spagopoulou F, Teplitsky C, Lind MI, Chantepie S, Gustafsson L, Maklakov AA. 2020 Silver-spoon upbringing improves early-life fitness but promotes reproductive ageing in a wild bird. *Ecol. Lett.* **23**, 994–1002. (doi:10.1111/ele.13501)
81. Sockman KW, Williams TD, Dawson A, Ball GF. 2004 Prior experience with photostimulation enhances photo-induced reproductive development in female European starlings: a possible basis for the age-related increase in avian reproductive performance. *Biol. Reprod.* **71**, 979–986. (doi:10.1095/biolreprod.104.029751)
82. Salvante KG, Dawson A, Aldredge RA, Sharp PJ, Sockman KW. 2013 Prior experience with photostimulation enhances photo-induced reproductive response in female house finches. *J. Biol. Rhythms* **28**, 38–50. (doi:10.1177/0748730412468087)
83. Holmes DJ, Thomson SL, Wu J, Ottinger MA. 2003 Reproductive aging in female birds. *Exp. Gerontol.* **38**, 751–756. (doi:10.1016/S0531-5565(03)00103-7)
84. Brown ME, Keefer CL, Songsasen N. 2019 Factors affecting captive whooping crane egg fertility: a retrospective analysis. *J. Wildl. Manage.* **83**, 1377–1386. (doi:10.1002/jwmg.21717)
85. Ottinger MA. 2007 Neuroendocrine aging in birds: comparing lifespan differences and conserved mechanisms. *Ageing Res. Rev.* **6**, 46–53. (doi:10.1016/j.arr.2007.02.006)
86. Finch CE, Holmes DJ. 2010 Ovarian aging in developmental and evolutionary contexts. *Ann. N. Y. Acad. Sci.* **1204**, 82–94. (doi:10.1111/j.1749-6632.2010.05610.x)
87. Ottinger MA, Abdelnabi M, Quinn M, Golden N, Wu J, Thompson N. 2002 Reproductive consequences of EDCs in birds. *Neurotoxicol. Teratol.* **24**, 17–28. (doi:10.1016/S0892-0362(01)00195-7)
88. Borgeest C, Greenfield C, Tomic D, Flaws JA. 2002 The effects of endocrine disrupting chemicals on the ovary. *Front. Biosci.* **7**, 1941–1948. (doi:10.2741/borgees)
89. Patisaul HB, Fenton SE, Aylor D. 2018 Animal models of endocrine disruption. *Best Pract. Res. Clin. Endocrinol. Metab.* **32**, 283–297. (doi:10.1016/j.beem.2018.03.011)
90. Crews D, Willingham E, Skipper JK. 2000 Endocrine disruptors: present issues, future directions. *Q. Rev. Biol.* **75**, 243–260. (doi:10.1086/393498)
91. Ainley DG, Grau CR, Roudybush TE, Morrell SH, Utts JM. 1981 Petroleum ingestion reduces reproduction in Cassin's auklets. *Mar. Pollut. Bull.* **12**, 314–317. (doi:10.1016/0025-326X(81)90083-7)
92. Fry DM. 1995 Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **103**, 165–171.
93. Fernie KJ, Shutt JL, Letcher RJ, Ritchie IJ, Bird DM. 2009 Environmentally relevant concentrations of DE-71 and HBCD alter eggshell thickness and reproductive success of American kestrels. *Environ. Sci. Technol.* **43**, 2124–2130. (doi:10.1021/es8027346)
94. Rahman MS, Sasanami T, Mori M. 2007 Effects of cadmium administration on reproductive performance of Japanese quail (*Coturnix japonica*). *J. Poult. Sci.* **44**, 92–97. (doi:10.2141/jpsa.44.92)
95. Gasparik J, Venglarcik J, Slamecka J, Kropil R, Smehyl P, Kopecky J. 2012 Distribution of lead in selected organs and its effect on reproduction parameters of pheasants (*Phasianus colchicus*) after an experimental per oral administration. *J. Environ. Sci. Heal. Part A* **47**, 1267–1271. (doi:10.1080/10934529.2012.672127)
96. He Y, Wang L, Li X, Zhao H. 2020 The effects of chronic lead exposure on the ovaries of female juvenile Japanese quails (*Coturnix japonica*): developmental delay, histopathological alterations, hormone release disruption and gene expression disorder. *Ecotoxicol. Environ. Saf.* **205**, 111338. (doi:10.1016/j.ecoenv.2020.111338)
97. Yuan C, Song HH, Jiang YJ, Azzam MMM, Zhu S, Zou XT. 2013 Effects of lead contamination in feed on laying performance, lead retention of

organs and eggs, protein metabolism, and hormone levels of laying hens. *J. Appl. Poult. Res.* **22**, 878–884. (doi:10.3382/japr.2013-00801)

98. Varian-Ramos CW, Swaddle JP, Cristol DA. 2014 Mercury reduces avian reproductive success and imposes selection: an experimental study with adult- or lifetime-exposure in zebra finch. *PLoS ONE* **9**, e95674. (doi:10.1371/journal.pone.0095674)
99. Zala SM, Penn DJ. 2004 Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Anim. Behav.* **68**, 649–664. (doi:10.1016/j.anbehav.2004.01.005)
100. Giesy JP, Feyk LA, Jones PD, Kannan K, Sanderson T. 2003 Review of the effects of endocrine-disrupting chemicals in birds. *Pure Appl. Chem.* **75**, 2287–2303. (doi:10.1351/pac200375112287)
101. Fidler AE, Zwart S, Pharis RP, Weston RJ, Lawrence SB, Jansen P, Elliott G, Merton DV. 2000 Screening the foods of an endangered parrot, the kakapo (*Strigops habroptilus*), for oestrogenic activity using a recombinant yeast bioassay. *Reprod. Fertil. Dev.* **41**, 2. (doi:10.1071/RD00041)
102. Bernanke J, Köhler H-R. 2009 The impact of environmental chemicals on wildlife vertebrates. *Rev. Environ. Contam. Toxicol.* **198**, 1–47. (doi:10.1007/978-0-387-09647-6_1)
103. Ottinger MA, Bohannon M, Carpenter L, Carro T, Rochester JR, Dean K. 2014 Actions of toxicants and endocrine-disrupting chemicals in birds. In *Sturkie's avian physiology* (ed. CG Scanes), pp. 979–1001. Amsterdam, The Netherlands: Elsevier Science & Technology.
104. Ottinger MA, Abdelnabi MA, Henry P, McGary S, Thompson N, Wu JM. 2001 Neuroendocrine and behavioral implications of endocrine disrupting chemicals in quail. *Horm. Behav.* **40**, 234–247. (doi:10.1006/hbeh.2001.1695)
105. Ottinger MA, Quinn MJ, Lavoie E, Abdelnabi MA, Thompson N, Hazelton JL, Wu JM, Beavers J, Jaber M. 2005 Consequences of endocrine disrupting chemicals on reproductive endocrine function in birds: establishing reliable end points of exposure. *Domest. Anim. Endocrinol.* **29**, 411–419. (doi:10.1016/j.domaniend.2005.02.038)
106. Ottinger MA, Dean KM. 2011 Neuroendocrine impacts of endocrine-disrupting chemicals in birds: life stage and species sensitivities. *J. Toxicol. Environ. Heal – Part B Crit. Rev.* **14**, 413–422. (doi:10.1080/10937404.2011.578560)
107. Rangel PL, Gutierrez CG. 2014 Reproduction in hens: is testosterone necessary for the ovulatory process? *Gen. Comp. Endocrinol.* **203**, 250–261. (doi:10.1016/j.ygcen.2014.03.040)
108. Mishra B, Sah N, Wasti S. 2019 Genetic and hormonal regulation of egg formation in the oviduct of laying hens. In *Poultry*, pp. 5–6. London, UK: IntechOpen.
109. Nakao N *et al.* 2007 Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology* **148**, 3031–3038. (doi:10.1210/en.2007-0044)
110. Rissman EF, Ascenzi M, Johnson P, Adkins-Regan E. 1984 Effect of embryonic treatment with oestradiol benzoate on reproductive morphology, ovulation and oviposition and plasma LH concentrations in female quail (*Coturnix coturnix japonica*). *J. Reprod. Fertil.* **71**, 411–417. (doi:10.1530/jrf.0.0710411)
111. Soliman KFA, Huston TM. 1978 Inhibitory effect of serotonin on ovulation in the domestic fowl, *Gallus domesticus*. *Anim. Reprod. Sci.* **1**, 69–73. (doi:10.1016/0378-4320(78)90011-8)
112. Rangel PL, Sharp PJ, Gutierrez CG. 2006 Testosterone antagonist (flutamide) blocks ovulation and preovulatory surges of progesterone, luteinizing hormone and oestradiol in laying hens. *Reproduction* **131**, 1109–1114. (doi:10.1530/rep.1.01067)
113. Ritchie M. 2014 Neuroanatomy and physiology of the avian hypothalamic/pituitary axis: clinical aspects. *Vet. Clin. North Am.–Exot. Anim. Pract.* **17**, 13–22. (doi:10.1016/j.cvex.2013.09.005)
114. Gaskins LA, Bergman L. 2011 Surveys of avian practitioners and pet owners regarding common behavior problems in psittacine birds. *J. Avian Med. Surg.* **25**, 111–118. (doi:10.1647/2010-027.1)
115. Zhao Q *et al.* 2017 Decreased egg production in laying hens associated with infection with genotype 3 avian hepatitis E virus strain from China. *Vet. Microbiol.* **203**, 174–180. (doi:10.1016/j.vetmic.2017.03.005)
116. Bonfante F *et al.* 2018 A G1-lineage H9N2 virus with oviduct tropism causes chronic pathological changes in the infundibulum and a long-lasting drop in egg production. *Vet. Res.* **49**, 83. (doi:10.1186/s13567-018-0575-1)
117. Crinion RAP, Ball RA, Hofstad MS. 1971 Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day old. *Avian Dis.* **15**, 32–41.
118. Srinivasan P, Balasubramaniam GA, Murthy TRGK, Balachandran P. 2013 Bacteriological and pathological studies of egg peritonitis in commercial layer chicken in Namakkal area. *Asian Pac. J. Trop. Biomed.* **3**, 988–994. (doi:10.1016/S2221-1691(13)60191-4)
119. Apperson K, Bird K, Cherian G, Löhr C. 2017 Histology of the ovary of the laying hen (*Gallus domesticus*). *Vet. Sci.* **4**, 66. (doi:10.3390/vetsci4040066)
120. Murdoch WJ, Van Kirk EA, Alexander BM. 2005 DNA damages in ovarian surface epithelial cells of ovulatory hens. *Exp. Biol. Med.* **230**, 429–433. (doi:10.1177/15353702-0323006-11)
121. Barnes MN, Berry WD, Straughn JM, Kirby TO, Leath CA, Huh WK, Grizzle WE, Partridge EE. 2002 A pilot study of ovarian cancer chemoprevention using medroxyprogesterone acetate in an avian model of spontaneous ovarian carcinogenesis. *Gynecol. Oncol.* **87**, 57–63. (doi:10.1006/gyno.2002.6806)
122. Rodriguez GC *et al.* 2002 Progestin-induced apoptosis in the macaque ovarian epithelium: differential regulation of transforming growth factor-β. *J. Natl. Cancer ilstitute* **94**, 50–60. (doi:10.1093/jnci/94.1.50)
123. Fasenko GM, Robinson FE, Danforth BL, Zelter I. 2000 An examination of fertility, hatchability, embryo mortality, and chick weight in double versus single-yolked broiler breeder eggs. *Can. J. Anim. Sci.* **80**, 489–493. (doi:10.4141/A99-090)
124. Salamon A, Kent J. 2014 Changes during incubation within double-yolked duck (*Anas platyrhynchos domesticus*) eggs: yolk position, mortality, hatchability and the importance of optimal egg size. *Int. J. Poult. Sci.* **13**, 6. (doi:10.3923/ijps.2014.695.702)
125. Briskie JV, Montgomerie R. 1997 Sexual selection and the intromittent organ of birds. *J. Avian Biol.* **28**, 73–86. (doi:10.2307/3677097)
126. Coker CR, McKinney F, Hays H, Briggs SV, Cheng KM. 2002 Intromittent organ morphology and testis size in relation to mating system in waterfowl. *Auk* **119**, 403–413. (doi:10.1093/auk/119.2.403)
127. Previatto DM, Gonçalves GL, Ortiz MC, de Melo-Soares CD, Posso SR. 2018 Morphology of the copulatory organ in *Ortalis canicollis* (Aves: Cracidae) and early evolution of the phallus in birds. *Acta Zool.* **99**, 42–51. (doi:10.1111/azo.12191)
128. Roberts V. 2006 Breeding and egg production. *Natl. Anim. Dis. Inf. Serv.*, Part 6. See http://www.nadis.org.uk/disease-a-z/poultry/diseases-of-farmyard-poultry/part-6-breeding-and-egg-production/ (accessed 31 January 2020).
129. Robinson FE, Wilson JL, Yu MW, Fasenko GM, Hardin RT. 1993 The relationship between body weight and reproductive efficiency in meat-type chickens. *Poult. Sci.* **72**, 912–922. (doi:10.3382/ps.0720912)
130. Török J, Michl G, Garamszegi LZ, Barna J. 2003 Repeated inseminations required for natural fertility in a wild bird population. *Proc. R. Soc. Lond. B* **270**, 641–647. (doi:10.1098/rspb.2002.2257)
131. Hunter FM, Petrie M, Otronen M, Birkhead T, Pape Møller A. 1993 Why do females copulate repeatedly with one male? *Trends Ecol. Evol.* **8**, 21–26. (doi:10.1016/0169-5347(93)90126-A)
132. Westneat DF, Rambo TB. 2000 Copulation exposes female red-winged blackbirds to bacteria in male semen. *J. AVIAN Biol.* **31**, 1–7. (doi:10.1034/j.1600-048X.2000.310101.x)
133. White J, Mirleau P, Danchin E, Mulard H, Hatch SA, Heeb P, Wagner RH. 2010 Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecol. Lett.* **13**, 1515–1524. (doi:10.1111/j.1461-0248.2010.01542.x)
134. Brillard JP. 2003 Practical aspects of fertility in poultry. *Worlds Poult. Sci. J.* **59**, 441–446. (doi:10.3184/175815509X431803)
135. Nestor KE, Brown KI. 1968 Method and frequency of artificial insemination and turkey fertility. *Poult. Sci.* **47**, 717–721. (doi:10.3382/ps.0470717)
136. Mohan J, Sharma SK, Kolluri G, Dhama K. 2018 History of artificial insemination in poultry, its components and significance. *Worlds Poult. Sci. J.* **74**, 1–14. (doi:10.1017/S0043933918000430)
137. Breque C, Surai P, Brillard JP. 2003 Roles of antioxidants on prolonged storage of avian spermatozoa in vivo and in vitro. *Mol. Reprod. Dev.* **66**, 314–323. (doi:10.1002/mrd.10347)
138. Yoshimura Y, Okamoto T, Tamura T. 1997 Localisation of MHC class II, lymphocytes and immunoglobulins in the oviduct of laying and

moulting hens. *Br. Poult. Sci.* **38**, 590–596. (doi:10.1080/00071669708418041)

139. Nii T, Isobe N, Yoshimura Y. 2014 Effects of avian infectious bronchitis virus antigen on eggshell formation and immunoreaction in hen oviduct. *Theriogenology* **81**, 1129–1138. (doi:10.1016/j.theriogenology.2014.02.002)
140. Hemmings N, Birkhead TR. 2015 Polyspermy in birds: sperm numbers and embryo survival. *Proc. R. Soc. B* **282**, 20151682. (doi:10.1098/rspb.2015.1682)
141. Brillard JP, Bakst MR. 1990 Quantification of spermatozoa in the sperm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. *Biol. Reprod.* **43**, 271–275. (doi:10.1095/biolreprod43.2.271)
142. Hemmings N, Birkhead TR, Brillard JP, Froment P, Briere S. 2015 Timing associated with oviductal sperm storage and release after artificial insemination in domestic hens. *Theriogenology* **83**, 1174–1178.e1. (doi:10.1016/j.theriogenology.2014.12.022)
143. Birkhead TR, Biggins JD. 1998 Sperm competition mechanisms in birds: models and data. *Behav. Ecol.* **9**, 253–260. (doi:10.1093/beheco/9.3.253)
144. Johnson A. 2014 Reproduction in the female. In *Sturkie's avian physiology* (ed. CG Scanes), pp. 635–666. Amsterdam, The Netherlands: Elsevier Science & Technology.
145. Birkhead TR. 1992 Sperm storage and the fertile period in the Bengalese finch. *Auk* **109**, 620–625.
146. Birkhead TR, Brillard JP. 2007 Reproductive isolation in birds: postcopulatory prezygotic barriers. *Trends Ecol. Evol.* **22**, 266–272. (doi:10.1016/j.tree.2007.02.004)
147. Sasanami T, Matsuzaki M, Mizushima S, Hiyama G. 2013 Sperm storage in the female reproductive tract in birds. *J. Reprod. Dev.* **59**, 334–338. (doi:10.1146/annurev-animal-021815-111350)
148. Pizzari T, Birkhead TR. 2000 Female feral fowl eject sperm of subdominant males. *Nature* **405**, 787–789. (doi:10.1038/35015558)
149. Møller AP, Mousseau TA, Rudolfsen G. 2008 Females affect sperm swimming performance: a field experiment with barn swallows *Hirundo rustica*. *Behav. Ecol.* **19**, 1343–1350. (doi:10.1093/beheco/arn068)
150. Huang A, Isobe N, Yoshimura Y. 2017 Changes in localization and density of CD63-positive exosome-like substances in the hen oviduct with artificial insemination and their effect on sperm viability. *Theriogenology* **101**, 135–143. (doi:10.1016/j.theriogenology.2017.06.028)
151. Bakst MR. 2011 Role of the oviduct in maintaining sustained fertility in hens. *J. Anim. Sci.* **89**, 1323–1329. (doi:10.2527/jas.2010-3663)
152. Van Krey HP, Schuppin GT, Denbow DM, Hulet RM. 1987 Turkey breeder hen infertility associated with plasma cells in the uterovaginal sperm storage glands. *Theriogenology* **27**, 913–921. (doi:10.1016/0093-691X(87)90213-5)
153. Higaki K, Yoshimura Y, Tamura TO. 1995 Localization of spermatozoa and leukocytes in vagina and uterovaginal junction after copulation in Japanese quail (*Coturnix coturnix japonica*). *Japanese Poult. Sci.* **32**, 387–393. (doi:10.2141/jpsa.32.387)
154. Khillare GS, Sastry KVH, Agrawal R, Prakash A, Mohan J, Singh RP. 2018 Molecular and biochemical events in sperm storage tubules of oviduct in birds. *Worlds Poult. Sci. J.* **74**, 35–44. (doi:10.1017/S004393391700085X)
155. Zeh JA, Zeh DW. 1997 The evolution of polyandry II: post-copulatory defences against genetic incompatibility. *Proc. R. Soc. Lond. B* **264**, 69–75. (doi:10.1098/rspb.1997.0010)
156. Løvlie H, Gillingham MAF, Worley K, Pizzari T, Richardson DS. 2013 Cryptic female choice favours sperm from major histocompatibility complex-dissimilar males. *Proc. R. Soc. B* **280**, 20131296. (doi:10.1098/rspb.2013.1296)
157. Hasson O, Stone L. 2009 Male infertility, female fertility and extrapair copulations. *Biol. Rev.* **84**, 225–244. (doi:10.1111/j.1469-185X.2008.00068.x)
158. Santos TC, Murakami AE, Oliveira CAL, Giraldelli N. 2013 Sperm-egg interaction and fertility of Japanese breeder quails from 10 to 61 weeks. *Poult. Sci.* **92**, 205–210. (doi:10.3382/ps.2012-02536)
159. Brillard JP, Beaumont C, Scheller MF. 1998 Physiological responses of hens divergently selected on the number of chicks obtained from a single insemination. *J. Reprod. Fertil.* **114**, 111–117. (doi:10.1530/jrf.0.1140111)
160. Holm L, Ekwall H, Wishart GJ, Ridderstråle Y. 2000 Localization of calcium and zinc in the sperm storage tubules of chicken, quail and turkey using X-ray microanalysis. *J. Reprod. Fertil.* **118**, 331–336. (doi:10.1530/reprod/118.2.331)
161. Matsuzaki M, Mizushima S, Hiyama G, Hirohashi N, Shiba K. 2015 Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. *Sci. Rep.* **5**, 1–12. (doi:10.1038/srep17643)
162. Huang A, Isobe N, Obitsu T, Yoshimura Y. 2016 Expression of lipases and lipid receptors in sperm storage tubules and possible role of fatty acids in sperm survival in the hen oviduct. *Theriogenology* **85**, 1334–1342. (doi:10.1016/j.theriogenology.2015.12.020)
163. Bakst MR, Bauchan G. 2015 Apical blebs on sperm storage tubule epithelial cell microvilli: their release and interaction with resident sperm in the turkey hen oviduct. *Theriogenology* **83**, 1438–1444. (doi:10.1016/j.theriogenology.2015.01.016)
164. Das S, Nagasaka N, Yoshimura Y. 2005 Effects of repeated artificial insemination on the structure and function of oviducal sperm storage tubules in hens. *J. Poult. Sci.* **42**, 39–47. (doi:10.2141/jpsa.42.39)
165. Das SC, Isobe N, Yoshimura Y. 2008 Mechanism of prolonged sperm storage and sperm survivability in hen oviduct: a review. *Am. J. Reprod. Immunol.* **60**, 477–481. (doi:10.1111/j.1600-0897.2008.00651.x)
166. Das SC, Nagasaka N, Yoshimura Y. 2006 Changes in the expression of estrogen receptor mRNA in the utero-vaginal junction containing sperm storage tubules in laying hens after repeated artificial insemination. *Theriogenology* **65**, 893–900. (doi:10.1016/j.theriogenology.2005.07.004)
167. Ciccone NA, Sharp PJ, Wilson PW, Dunn IC. 2005 Changes in reproductive neuroendocrine mRNAs with decreasing ovarian function in ageing hens. *Gen. Comp. Endocrinol.* **144**, 20–27. (doi:10.1016/j.ygcen.2005.04.009)
168. Mendonca T, Cadby AJ, Hemmings N. 2019 Sperm gatekeeping: 3D imaging reveals a constricted entrance to zebra finch sperm storage tubules. *Biophys. J.* **117**, 2180–2187. (doi:10.1101/451484)
169. Ito T, Yoshizaki N, Tokumoto T, Ono H, Yoshimura T, Tsukada A, Kansaku N, Sasanami T. 2011 Progesterone is as sperm-releasing factor from the sperm-storage tubules in birds. *Endocrinology* **152**, 3952–3962. (doi:10.1210/en.2011-0237)
170. Hiyama G, Matsuzaki M, Mizushima S, Dohra H, Ikegami K, Yoshimura T, Shiba K, Inaba K, Sasanami T. 2014 Sperm activation by heat shock protein 70 supports the migration of sperm released from sperm storage tubules in Japanese quail (*Coturnix japonica*). *Reproduction* **147**, 167–178. (doi:10.1530/REP-13-0439)
171. Rozenboim I, Tako E, Gal-Garber O, Proudman JA, Uni Z. 2007 The effect of heat stress on ovarian function of laying hens. *Poult. Sci.* **86**, 1760–1765. (doi:10.1093/ps/86.8.1760)
172. Allen T, Grigg G. 1957 Sperm transport in the fowl. *Aust. J. Agric. Res.* **8**, 788–789. (doi:10.1071/AR9570788)
173. Bakst MR, Donoghue AM, Yoho DE, Moyle JR, Whipple SM, Camp MJ, Liu GQ, Bramwell RK. 2010 Comparisons of sperm storage tubule distribution and number in 4 strains of mature broiler breeders and in turkey hens before and after the onset of photostimulation. *Poult. Sci.* **89**, 986–992. (doi:10.3382/ps.2009-00481)
174. Spargo SC, Hope RM. 2003 Evolution and nomenclature of the zona pellucida gene family. *Biol. Reprod.* **68**, 358–362. (doi:10.1095/biolreprod.102.008086)
175. Berlin S, Qu L, Ellegren H. 2008 Adaptive evolution of gamete-recognition proteins in birds. *J. Mol. Evol.* **67**, 488–496. (doi:10.1007/s00239-008-9165-6)
176. Nishio S, Kohno Y, Iwata Y, Arai M, Okumura H, Oshima K, Nadano D, Matsuda T. 2014 Glycosylated chicken ZP2 accumulates in the egg coat of immature oocytes and remains localized to the germinal disc region of mature eggs. *Biol. Reprod.* **91**, 1–10. (doi:10.1095/biolreprod.114.119826)
177. Perry MM, Gilbert AB, Evans AJ. 1978 The structure of the germinal disc region of the hen's ovarian follicle during the rapid growth phase. *J. Anat.* **127**, 379–392.
178. Bakst M, Howarth B. 1977 Hydrolysis of the hen's perivitelline layer by cock sperm *in vitro*. *Biol. Reprod.* **17**, 370–379. (doi:10.1095/BIOLREPROD17.3.370)
179. Bakst M, Howarth B. 1977 The fine structure of the hen's ovum at ovulation. *Biol. Reprod.* **17**, 361–369. (doi:10.1095/biolreprod17.3.361)
180. Robertson L. 1999 Sperm-egg interaction in birds: assays and mechanisms. PhD thesis, University of Abertay, Dundee, UK. See https://rke.abertay.ac.uk/en/studentTheses/sperm-egg-interaction-in-birds (accessed 30 September 2020).
181. Howarth B, Digby ST. 1973 Evidence for the penetration of the vitelline membrane of the hen's

ovum by a trypsin-like acrosomal enzyme. *J. Reprod. Fertil.* **33**, 123–125. (doi:10.1530/jrf.0.0330123)

182. Damaziak K, Kieliszek M, Bucław M. 2020 Characterization of structure and protein of vitelline membranes of precocial (ring-necked pheasant, gray partridge) and superaltricial (cockatiel parrot, domestic pigeon) birds. *PLoS ONE* **15**, 1–24. (doi:10.1371/journal.pone.0228310)
183. Springate L, Frasier TR. 2017 Gamete compatibility genes in mammals: candidates, applications and a potential path forward. *R. Soc. Open Sci.* **4**, 170577. (doi:10.1098/rsos.170577)
184. Noda T, Lu Y, Fujihara Y, Oura S, Koyano T, Kobayashi S, Matzuk MM, Ikawa M. 2020 Sperm proteins SOF1, TMEM95, and SPACA6 are required for sperm-oocyte fusion in mice. *Proc. Natl Acad. Sci. USA* **117**, 11 493–11 502. (doi:10.1073/pnas.1922650117)
185. Miyado K *et al.* 2000 Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321–324. (doi:10.1126/science.287.5451.321)
186. Naokazu I, Masaru O. 2005 The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* **434**, 234–238. (doi:10.1038/nature03362)
187. Bianchi E, Doe B, Goulding D, Wright GJ. 2014 Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* **508**, 483–487. (doi:10.1038/nature13203)
188. Grayson P. 2015 Izumo1 and Juno: The evolutionary origins and coevolution of essential sperm–egg binding partners. *R. Soc. Open Sci.* **2**, 150296. (doi:10.1098/rsos.150296)
189. Trebichalská Z, Holubcová Z. 2020 Perfect date—the review of current research into molecular bases of mammalian fertilization. *J. Assist. Reprod. Genet.* **37**, 243–256. (doi:10.1007/s10815-019-01679-4)
190. Bakst MR, Eastridge J, Malecki IA. 2014 The inner perivitelline layer sperm hole assay: use of filter paper rings for the isolation of the perivitelline layer overlying the germinal disc and new observations on its morphology. *J. Appl. Poult. Res.* **23**, 121–128. (doi:10.3382/japr.2013-00873)
191. Wishart GJ. 1987 Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. *J. Reprod. Fertil.* **80**, 493–498. (doi:10.1530/jrf.0.0800493)
192. Barbato GF, Cramer PG, Hammerstedt RH. 1998 A practical *in vitro* sperm-egg binding assay that detects subfertile males. *Biol. Reprod.* **58**, 686–699. (doi:10.1095/biolreprod58.3.686)
193. Christensen VL, Fairchild BD, Ort DT, Nestor KE. 2005 Dam and sire effects on sperm penetration of the perivitelline layer and resulting fecundity of different lines of turkeys. *J. Appl. Poult. Res.* **14**, 483–491. (doi:10.1093/japr/14.3.483)
194. Firman RC. 2018 Postmating sexual conflict and female control over fertilization during gamete interaction. *Ann. NY Acad. Sci.* **1422**, 48–64. (doi:10.1111/nyas.13635)
195. Hurley LL, Fanson KV, Griffith SC. 2017 Variation in the number of sperm trapped on the perivitelline layer of the egg in three species of estrildid finch. *AUK* **134**, 832–841. (doi:10.1642/AUK-17-41.1)
196. de Camargo C, Gibbs HL, Costa MC, Silveira LF, Rainho CA, Ribolla PEM, Wasko AP, Francisco MR. 2017 Triploidy in a sexually dimorphic passerine provides new evidence for the effect of the W chromosome on secondary sexual traits in birds. *J. Avian Biol.* **48**, 1475–1480. (doi:10.1111/jav.01504)
197. Ayers KL, Smith CA, Lambeth LS. 2013 The molecular genetics of avian sex determination and its manipulation. *Genesis* **51**, 325–336. (doi:10.1002/dvg.22382)
198. Forstmeier W, Ellegren H. 2010 Trisomy and triploidy are sources of embryo mortality in the zebra finch. *Proc. R. Soc. B* **277**, 2655–2660. (doi:10.1098/rspb.2010.0394)
199. Arlt D, Bensch S, Hansson B, Hasselquist D, Westerdahl H. 2004 Observation of a ZZW female in a natural population: Implications for avian sex determination. *Proc. R. Soc. Lond. B* **271**, 249–251. (doi:10.1098/rsbl.2003.0155)
200. Křiženecký J, Sajner J, Vančiková RJ. 1958 A case of a hen yolk with seven blastodiscs. *Poult. Sci.* **37**, 512–514. (doi:10.3382/ps.0370512)
201. Collins JK, Jones KT. 2016 DNA damage responses in mammalian oocytes. *Reproduction* **152**, R15–R22. (doi:10.1530/REP-16-0069)
202. Mihalas BP, Redgrove KA, McLaughlin EA, Nixon B. 2017 Molecular mechanisms responsible for increased vulnerability of the ageing oocyte to oxidative damage. *Oxid. Med. Cell. Longev.* 2017, 1–22. (doi:10.1155/2017/4015874)
203. Patel S, Zhou C, Rattan S, Flaws JA. 2015 Effects of endocrine-disrupting chemicals on the ovary. *Biol. Reprod.* **93**, 1–9. (doi:10.1095/biolreprod.115.130336)
204. García-Rodríguez A, Gosálvez J, Agarwal A, Roy R, Johnston S. 2019 DNA damage and repair in human reproductive cells. *Int. J. Mol. Sci.* **20**, 1–22. (doi:10.3390/ijms20010031)
205. Liu W *et al.* 2017 Dosage effects of ZP2 and ZP3 heterozygous mutations cause human infertility. *Hum. Genet.* **136**, 975–985. (doi:10.1007/s00439-017-1822-7)
206. Gorelick R, Derraugh LJ, Carpinone J, Bertram SM. 2011 Post-plasmogamic pre-karyogamic sexual selection: mate choice inside an egg cell. *Ideas Ecol. Evol.* **4**, 14–23. (doi:10.4033/iee.2011.4.3.n)
207. Carré D, Sardet C. 1984 Fertilization and early development in *Beroe ovata*. *Dev. Biol.* **105**, 188–195. (doi:10.1016/0012-1606(84)90274-4)
208. Stepińska U, Olszańska B. 2003 DNase I and II present in avian oocytes: a possible involvement in sperm degradation at polyspermic fertilisation. *Zygote* **11**, 35–42. (doi:10.1017/S0967199403001059)
209. Damaziak K, Paweska M, Gozdowski D, Niemiec J. 2018 Short periods of incubation, egg turning during storage and broiler breeder hens age for early development of embryos, hatching results, chicks quality and juvenile growth. *Poult. Sci.* **97**, 3264–3276. (doi:10.3382/ps/pey163)
210. Koyanagi F, Nishiyama H. 1981 Fate of spermatozoa that do not participate in fertilization in the domestic fowl. *Cell Tissue Res.* **214**, 89–95. (doi:10.1007/bf00235147)
211. Asersohn K, Brekke P, Hemmings N. 2021 Physiological factors influencing female fertility in birds. FigShare.

# Chapter 3:

# A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches

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*Zebra finch (Taeniopygia guttata) pair*

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# A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches

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Despite constituting an essential component of fitness, reproductive success can vary remarkably between individuals and the causes of such variation are not well understood across taxa. In the zebra finch—a model songbird, almost all the variation in sperm morphology and swimming speed is maintained by a large polymorphic inversion (commonly known as a supergene) on the Z chromosome. The relationship between this polymorphism and reproductive success is not fully understood, particularly for females. Here, we explore the effects of female haplotype, and the combination of male and female genotype, on several primary reproductive traits in a captive population of zebra finches. Despite the inversion polymorphism's known effects on sperm traits, we find no evidence that inversion haplotype influences egg production by females or survival of embryos through to hatching. However, our findings do reinforce existing evidence that the inversion polymorphism is maintained by a heterozygote advantage for male fitness. This work provides an important step in understanding the causes of variation in reproductive success in this model species.

## 1. Introduction

Reproductive success is a central determinant of evolutionary fitness, so we expect traits important for reproduction to be under strong directional selection. Despite this, reproductive output often varies considerably between individuals, and the genetic basis of this variation is not well understood [1–3]. Chromosomal inversions are widespread across taxa, and inversion polymorphisms are increasingly recognized as important in the maintenance of genetic variation [4,5]. Estrildidae finches like the zebra finch (*Taeniopygia guttata*) are particularly prone to inversions [6,7], making them useful for studying the relationship between inversions and the maintenance of reproductive trait variation.

Like all birds, zebra finches have a ZZ/ZW sex chromosome system whereby females are the heterogametic sex (ZW). The zebra finch Z chromosome houses a polymorphic inversion consisting of (at least) three segregating haplotypes: A, B and C (figure 1*a*,*b*) [8,9]. Recombination is highly suppressed within the inverted region in heterozygous males, so mutations that arise on a given genetic background (i.e. haplotype) cannot recombine onto other haplotypes, but instead are preserved and inherited together as a single unit [10,11]. When inversions link multiple loci that act jointly to encode complex phenotypes in a balanced polymorphism, they are commonly referred to as a 'supergene' [8,10,12].

The zebra finch Z inversion polymorphism is large, containing over 600 genes and spanning 86% of the chromosome [6,13]. The inversion's molecular evolution is yet to be fully determined, and while A is likely to be the ancestral haplotype, it is unclear the order in which B and C arose, and whether they were both generated from A or sequentially from one another. Each haplotype has accumulated large genetic differences over time, and all three are maintained at stable frequencies

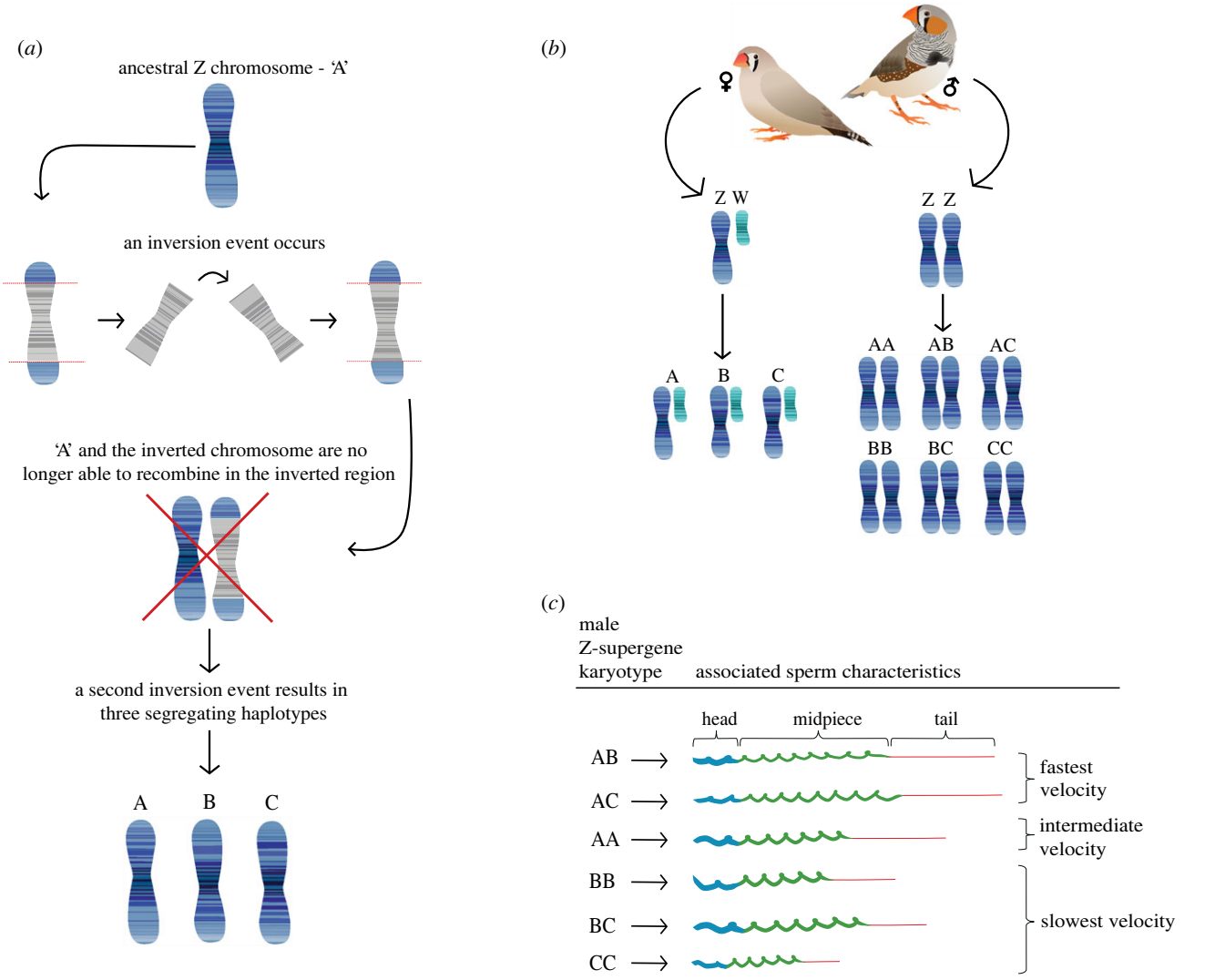


**Figure 1.** (*a*) Schematic illustrating the formation of the zebra finch Z chromosome inversion polymorphism. A large inversion (i.e. the complete end-to-end reversal of gene order) occurred on the ancestral Z chromosome 'A', generating an alternate Z chromosome haplotype. The ancestral and inverted haplotype were then unable to recombine in the inverted region (commonly referred to as a 'supergene'), resulting in a build-up of genetic differences over time. A second inversion independently generated a third haplotype. The grey region illustrates the inverted non-recombining region. Note that we currently do not know the order in which B and C were generated, whether all haplotypes were generated from A or sequentially from one another, or whether additional inversions were involved. Chromosome illustrations do not represent accurate maps. (*b*) Schematic illustrating all inversion genotypes carried by females and males. Female birds are heterogametic, and so female zebra finches carry a single Z haplotype (inherited from their father). Males are homogametic and carry one of six possible karyotypes. (*c*) Schematic illustrating the effect of Z karyotype for male sperm characteristics (scale is approximate). Differences between Z haplotypes almost entirely explain the variation in sperm morphology and swimming speed in the zebra finch [8]. All three haplotypes are maintained at stable frequencies in the population, probably because heterokaryotypic males carrying one A haplotype and one alternative haplotype (i.e. AB or AC males) have the fastest and most successful sperm, which are associated with longer midpieces and relatively long overall length. AA males have intermediate velocity associated with long tails but short midpieces, whereas BB, BC and CC males have sperm characterized by relatively short tails, short overall length, and the slowest velocity. Sperm speed categories are based on velocity data from Kim *et al.* [8].

in both captive and wild populations (frequencies of A, B and C respectively: 0.378, 0.408 and 0.215 in the captive population studied here; and 0.730, 0.199 and 0.122 in the wild) [8]. Importantly, the male inversion karyotype is known to be responsible for almost all the variation in sperm morphology and swimming speed in this species (figure 1*c*), with important consequences for male fertilization success under sperm competition [8]. Sperm morphology is extremely variable between inversion karyotypes, and heterokaryotypic males (AB or AC karyotypes) have the fastest and most successful sperm under a competitive scenario (figure 1*c*). This suggests that all three haplotypes, including those associated with relatively poorly performing sperm (e.g. BB, BC and CC males), are being maintained in the population at least in part by a heterozygote advantage for male reproductive success (figure 1*c*) [8].

Whether the observed overdominance for male reproductive success is the only force maintaining all three inversion haplotypes in this system remains unknown. Females may also influence the maintenance of the inversion polymorphism, some (non-exhaustive and non-mutually exclusive) examples include sexually antagonistic selection, variation in male-female compatibility, or through paternity bias based on male karyotype [4,10]. Females could bias paternity either before copulation via assortative mating; or post-copulation, for example via selective sperm storage, reduced egg investment, reduced incubation effort, or by modifying brood sex ratio [14–19]. Female zebra finches have been known to modify egg investment in response to partner quality [20,21], and can facultatively adjust offspring sex ratio depending on female condition [22]. In some other passerines, females have also been shown to adjust clutch sex ratio depending on mate quality [16,23]. These mechanisms of paternity bias could potentially act to maintain the inversion polymorphism by reinforcing the existing heterozygote advantage, either through propagating the benefits associated with certain male

karyotypes, or by offsetting the disadvantages associated with other karyotypes, or both. However, while previous work has found evidence for strong additive effects of Z-chromosome inversion genotype on several morphological traits [6], whether females can detect and respond to these signals of male inversion karyotype is unknown.

The direct effects of the inversion polymorphism on female fitness have also not yet been fully explored. Sexually antagonistic loci–loci that contain alleles which benefit one sex at the detriment of the other, are predicted to be particularly prevalent in sex-linked regions, owing to how the unique pattern(s) of sex chromosome inheritance affect the evolutionary dynamics of sexually antagonistic selection [24–26]. Whether a Z-linked sexually antagonistic allele will reach fixation is dependent on both the sex in which it benefits, and its dominance coefficient [24–29]. For example, if recessive sexually antagonistic alleles have accumulated within the Z chromosome inversion, they could potentially act to maintain the polymorphism because haplotypes with alleles that are detrimental to males but beneficial in females can reach high frequencies (recessive alleles are always expressed in females). Conversely, the higher dose of the Z in males, coupled with the fact that dosage compensation (the equalization of gene expression between the autosomes and the sex chromosomes in the heterogametic sex) is incomplete on the avian Z, suggests that fully or partially dominant Z-linked male-beneficial alleles could be positively selected, even if sexually antagonistic [24,25,27,30–33]. To determine whether sexually antagonistic selection could be contributing to the maintenance of the Z-chromosome inversion polymorphism, it is necessary to determine whether female reproductive traits also vary based on inversion haplotype, as well as measure the survival probability of offspring with different combinations of haplotypes.

Here, we use an extensive long-term breeding database with data from 1716 genotyped captive zebra finch individuals to analyse the effects of both male and female inversion haplotype (individually and in combination) on reproductive traits including egg production (a female-specific reproductive trait) and survival rates of embryos through to hatching. This work provides an important step in improving our understanding of the consequences of inversion polymorphisms for reproduction, and more broadly the causes of variation in reproductive success in birds from both the male and female perspective.

## 2. Methods

### (a) Animals

All zebra finches belonged to a domesticated population maintained at The University of Sheffield between 1985 and 2016. Birds from this population were separated physically (but not visually or acoustically) by sex unless breeding, in which case a single pair was housed together without access to other individuals and with no opportunity to engage in extra pair copulations. Pairs were selected by researchers and no natural mate choice was permitted. Previously mated females were rested for at least two weeks before re-pairing with a new male to ensure sperm from the previous male was fully depleted from her sperm storage organs (the maximum duration of sperm storage is 13 days in the zebra finch [34]). Paternity was therefore known conclusively for every egg, and sperm did not have to compete for fertilization of the ova. All deceased birds were preserved in −20°C freezers and accessed later for tissue sampling and DNA extraction. All sampled birds were adults.

### (b) DNA extraction

Z chromosome inversion karyotype was already known for 197 male birds from a previous study [8]. Tissue samples (brain or toe tissue) were dissected from an additional 1785 frozen specimens (1071 females and 714 males). Tissue samples were stored at room temperature in 100% ethanol until extraction. DNA was extracted using a standard (plated) ammonium acetate method [35], and DNA samples were stored in a low TE buffer (Tris-HCL (1 M) and EDTA (0.5 M)) at −4°C and later quantified using a FLUOStar fluorometer before diluting to a concentration of 5 ng $\mu l^{-1}$.

### (c) Single nucleotide polymorphism typing

Samples were typed for their single nucleotide polymorphism (SNP) genotypes using kompetitive allele specific polymerase chain reaction (KASP)-genotyping chemistry on an LGC SNPLine system [36] in The University of Sheffield's Molecular Ecology Laboratory. A total of nine SNPs were chosen from a zebra finch high density 600k SNP chip. These SNPs demonstrated the highest fixed allelic differences between haplotypes (see Kim *et al*. [8] for details). Individual assays for each SNP were designed using LGC Genomics Ltd guidelines. Genotype calling was performed using cluster analysis in the software KRAKEN [37]. Diagnostic SNP data obtained from Kim *et al*. [8] was used to call SNP genotypes for each individual [38], and genotype calling was successful for 98% of samples. Unsuccessful calling for 2% of samples was most often owing to amplification failure, indicating insufficient DNA present in the sample. A total of 1753 individuals were typed (1950 including the additional males from the Kim *et al*., dataset). Owing to some uncertainty in the breeding record (missing or ambiguous data), 12% of individuals (233) were removed, leaving a total of 1716 individuals, including 792 males (AA: 139; AB: 229; AC: 111; BB: 168; BC: 115; CC: 30), 924 females (A: 340; B: 376; C: 208) and 1319 unique pairings in the final datasets (see the electronic supplementary material, table S2 for sample sizes of parental genotype combinations) [38].

### (d) Measures of reproductive success

We investigated the effect of female inversion haplotype, male inversion karyotype and the combination of male and female genotype on five reproductive traits:

(i) egg production: measured as the number of eggs laid per clutch (all eggs laid including infertile eggs) (n clutches: 3041; n pairs: 1319; n eggs: 12292);

(ii) egg fertility and early embryo development: measured as the proportion of eggs (per pair) that were fertilized and survived past 3 days of incubation, relative to eggs that were either unfertilized or died within the first 3 days of development (*n* pairs: 454). Failed fertilization and death during early development were not distinguished from one another because, prior to 2008 (when microscopic techniques were developed to accurately determine the fertility status of undeveloped eggs [39]), egg fertility was primarily determined by candling (i.e. shining a light through the eggshell to visualise the contents without opening the egg). Zebra finch embryonic development is only reliably visible via candling after 3 days of incubation, therefore eggs with no visible sign of development by 3 days could have either been unfertilized, or fertilized but the embryo died very early. Eggs with visible signs of development by day 3 of incubation

could be definitively classified as fertilized and had survived the very early stages of development (prior to the formation of blood vessels). Both fertilization failure and early-stage embryo mortality are more likely to be linked to genetic problems (e.g. sperm-egg incompatibility, aneuploidy), parental age effects or variation in female receptivity to sperm, compared to later stage mortality [40];

(iii) hatching success of developing eggs: measured as the proportion of eggs (per clutch) that showed obvious signs of development on candling (i.e. were fertilized and developed to at least day 3 of incubation) and went on to successfully hatch, relative to those in which the embryos died at a relatively late stage of development (i.e. after day 3 of incubation), resulting in hatching failure (*n* pairs: 460; *n* clutches: 1528). Compared to early-stage embryo mortality, late-stage mortality is more likely owing to environmental conditions in the nest and/or inadequate parental care during incubation [40]. Any effect of inversion haplotype or parental haplotype combination on late-stage embryo development is therefore likely to occur through selective parental investment, rather than direct genetic effects;

(iv) offspring sex ratio: measured as the deviation of offspring sex ratio from a 50 : 50 expectation (*n* clutches: 1769, *n* offspring: 880). Only offspring that survived to 100 days were included in the sex ratio data as sex was assigned based on plumage traits at sexual maturity; and

(v) offspring genotype: measured as the consistency of offspring genotypes with Mendelian expectations. For example, under Mendelian expectations, an A female mated to an AB male should produce male offspring that carry either the AA or AB karyotype at a 50 : 50 ratio, and female offspring that carry the A or B haplotype at a 50 : 50 ratio (*n* offspring: 683).

### (e) Statistical analysis

All statistical analyses were conducted in R (v. 4.2.1) [41]. We adopted a Bayesian framework using the 'brms' package [42] for the first four reproductive measures described above: (i) egg production; (ii) egg fertility and early embryo development; (iii) hatching success of developing eggs; and (iv) offspring sex ratio.

For each reproductive measure, we fitted two separate multilevel models. The first model functioned to test the effects of female haplotype and parental haplotype combination on reproduction. This model included female haplotype as a fixed effect, and a second fixed effect variable termed 'haplotypes shared', which refers to the number of inversion haplotypes shared between the male and female, and therefore the degree of genetic similarity at the inversion. For example, a female with haplotype A shares two haplotypes with an AA male, one with AB/AC males, and none with BB/BC/CC males. Attempting to include a direct interaction between male karyotype and female haplotype would be inappropriate owing to the sheer number of possible contrasts and our limited *a priori* knowledge regarding how specific combinations would perform. However, if parental haplotype combination affects reproductive success, we would expect this effect to vary based on the number of haplotypes shared between parents. This model also allows us to interpret the main effect of female haplotype on reproduction, and whether any effect of parental haplotype sharing varies by female haplotype.

The model described above may miss some variation explained by specific male karyotypes. To account for this, we performed a second model including male karyotype as a separate fixed effect (with female haplotype included as a control). For every model, male and female age (scaled and mean centred) were also included as fixed effects, and male and female identity (ID) included as hierarchical groups. Whilst housing conditions were kept relatively consistent across the lifetime of the study population, the large timescales over which this population was kept meant that certain aspects of husbandry varied over time (such as food, conspecific identity and keeper identity). To ensure any effects of husbandry/environmental conditions were controlled for, we included a categorical variable of female birth year as an additional hierarchical group (17 levels, spanning 1997–2013).

Choice of family for Bayesian models was based on a combination of prior knowledge of the data and assessment of model fit using posterior predictive checks. For the 'egg fertility and early embryo development', 'hatching success of developed eggs', and 'sex ratio' models, a binomial family was chosen. For the 'egg production' analysis, all variations of a Poisson distribution, negative binomial and negative binomial hurdle models fit the data very poorly with an extremely poor recovery of the tail ends of the distribution. A continuation ratio family (cRatio) (a highly flexible ordinal model) can be considered appropriate when used with data that is bounded and sequential (the attainment of one level is required for the attainment of the next). cRatio models have been proposed and used in the past for similar ordinal-like count data [43–45], and make logical sense in our case (see the electronic supplementary material, S5 for details). The use of a cRatio model also allows us to tease apart the separate probabilities associated with the attainment of each level, providing us with superior inference over a Poisson or negative binomial model. We present here the results of the cRatio model (see the electronic supplementary material, S5A-C for a summary of additional modelling attempts using more traditional approaches).

Regularizing zero-centred diffuse priors were chosen for all parameters. Models were run across four chains with 2000 warmup and 1000 post warmup iterations, except for the 'hatching success of developed eggs' models which required 5000 warmup and 2500 post warmup iterations to reach a sufficient effective sample size. Model convergence was assessed visually using trace plots and parameter $\hat{R}$ values, none of which exceeded 1.01 indicating good convergence of between- and within-chain estimates. Posterior predictive checks were used to assess the adequacy of model fit.

We used the probability of direction (PD) to determine effect presence and direction, and the 90% highest density interval (HDI) to determine the degree of effect uncertainty. We chose a 90% rather than 95% HDI, because 90% has been suggested to be more stable [46]. The PD describes the proportion of the posterior distribution that is of the median's sign (i.e. a PD of 100% indicates all iterations were either positive or negative). The PD therefore provides the probability that a parameter is either positive or negative. As per convention, we consider PD values greater than 97.5% as substantial evidence of an effect (in combination with an assessment of the 90% HDI), because a PD of 97.5 is highly correlated with a two-sided *p*-value of 0.05. Effect direction is then evident by the sign of the median. All model checks and PD plots can be found in the electronic supplementary material. We performed a variance ratio analysis for each model, to calculate the variance explained by the hierarchical structure of the models (i.e. the 'random effects' – mother ID, father ID and mother birth year).

For the final reproductive measure, 'offspring genotype', we performed an exact binomial test for each combination of parental genotypes, to determine whether offspring genotype differed significantly from Mendelian expectations. Significance from binomial tests was determined by *p*-values of less than 0.05.

## 3. Results

### (a) Egg production

We found no evidence of an effect of either female inversion haplotype, haplotype sharing (the number of haplotypes shared between the male and female, i.e. their genetic similarity at the inversion) or male karyotype on egg production (figure 2; electronic

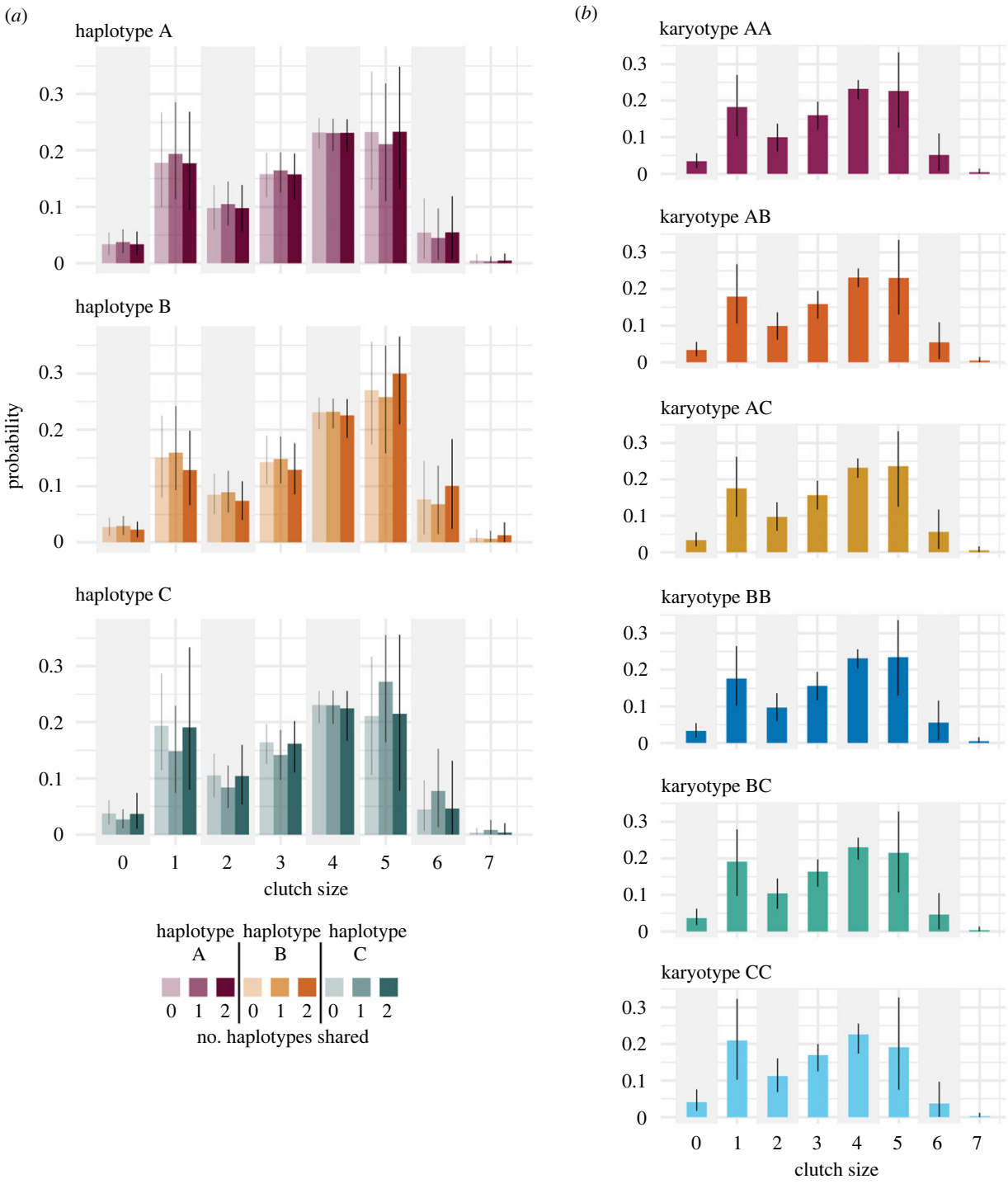


**Figure 2.** (*a*) The probability of laying different clutch sizes with respect to female inversion haplotype: A (purple); B (orange) and C (green), and the degree of parental haplotype sharing: no haplotypes shared (lightest tone), one haplotype shared (medium tone) and two haplotypes shared (darkest tone). Separate probabilities are given for each step-increase in clutch size. (*b*) Variation in the probability of laying different clutch sizes with respect to male inversion karyotype. Panels are split by male karyotype: AA (purple); AB (orange); AC (yellow); BB (dark blue); BC (green) and CC (light blue). Bars represent the posterior medians and error bars are the 90% HDI for the average individual. For a boxplot of the raw data see the electronic supplementary material, figure S1*d*.

supplementary material, figure S1*a–h*). The effect of haplotype sharing was not influenced by female haplotype (figure 2*a*; electronic supplementary material, figure S1*c*).

Across all haplotypes, the most probable clutch size was four or five eggs, after which a single-egged clutch was more probable than any other clutch size. Very few clutches produced more than seven eggs (figure 2*a*). The high number of single-egged clutches is probably the result of an age-related decline in clutch size: we found a strong negative effect of female (but not male) age on egg production (est. = –0.67; 90% HDI: –0.79, –0.52; PD = 100%; electronic supplementary material, figure S1*e,f*). As females aged, there was an increase in the probability of laying a single-egged clutch, and a strong (but smaller) increase in the probability of laying no eggs, while the probability of each step-increase in clutch size also declined with age (none of these age effects were dependant on female inversion haplotype). A summary of the variance decomposition analysis is presented in the electronic supplementary material, table S1.

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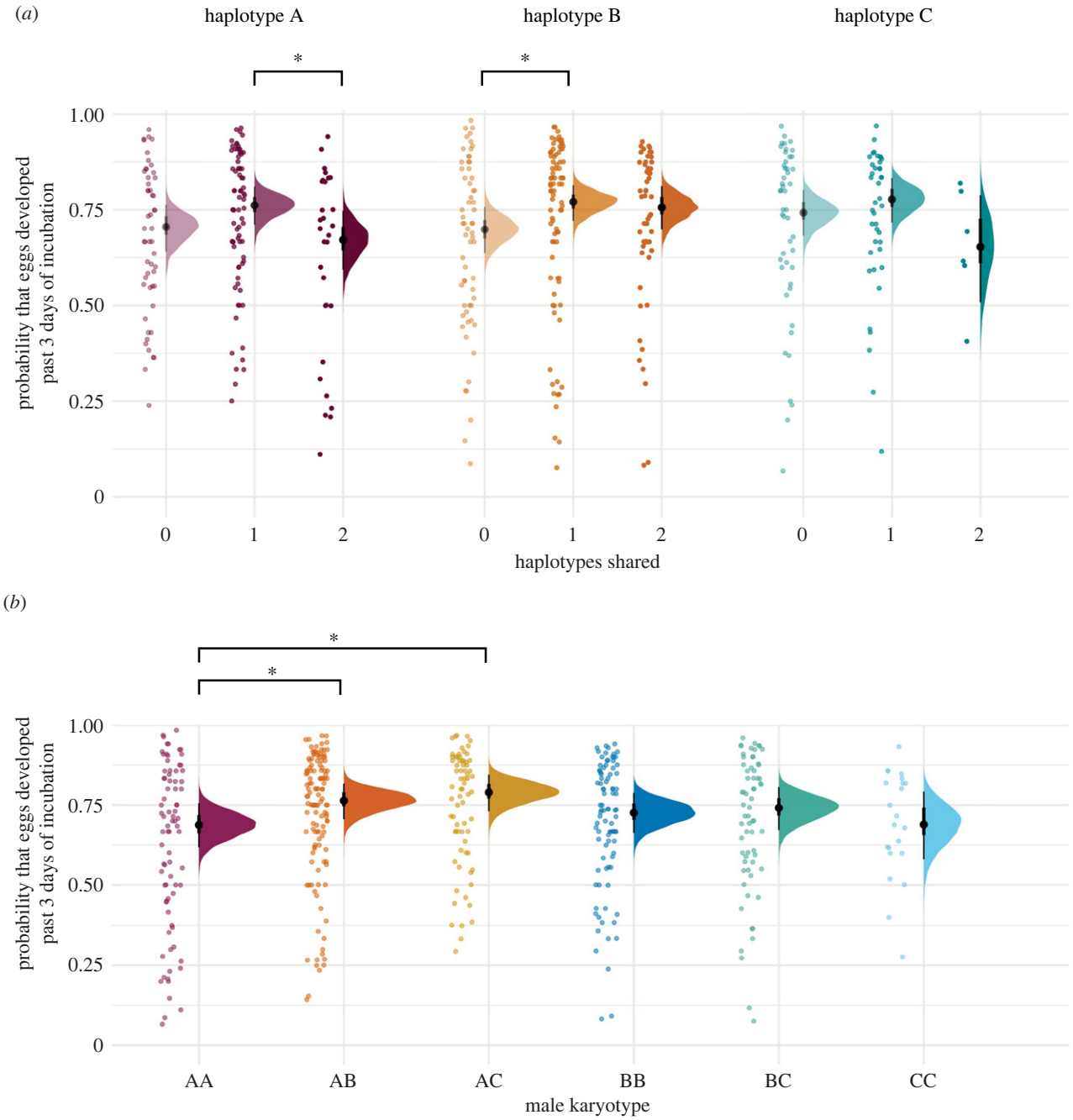


**Figure 3.** (*a*) The probability that eggs will survive early development (relative to being either infertile or dying during early development), with respect to female inversion haplotype: A (purple), B (orange) and C (green), and the degree of parental haplotype sharing: no haplotypes shared (lightest tone), one haplotype shared (medium tone) and two haplotypes shared (darkest tone). Asterisks indicate that the model observed a substantial difference between connections (PD > 97.5%) (A females sharing two haplotypes relative to one haplotype, B females sharing one haplotype relative to no haplotypes). (*b*) The probability that eggs will be fertilized and survive early development, with respect to male inversion karyotype: AA (maroon), AB (orange), AC (yellow), BB (dark blue), BC (green) and CC (light blue). Asterisks indicate that the model observed a substantial difference between connections (PD > 97.5%) (AB and AC relative to AA male karyotypes). Points show the raw observed data, density plots show the posterior distributions (and median and 90% HDI) for the average individual. Points have been jittered to aid visualization and prevent overlaying points.

## (b) Egg fertility and early embryo development

We found no evidence of an effect of female haplotype on the probability of an egg developing past day 3 of incubation (figure 3*a*; electronic supplementary material, figure S2*a*,*b*). Overall, we found no consistent effect of parental haplotype sharing on early embryo development. There were, however, two exceptions: firstly, a clear but small positive effect for B females that shared one haplotype with the male, relative to B females that shared no haplotypes with the male (median = 0.37; 90% HDI: 0.06, 0.66; PD = 97.9%). Specifically, B : AB and B : BC parental combinations produced eggs that were around 40% more likely to be fertilized and survive early development relative to B : AA, B : CC or B : AC parental combinations ($\text{median}_{(\text{odds ratio})} = 1.44$). Secondly, we found a clear negative effect for A females that shared two haplotypes with the male, relative to A females that shared one haplotype with the male (median = −0.45; 90% HDI: −0.81, −0.08; PD = 97.8%). Specifically, A : AA parental combinations were around half as likely to produce eggs that were fertilized and survived early development relative to A : AB and A:AC parental combinations ($\text{median}_{(\text{odds ratio})} = 0.63$) (figure 3*a*; electronic supplementary material, figure S2*c*).

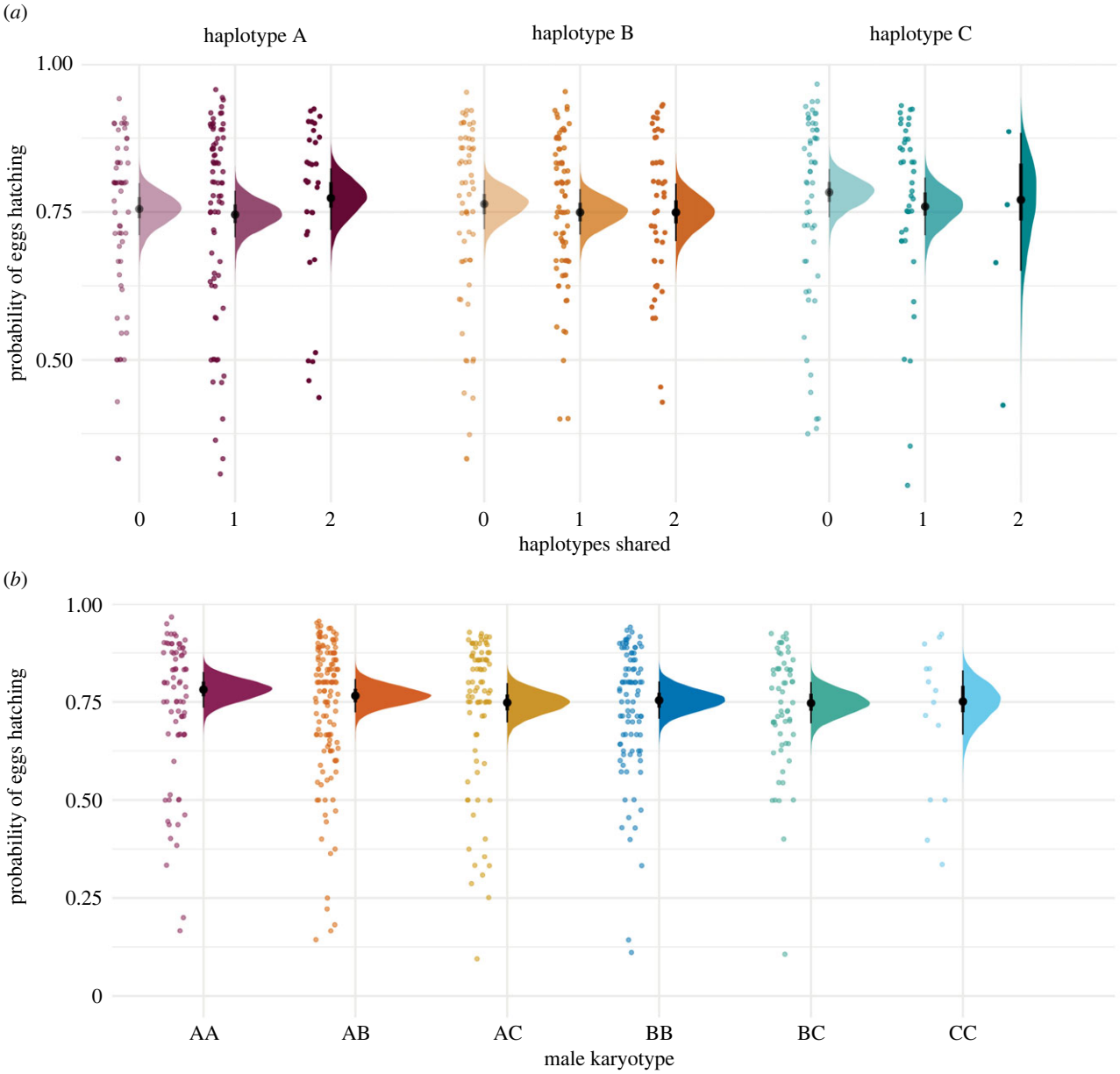


**Figure 4.** (*a*) Variation in the probability that a developing egg (containing an embryo at day 3 of incubation) will survive to hatch, with respect to female inversion haplotype: A (purple), B (orange) and C (green), and the degree of parental haplotype sharing: no haplotypes shared (lightest tone), one haplotype shared (medium tone) and two haplotypes shared (darkest tone). (*b*) Variation in the probability that a developing egg will survive to hatch, with respect to male inversion karyotype: AA (maroon), AB (orange), AC (yellow), BB (dark blue), BC (green) and CC (light blue). Points show the raw observed data, density plots show the posterior distribution (and median and 90% HDI) for the average individual. Points have been jittered to aid visualization and prevent overlaying points.

We found no consistent effect of male karyotype on egg fertility and early embryo development. However, we did find a small but clear negative effect of AA males relative to AB males (median = −0.38; 90% HDI: −0.66, −0.07; PD = 97.8%) and AC males (median = −0.54; 90% HDI: −0.86, −0.17; PD = 99.6%) (figure 3*b*; electronic supplementary material, figure S2*f*,*g*). Specifically, eggs from females paired to AA males were around half as likely to be fertilized or survive early development, relative to eggs from females paired with AB and AC males ($\text{median}_{(\text{odds ratio})}$ = 0.68 and 0.58 respectively). AB and AC carrying males did not differ substantially from any other male karyotype. There was no clear effect of male or female age on egg fertility and early embryo development (electronic supplementary material, figure S2*d*–*e*). A summary of the variance decomposition analysis is presented in the electronic supplementary material, table S1.

## (c) Hatching success of developing eggs

We found no evidence of an effect of female haplotype, haplotype sharing or male karyotype on the hatching success of developing eggs (i.e. those that showed evidence of development at day 3 of incubation) (figure 4; electronic supplementary material, figure S3*a*–*g*). The effect of haplotype sharing was also not influenced by female haplotype (figure 4*a*; electronic supplementary material, figure S3*c*). We also found no evidence of an effect of male or female age on hatching success (electronic supplementary material, figure S3*d*,*e*). A summary of the variance decomposition analysis is presented in the electronic supplementary material, table S1.

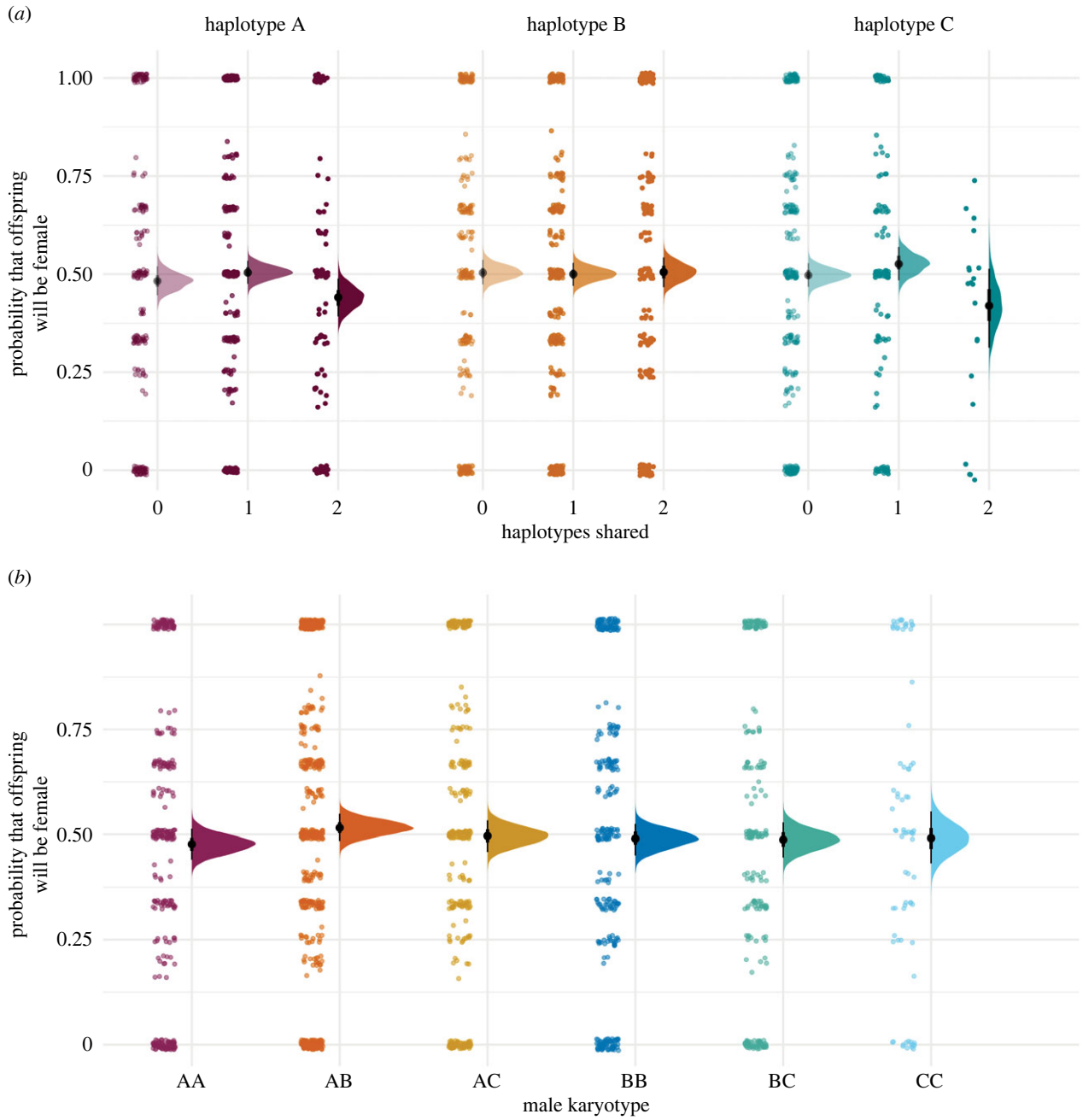


**Figure 5.** (*a*) Variation in the probability that offspring will be female (relative to male) with respect to female inversion haplotype: A (purple), B (orange) and C (green), and parental haplotype sharing: no haplotypes shared (lightest tone), one haplotype shared (medium tone) and two haplotypes shared (darkest tone). (*b*) Variation in the probability that offspring will be female (relative to male), with respect to male inversion karyotype: AA (maroon), AB (orange), AC (yellow), BB (dark blue), BC (green) and CC (light blue). Points show the raw observed data, density plots show the posterior distribution (and median and 90% HDI) for the average individual. Points have been jittered to aid visualization and prevent overlaying points.

### (d) Offspring sex ratio

We found no evidence of an effect of female haplotype, the degree of haplotype sharing or male karyotype on the sex ratio of offspring (figure 5; electronic supplementary material, figure S4*a–g*). The effect of haplotype sharing was not influenced by female haplotype (figure 5*a*; electronic supplementary material, figure S4*c*), and there was also no evidence of an effect of male or female age (electronic supplementary material, figure S4*d*,*e*)). A summary of the variance decomposition analysis is presented in the electronic supplementary material, table S1.

### (e) Offspring genotype ratios

There was no evidence of an effect of parent genotypes on expected offspring genotype ratios, with Mendelian expectations being met for both female and male offspring, and for all combinations of parent genotype (table 1).

## 4. Discussion

Despite the strong effects of the Z chromosome inversion polymorphism for male fertility in zebra finches, we found no evidence that inversion haplotypes contain alleles with differential effects on egg production by females or survival of embryos through to

**Table 1.** Expected and observed offspring genotype ratios for each combination of parental genotypes. (Also provided are the *p*-values and 95% confidence intervals (CI) from binomial exact tests assuming the Mendelian expectation of a 50 : 50 ratio within each combination.)

| parent genotype combination (female : male) | offspring sex | expected offspring genotype | number of offspring | observed proportion of offspring genotypes | exact binomial test for Mendelian expectations | |
|---|---|---|---|---|---|---|
| | | | | | *p* | 95% CI |
| A : AB | male | AA, AB | 29, 21 | 0.58, 0.42 | $p = 0.32$ | 0.43–0.72 |
| | female | A, B | 42, 42 | 0.5, 0.5 | $p = 1$ | 0.39–0.61 |
| A : AC | male | AA, AC | 21, 20 | 0.51, 0.49 | $p = 1$ | 0.35–0.67 |
| | female | A, C | 15, 28 | 0.35, 0.65 | $p = 0.07$ | 0.21–0.51 |
| A : BC | male | AB, AC | 13, 8 | 0.62, 0.38 | $p = 0.38$ | 0.38–0.82 |
| | female | B, C | 26, 23 | 0.41, 0.59 | $p = 0.34$ | 0.26–0.58 |
| B : AB | male | AB, BB | 29, 31 | 0.48, 0.52 | $p = 0.90$ | 0.35–0.62 |
| | female | A, B | 28, 38 | 0.42, 0.58 | $p = 0.27$ | 0.30–0.55 |
| B : AC | male | AB, BC | 16, 7 | 0.7, 0.3 | $p = 0.10$ | 0.47–0.87 |
| | female | A, C | 14, 14 | 0.5, 0.5 | $p = 1$ | 0.31–0.69 |
| B : BC | male | BB, BC | 24, 13 | 0.65, 0.35 | $p = 0.10$ | 0.47–0.80 |
| | female | B, C | 18, 14 | 0.56, 0.44 | $p = 0.60$ | 0.38–0.74 |
| C : AB | male | AC, BC | 14, 11 | 0.56, 0.44 | $p = 0.69$ | 0.35–0.76 |
| | female | A, B | 22, 28 | 0.44, 0.56 | $p = 0.48$ | 0.30–0.59 |
| C : AC | male | AC, CC | 6, 12 | 0.33, 0.67 | $p = 0.24$ | 0.13–0.59 |
| | female | A, C | 18, 24 | 0.43, 0.57 | $p = 0.44$ | 0.28–0.59 |
| C : BC | male | BC, CC | 9, 5 | 0.64, 0.36 | $p = 0.42$ | 0.35–0.87 |
| | female | B, C | 5, 5 | 0.5, 0.5 | $p = 1$ | 0.19–0.81 |

hatching. Our results indicate it is unlikely that sexually antagonistic alleles acting upon egg production (a female-specific reproductive trait) have accumulated within the inversion. Instead, our findings suggest either: (i) the Z chromosome inversion haplotypes explain little variation in egg production, because any between-haplotype variation results in very similar mean phenotypes; (ii) prior sexual antagonism associated with egg production has already been resolved (for example through sex-biased gene expression [47]); (iii) there are relatively few genes responsible for variation in egg production located in the inversion, but instead they are located elsewhere on the Z or on the autosomes; (iv) egg production is a highly polygenic trait, and any associated loci located in the inversion exhibit a small proportion of genetic variation relative to loci elsewhere in the genome; or egg production may simply exhibit very little genetic variation.

Given the accumulated differences between inversion haplotypes, we analysed whether parental genotype combination is likely to contribute to variation in reproductive traits. We found no evidence that inversion haplotype sharing has a consistent effect on reproductive traits. We did, however, find that the probability of offspring surviving early development (relative to an egg being unfertilized or an embryo dying before 3 days of development), varied depending on the number of haplotypes shared and female haplotype. Specifically, 'B' females sharing one haplotype with the male (B : AB and B : BC combinations) had a higher likelihood of producing eggs that developed beyond 3 days of incubation, relative to 'B' females sharing no haplotypes with the male (B : AA, B : CC and B : AC combinations). Additionally, 'A' females sharing two haplotypes with the male (A : AA combinations) had a higher chance of eggs failing to develop, relative to 'A' females sharing one haplotype with the male (A : AB and A : AC combinations) (figure 3*a*). However, if parental haplotype sharing was consistently important, we would accordingly expect to observe a consistent effect associated with each degree of haplotypes shared. Our results do suggest that some specific combinations of parental genotypes perform better than others, but we suspect this is predominantly driven by the effect of male karyotype, because we also found that relative to homokaryotypic (AA carrying) males, heterokaryotypic (AB and AC carrying) males are more likely to produce fertilized eggs that survive beyond 3 days of development (figure 3*b*). This is additional to the prior evidence that AB and AC males have the fastest and most successful sperm under sperm competition [8,9]. Since multiple mating was not permitted in our population, the improved egg survival we observed must be independent of sperm competition, instead suggesting an additional non-competitive fertilization and/or developmental advantage. Our data do not allow us to discriminate between fertilization failure and embryo death as the cause of early reproductive failure in undeveloped eggs, but our findings do suggest that variation in male fertilization success may underpin the effects of haplotype sharing on egg development that we observed here. While female inversion haplotype does not appear to affect our measured reproductive traits, whether it influences other aspects of fitness remains unknown.

In their original study, despite the strong effects of the male karyotype for sperm traits, Kim *et al.* [8] found no systematic evidence of an effect of male inversion karyotype on hatching success, although they were unable to account for the influence of CC males. Conversely, Knief *et al.* [6] presented tentative evidence that mortality rates may be slightly higher in the offspring of heterokaryotypic males. Neither study accounted for the influence of female haplotype, or the interaction between male and female genotypes. Additionally, in both studies, measures of unhatched eggs represented those that failed at any stage from fertilization to late development. Our study—which does consider the influence of female haplotype, their interaction with male karyotype, and CC males, as well as partitioning hatching success into two distinct functional categories (representing separate and mechanistically divergent stages of hatching failure)—finds that aside from the positive effect of AC and AB males relative to AA males, there is no further evidence that male karyotype has a systematic influence over any other measures of reproductive success. Our finding that AB and AC males may actually carry an advantage during fertilization and/or early embryo development, lends additional support to the evidence put forward by both Kim *et al.* [8] and Knief *et al.* [9] that variation between haplotypes is maintained in the population by a heterozygote advantage for male fitness.

In some systems, females employ post-copulatory reproductive strategies to offset the costs associated with mating with less fit males [16,20,21] (e.g. those homozygous for the inversion, who have more poorly performing sperm). We explored this here by analysing whether offspring sex and genotype ratios varied by parental haplotype sharing. For example, we might expect females to favour producing a female-biased brood when mated to homozygous males that share her haplotype (i.e. in which case all her male offspring would be less fit). However, we found no evidence that offspring sex ratio was influenced by inversion haplotype, or the number of haplotypes shared between parents. Similarly, offspring genotype ratios conformed to Mendelian expectations under all parental combinations. It is therefore unlikely in this system that females employ post-reproductive differential offspring investment based on the father's inversion haplotype. Differential investment in offspring sex ratio is predicted to occur where the costs and benefits of producing offspring differ between sons and daughters [16,48,49]. In the zebra finch, females have previously been shown to employ facultative offspring sex ratio adjustments based on female condition [22], and differential offspring investment has also been observed based on partner quality [20]. It is possible that we do not observe differential investment here because conflict over optimal trait values limits the ability of females to drive such traits to fixation. Alternatively, because male karyotype has no systematic influence on female reproductive traits or on offspring survival, instead primarily influencing sperm success under sperm competition, the strength of selection for such a trait will also probably depend on the degree of extra pair paternity. Finally, if inversion haplotype is linked with genes for phenotypic or behavioural signals associated with attractiveness in males (for example male band colour [50]) females may select the more suitable males based on pre-copulation cues, providing little need to evolve a mechanism for adjusting investment post-mating. Beak length has already been found to be linked with male Z inversion karyotype in this species, providing one potential mechanism for pre-copulatory female choice [6]. However, since natural mate choice was not permitted in our study population, we were unable to test this.

Finally, although not related to the inversion polymorphism, we observed a clear decline in egg production with female age, which is consistent with general trends of reproductive senescence across many (though not all) female birds [51]. We also observed a marked increase in the number of single-egged clutches with female age (electronic supplementary material, figure S1*f*). It may be that for older females, reproductive fitness is maximized by concentrating effort into the formation and post-laying care of a single egg, rather than spreading limited energy over a larger clutch. Laying larger clutches may be physically very challenging for older birds that have probably experienced a reduction in their follicular reserve, a decline in normal immunological and hormonal functioning, and an increase in the energy required for somatic maintenance [52]. This effect became apparent in our population when female birds reached between 3 and 4 years of age. In the wild, mortality is high, and the median life expectancy is only approximately four months [53]. It therefore seems unlikely that this phenomenon would be observed in the wild, but whether it is a specific feature of our population, or a more general pattern observed across captive birds is also unknown.

Understanding the factors that contribute to variation in reproductive success is a key goal of evolutionary research. Our findings provide a thorough test of the effects of a sex-linked inversion polymorphism for reproductive traits. We have shown that this inversion polymorphism, or 'supergene', despite its important effects for male fertility, does not appear to influence egg production by females or survival of embryos through to hatching. We have also provided additional evidence that the zebra finch Z inversion is probably being maintained in a stable polymorphism owing to heterozygote advantage for male fitness, whereby heterokaryotypic males carrying one copy of the ancestral inversion not only have faster and more successful sperm under sperm competition, but may also have an increased likelihood of fertilization and/or early embryo survival relative to homokaryotypes.

Ethics. The data used for this study was collected following approval by the University of Sheffield, UK. All procedures used to collect the data conformed to the legal requirements for animal research in the UK, and were conducted under a project licence (PPL 40/3481) issued by the Home Office. All animals were humanely killed under Schedule 1 (Animals (Scientific Procedures) Act 1986).

Data accessibility. Data and code are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.cz8w9gj7w [38]. Electronic supplementary material is provided online [54].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. K.A.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, validation, visualization, writing—original draft, writing—review and editing; O.M.: formal analysis, methodology, software, validation, visualization, writing—review and editing; J.S.: conceptualization, funding acquisition, methodology, resources, supervision, writing—review and editing; N.H.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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## References


1. Morrow EH, Arnqvist G, Pitcher TE. 2002 The evolution of infertility: does hatching rate in birds coevolve with female polyandry? *J. Evol. Biol.* **15**, 702–709. (doi:10.1046/j.1420-9101.2002.00445.x)
2. Pei Y, Forstmeier W, Wang D, Martin K, Rutkowska J, Kempenaers B. 2020 Proximate causes of infertility and embryo mortality in captive zebra finches. *Am. Nat.* **196**, 577–596. (doi:10.1086/710956)
3. Clutton-Brock T. 1988 *Reproductive success: studies of individual variation in contrasting breeding systems*. Chicago, IL: University of Chicago Press.
4. Pei Y, Forstmeier W, Knief U, Kempenaers B. 2023 Weak antagonistic fitness effects can maintain an inversion polymorphism. *Mol. Ecol.* **32**, 3575–3585. (doi:10.1111/mec.16963)
5. Nosil P, Soria-Carrasco V, Villoutreix R, De-la-Mora M, De Carvalho CF, Parchman T, Feder JL, Gompert Z. 2023 Complex evolutionary processes maintain an ancient chromosomal inversion. *Proc. Natl Acad. Sci. USA* **120**, e2300673120. (doi:10.1073/pnas.2300673120)
6. Knief U, Hemmrich-Stanisak G, Wittig M, Franke A, Griffith SC, Kempenaers B, Forstmeier W. 2016 Fitness consequences of polymorphic inversions in the zebra finch genome. *Genome Biol.* **17**, 1–22. (doi:10.1186/s13059-016-1056-3)
7. Christidis L. 1986 Chromosomal evolution within the family Estrildidae (Aves) I. The Poephilae. *Genetica* **71**, 81–97. (doi:10.1007/BF00058691)
8. Kim K-W, Bennison C, Hemmings N, Brookes L, Hurley LL, Griffith SC, Burke T, Birkhead TR, Slate J. 2017 A sex-linked supergene controls sperm morphology and swimming speed in a songbird. *Nat. Ecol. Evol.* **1**, 1168–1176. (doi:10.1038/s41559-017-0235-2)
9. Knief U *et al.* 2017 A sex-chromosome inversion causes strong overdominance for sperm traits that affect siring success. *Nat. Ecol. Evol.* **1**, 1177–1184. (doi:10.1038/s41559-017-0236-1)
10. Berdan EL, Flatt T, Kozak GM, Lotterhos KE, Wielstra B. 2022 Genomic architecture of supergenes: connecting form and function. *Phil. Trans. R. Soc. B* **377**, 20210192. (doi:10.1098/rstb.2021.0192)
11. Funk ER, Mason NA, Pálsson S, Albrecht T, Johnson JA, Taylor SA. 2021 A supergene underlies linked variation in color and morphology in a Holarctic songbird. *Nat. Commun.* **12**, 6833. (doi:10.1038/s41467-021-27173-z)
12. Thompson MJ, Jiggins CD. 2014 Supergenes and their role in evolution. *Heredity* **113**, 1–8. (doi:10.1038/hdy.2014.20)
13. Itoh Y, Kampf K, Balakrishnan CN, Arnold AP. 2011 Karyotypic polymorphism of the zebra finch Z chromosome. *Chromosoma* **120**, 255–264. (doi:10.1007/s00412-010-0308-3)
14. Jennions MD, Petrie M. 2000 Why do females mate multiply? A review of the genetic benefits. *Biol. Rev.* **75**, 21–64. (doi:10.1111/j.1469-185X.1999.tb00040.x)
15. Uller T, Eklöf J, Andersson S. 2005 Female egg investment in relation to male sexual traits and the potential for transgenerational effects in sexual selection. *Behav. Ecol. Sociobiol.* **57**, 584–590. (doi:10.1007/s00265-004-0886-2)
16. Bowers EK, Munclinger P, Bureš S, Kučerová L, Nádvorník P, Krist M. 2013 Cross-fostering eggs reveals that female collared flycatchers adjust clutch sex ratios according to parental ability to invest in offspring. *Mol. Ecol.* **22**, 215–228. (doi:10.1111/mec.12106)
17. Wagner RH, Helfenstein F, Danchin E. 2004 Female choice of young sperm in a genetically monogamous bird. *Proc. R. Soc. B* **271**, S134–S137. (doi:10.1098/rsbl.2003.0142)
18. Cunningham EJA, Russell AF. 2000 Egg investment is influenced by male attractiveness in the mallard. *Nature* **404**, 74–77. (doi:10.1038/35003565)
19. Hasegawa M, Arai E, Watanabe M, Nakamura M. 2012 High incubation investment of females paired to attractive males in barn swallows. *Ornithol. Sci.* **11**, 1–8. (doi:10.2326/osj.11.1)
20. Bolund E, Schielzeth H, Forstmeier W. 2009 Compensatory investment in zebra finches: females lay larger eggs when paired to sexually unattractive males. *Proc. R. Soc. B* **276**, 707–715. (doi:10.1098/rspb.2008.1251)
21. Gorman HE, Arnold KE, Nager RG. 2005 Incubation effort in relation to male attractiveness in zebra finches *Taeniopygia guttata*. *J. Avian Biol.* **36**, 413–420. (doi:10.1111/j.2005.0908-8857.03464.x)
22. Bradbury RB, Blakey JK. 1998 Diet, maternal condition, and offspring sex ratio in the zebra finch, *Poephila guttata*. *Proc. R. Soc. Lond. B* **265**, 895–899. (doi:10.1098/rspb.1998.0375)
23. Pike TW, Petrie M. 2003 Potential mechanisms of avian sex manipulation. *Biol. Rev.* **78**, 553–574. (doi:10.1017/S1464793103006146)
24. Rice W. 1984 Sex chromosomes and the evolution of sexual dimorphism. *Evolution* **38**, 735–742. (doi:10.2307/2408385)
25. Mank JE, Ellegren H. 2009 Sex-linkage of sexually antagonistic genes is predicted by female, but not male, effects in birds. *Evolution* **63**, 1464–1472. (doi:10.1111/j.1558-5646.2009.00618.x)
26. Ellegren H, Parsch J. 2007 The evolution of sex-biased genes and sex-biased gene expression. *Nat. Rev. Genet.* **8**, 689–698. (doi:10.1038/nrg2167)
27. Ellegren H. 2011 Emergence of male-biased genes on the chicken Z-chromosome: sex-chromosome contrasts between male and female heterogametic systems. *Genome Res.* **21**, 2082–2086. (doi:10.1101/gr.119065.110)
28. 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. (doi:10.1186/1471-2164-12-37)
29. Parsons PA, Bodmer WF. 1961 The evolution of overdominance: natural selection and heterozygote advantage. *Nature* **190**, 7–12. (doi:10.1038/190007a0)
30. Höök L, Leal L, Talla V, Backström N. 2019 Multilayered tuning of dosage compensation and Z-chromosome masculinization in the wood white (*Leptidea sinapis*) butterfly. *Genome Biology and Evolution* **11**, 2633–2652. (doi:10.1093/gbe/evz176)
31. Itoh Y *et al.* 2007 Dosage compensation is less effective in birds than in mammals. *J. Biol.* **6**, 2. (doi:10.1186/jbiol53)
32. Naurin S, Hansson B, Bensch S, Hasselquist D. 2010 Why does dosage compensation differ between XY and ZW taxa? *Trends Genet.* **26**, 15–20. (doi:10.1016/j.tig.2009.11.006)
33. 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. (doi:10.1534/genetics.112.145102)
34. Birkhead TR, Hunter FM, Pellatt JE. 1989 Sperm competition in the zebra finch, *Taeniopygia guttata*. *Anim. Behav.* **38**, 935–950. (doi:10.1016/S0003-3472(89)80135-6)
35. Bruford MW, Hanotte O, Brookfield JFY, Burke T. 1998 *Molecular genetic analysis of populations: a practical approach*. Oxford, UK: IRL Press.
36. LGC Group. 2024 SNPline workflow. See https://www.biosearchtech.com/support/resources/snpline-instrument-genotyping-workflow.
37. LGC Genomics. 2011 Kraken. LGCgenomics. See https://results.lgcgenomics.com/software/kraken_manual/frames/f=advanced.htm.

38. Assersohn K, Morton O, Slate J, Hemmings N. 2023 Data from: A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches. Dryad Digital Repository. (doi:10.5061/dryad.cz8w9gj7w)
39. Birkhead TR, Hall J, Schut E, Hemmings N. 2008 Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis* **150**, 508–517. (doi:10.1111/j.1474-919X.2008.00813.x)
40. Assersohn K, Marshall AF, Morland F, Brekke P, Hemmings N. 2021 Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. *Anim. Conserv.* **24**, 1–12. (doi:10.1111/acv.12674)
41. R Core Team. 2022 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
42. Bürkner P-C. 2017 brms: an *R* package for Bayesian multilevel models using Stan. *J. Stat. Soft.* **80**, 1–28. (doi:10.18637/jss.v080.i01)
43. Berger M, Tutz G. 2021 Transition models for count data: a flexible alternative to fixed distribution models. *Stat. Methods Appl.* **30**, 1259–1283. (doi:10.1007/s10260-021-00558-6)
44. Chambers M, Drovandi C. 2023 Many-levelled continuation ratio models for frequency of alcohol and drug use data. *Am. J. Drug Alcohol Abuse* **49**, 566–575. (doi:10.1080/00952990.2023.2213868))
45. Valle D, Ben Toh K, Laporta GZ, Zhao Q. 2019 Ordinal regression models for zero-inflated and/or over-dispersed count data. *Sci. Rep.* **9**, 3046. (doi:10.1038/s41598-019-39377-x)
46. Makowski D, Ben-Shachar M, Lüdecke D. 2019 bayestestR: describing effects and their uncertainty, existence and significance within the Bayesian framework. *J. Open Source Softw.* **4**, 1541. (doi:10.21105/joss.01541)
47. Cox RM, Calsbeek R. 2009 Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. *Am. Nat.* **173**, 176–187. (doi:10.1086/595841)
48. Bartlow AW, Jankowski MD, Hathcock CD, Ryti RT, Reneau SL, Fair JM. 2021 Sex ratio of western bluebirds (*Sialia mexicana*) is mediated by phenology and clutch size. *Ibis* **163**, 977–989. (doi:10.1111/ibi.12935)
49. Trivers RL, Willard DE. 1973 Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**, 90–92. (doi:10.1126/science.179.4068.90)
50. Burley N, Enstrom DA, Chitwood L. 2002 Extra-pair relations in zebra finches: differential male success results from female tactics. *Anim. Behav.* **48**, 1031–1041. (doi:10.1006/anbe.1994.1336)
51. Holmes DJ, Thomson SL, Wu J, Ottinger MA. 2003 Reproductive aging in female birds. *Exp. Gerontol.* **38**, 751–756. (doi:10.1016/S0531-5565(03)00103-7)
52. Assersohn K, Brekke P, Hemmings N. 2021 Physiological factors influencing female fertility in birds. *R. Soc. Open Sci.* **8**, 202274. (doi:10.1098/rsos.202274)
53. Williams TD, Christians JK. 2003 Experimental dissociation of the effects of diet, age and breeding experience on primary reproductive effort in zebra finches *Taeniopygia guttata*. *J. Avian Biol.* **34**, 379–386. (doi:10.1111/j.0908-8857.2003.03080.x)
54. Assersohn K, Morton O, Slate J, Hemmings N. 2024 A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches. Figshare. (doi:10.6084/m9.figshare.c.7109028)

# Chapter 4:

# The surprising complexity and diversity of sperm storage structures across Galliformes

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*Mountain quail (Oreortyx pictus), the folds of the utero-vaginal junction, and a sperm storage tubule.*

# The surprising complexity and diversity of sperm storage structures across Galliformes

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## Abstract

In internal fertilisers, the precise timing of ovulation with the arrival of sperm at the site of fertilisation is essential for fertilisation success. In birds, mating is often not synchronised with ovulation, but instead females utilise specialised sperm storage tubules (SSTs) in the reproductive tract, which can ensure sperm are always available for fertilisation at the time of ovulation, whilst simultaneously providing a mechanism of post-copulatory sexual selection. Despite the clear importance of SSTs for fertilisation success, we know little about the mechanisms involved in sperm acceptance, storage, and release. Furthermore, most research has been conducted on only a small number of species, based on which SSTs are usually assumed to look and function in the same way across all species. Here, we conduct a comparative exploration of SST morphology across 26 species of Galliformes. We show that SSTs, and the surrounding tissue, can vary significantly in morphology across species. We provide observational evidence that Galliformes exhibit at least 5 distinct categories of tubule types, including distinctive coiled and multi-branched tubules, and describe 2 additional features of the surrounding tissue. We suggest functional explanations for variation in tubule morphology and propose next steps for future research. Our findings indicate that SSTs are likely to be far more variable than has previously been assumed, with potentially important consequences for our understanding of sperm storage in birds and post-copulatory sexual selection in general.

Keywords: fertility, sperm selection, post-copulatory sexual selection, cryptic female choice, uterovaginal junction, reproduction

# 1 Introduction

In internal fertilisers, successful fertilisation relies on the arrival of sperm at the site of fertilisation at the precise time of ovulation. In many species, ensuring sufficient sperm are available for fertilisation requires insemination to be precisely timed with the release of the ovum. In others, insemination and ovulation are not synchronised, sometimes occurring many days or even months apart (Birkhead, 1992; Birkhead and del Nevo, 1987; Hatch, 1983; Hemmings and Birkhead, 2020; Wanless and Harris, 1986). To compensate for this, females may store sperm within the reproductive tract, maintaining it in a viable state to be released at ovulation (Bakst, 2011).

Female sperm storage is a widespread phenomenon, occurring in many invertebrates and all major vertebrate groups (Holt and Fazeli, 2016; Shankar et al., 2022). In species exhibiting sperm storage (as opposed to just sperm longevity), females can store sperm for a few hours to months or even years (Holt and Fazeli, 2016; Levine et al., 2021; Orr and Zuk, 2012). This is achieved through providing an environment favourable for sperm survival, often in the form of specialised morphological structures e.g., seminal receptacles, (gastropods and arthropods), spermathecae (many invertebrates), tubules (birds, reptiles, fish and amphibians), or a combination of both spermathecae and seminal receptacles (*Drosophila melanogaster*) (reviewed in; Holt, 2011 and Orr and Brennan, 2015). Sperm storage structures not only guard against a lack of sperm at fertilisation, but may also allow females to preferentially store/release sperm from preferred males, thereby providing a mechanism of postcopulatory sexual selection (i.e., cryptic female choice) (Bakst, 2011; Eberhard, 1996; Mendonca et al., 2019; Sasanami, 2017).

Sperm storage has been particularly well-studied in birds, beginning with speculation in the early-1900s that viable sperm were capable of surviving in the oviduct long after insemination (Payne, 1914). It wasn't until the mid-1900s that the first specialised storage sites, then termed 'sperm nests', were identified and suggested to be responsible for sustained fertility in female birds (Van Drimmelen, 1946). 'Sperm nests' were initially described as being located within shallow crypts of the infundibulum (the site of fertilisation), but in a series of histological studies, sperm storage was later suggested to occur principally at the utero-vaginal junction (UVJ), a small non-distinct section of the vagina bordering the uterus (Bobr et

al., 1964a, 1964b; Verma and Cherms, 1964, 1965). Today, these structures are commonly known as 'sperm storage tubules' (SSTs), occurring primarily in the UVJ, with evidence for infundibular storage sites remaining equivocal (but still widely cited) (Assersohn et al., 2021; Bakst, 2011; Sasanami, 2017).

Although commonly defined as 'simple tubular invaginations', SSTs are increasingly appreciated as being highly specialised, with an expanding body of evidence to suggest that the molecular and biochemical processes occurring within SSTs are complex and highly regulated (Bakst, 2011; Freedman et al., 2001; Hemmings et al., 2015; Holm et al., 2000; Khillare et al., 2018; Mendonca et al., 2019; Sasanami, 2017). The vagina is typically hostile to sperm: only 1% of inseminated sperm make it into storage (Bakst, 2011) and it is thought to be a selective process that probably ensures sperm with atypical morphology and physiology are inhibited from participating in fertilisation (Bakst, 1994; Bobr et al., 1964a; Khillare et al., 2018; Ogasawara et al., 1966). Once accepted into SSTs, numerous compounds are produced that likely act to suppress sperm motility and provide protection from structural damage and oxidative stress (Bakst and Bauchan, 2015; Freedman et al., 2001; Holm et al., 2000; Huang et al., 2017, 2016; Khillare et al., 2018; Matsuzaki et al., 2015; Mendonca et al., 2019; Sasanami, 2017). At ovulation, sperm are re-mobilised and released from storage (Hiyama et al., 2014; Ito et al., 2011). In birds, there is a very narrow window of time (around 15 minutes in domestic fowl; (Bakst, 1994)) between ovum release and the laying down of the outer perivitelline layer (a matrix of glycoproteins that surrounds the ovum and blocks further penetration by sperm) (Hemmings and Birkhead, 2015). The timing of acceptance and release of sperm is therefore highly regulated, probably through fine hormonal and possibly nervous control (Hemmings et al., 2015). Furthermore, 3D imaging of SSTs in zebra finches (*Taeniopygia guttata*) has identified gate-like constricted openings that may act to limit the ability of sperm swimming below a certain velocity to enter storage, providing an additional mechanism of selection for high quality sperm (Hiyama et al., 2014; Ito et al., 2011; Mendonca et al., 2019). Despite these advances, we still lack a comprehensive understanding of the mechanisms by which SSTs accept, store/maintain and release sperm (Khillare et al., 2018; Sasanami, 2017; Shankar et al., 2022). We have long known that even among commercial birds selected for consistent and high fertility, sperm storage duration varies greatly. For example, sperm are stored for just 5-10 days in Japanese quail (*Coturnix japonica*)

but up to 15 weeks in turkeys (*Meleagris gallopavo*) (Birkhead and Møller, 1990; Sasanami, 2017). However, the causes of such variation are not known or generally discussed in the context of SST diversity across avian species.

Perhaps an even more fundamental barrier to our understanding of sperm storage in birds is our lack of knowledge of inter-specific variation in SST morphology. Sperm are some of the most diverse cells in the animal kingdom (Pitnick et al., 2009), and so it stands to reason that the structures that selectively store them might also vary considerably in both structure and function (Cramer et al., 2023). Sperm length has been found to correlate negatively with SST number and positively with SST length in passerines (Briskie and Montgomerie, 1993), suggesting that the co-evolutionary dynamics between male and female post-copulatory sexual selection may be a driving factor in the evolution of sperm morphology (Kustra and Alonzo, 2023). Sperm morphology and female sperm storage organ morphology have been shown to correlate in other taxa as well (mainly invertebrates) (Higginson et al., 2012; Miller and Pitnick, 2002). Within-population variation in sperm storage organ morphology and function has also been documented in *Drosophila* (Lüpold et al., 2013). However, the general assumption in birds is that SSTs always look- and function in- the same way as those observed in the species in which SSTs have been most well-studied (e.g., domestic chickens (*Gallus gallus domesticus*), turkey, Japanese quail, and the zebra finch). In studies that have identified SSTs, there is also inconsistency in how quantitative variables such as SST number and length are measured, making cross study comparison difficult. For example, there is no consensus on whether branched tubules should be considered (and measured as) one tubule with great total length, or many distinct shorter tubules. An important step in determining the degree of variation in SST form and function across species, and how this correlates with post-copulatory processes, will be to develop more consistent and reproducible descriptions and measures of SST traits.

Here, we report and present the discovery of remarkable variation in morphological structure of SSTs across species of Galliformes. We also present methods for dissecting and examining the folds of the utero-vaginal junction in birds, suggest criteria for categorising tubule morphology based on our observations, and discuss the potential for future research in Galliformes and across birds in general.

# 2 Materials and Methods

## 2.1 Animals

Galliformes are a very large, diverse, and well-studied bird group in which body size and mating system vary greatly, and samples are easily accessible, making them ideal for exploring interspecific variation in SST morphology. We collected the whole oviduct of a single female from 28 different species of Galliformes. Dead birds were sourced in 2016 from breeders in the process of disposing of excess stock, and dissections took place on site within 30 minutes of each bird being killed. Females were included in the study only if they were in breeding condition (confirmed by the presence of an ovum in the oviduct and/or a hierarchy of developed ova in the ovary), since the avian oviduct is known to regress in size outside of the reproductive period. Males and females were housed together, allowed free access to each other, and all copulations were natural. Females were dissected at most within 1-2 days of commencing egg-laying, and all females were confirmed to have laid fertile eggs (demonstrating that they had recently accepted and stored sperm).

The oviduct was removed intact (including the cloaca and ovary), unravelled, stripped of connective tissue, and briefly cleaned in phosphate buffered saline (PBS). The oviduct was then pinned out lengthways in a long, shallow wax-based tray, where it was photographed and measured. The length of each individual section of the oviduct, as well as its entire length, was measured. We also recorded the wet mass of the oviduct after briefly dabbing off excess liquid with absorbent tissue. Once all measurements were complete, the oviduct was transferred into a deeper tray, pinned so that each section was straight, and submerged in 10% formalin solution to fix the tissue. After at least 48 hours in fixative, a segment of the vagina containing the UVJ was then cut away from the rest of the oviduct (Figure 1; step 1) (Briskie and Birkhead, 1993). Since the UVJ has previously been reported to be located at the uterus end of the vagina, the segment was always cut at the beginning of the uterus. However, due to the use of vaginal tissue for another study, the degree to which the segment extended along the vagina varied from 19-30% of the total vaginal length. Upon microscopic examination of each sample, the start of the UVJ segment was designated as the point at which sperm storage tubules first appeared, and the end of the UVJ segment was where uterine tissue started. Occasionally, it appeared that the distribution of tubules continued

slightly further down the vagina than the length of our sample allowed us to visualise, however this was uncommon (only occurring in 4 species; California quail (*Callipepla californica)*, European quail (*Coturnix coturnix*), mountain quail (*Oreortyx pictus*) and Temminck's tragopan (*Tragopan temminckii*)), and the dissected segments were expected to contain the majority (if not all) of the SSTs in the sample.

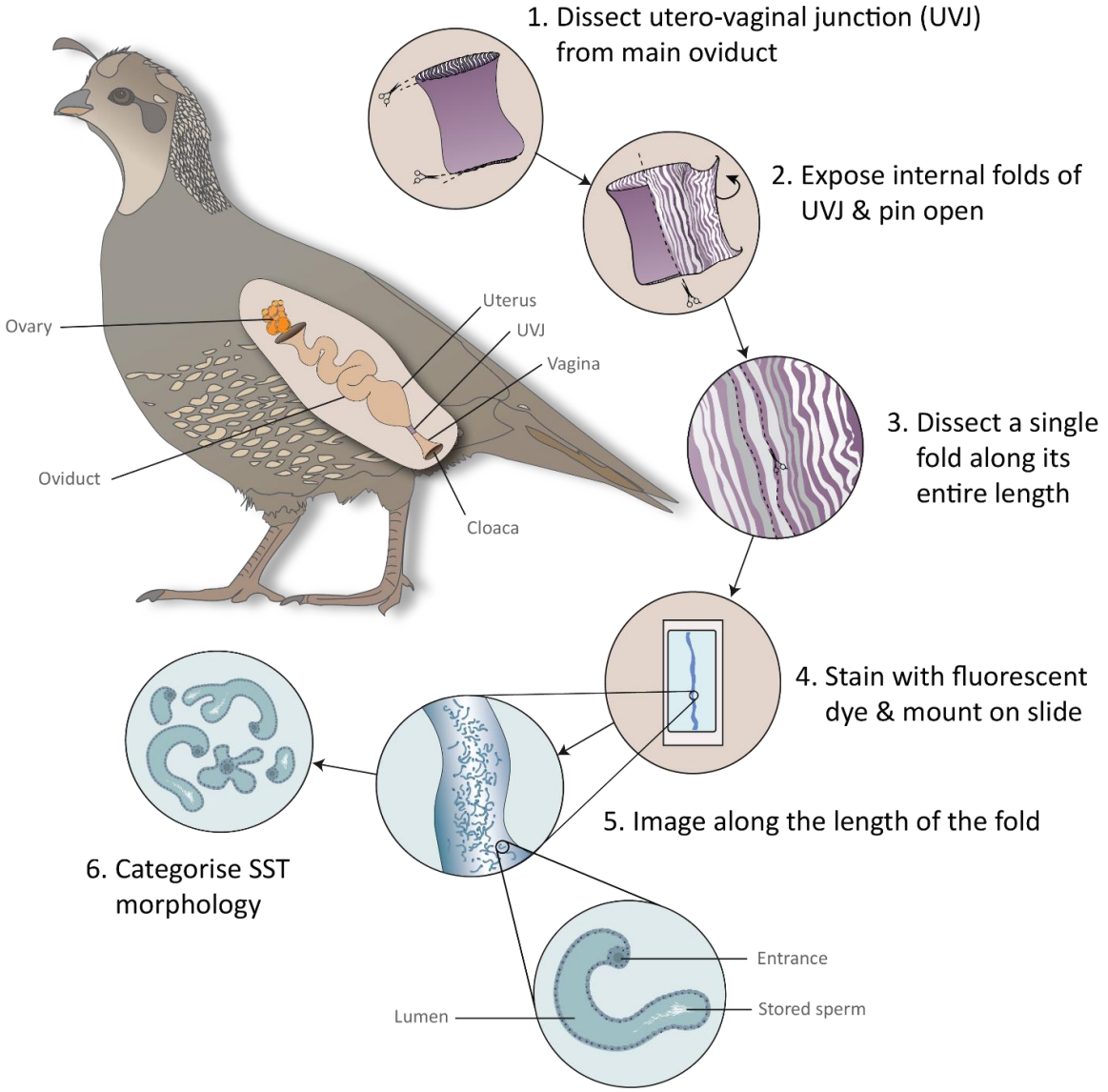


***Figure 1*** *Schematic illustrating the avian oviduct and ovary, and the methods of UVJ dissection, fold dissection and SST categorisation. Within the oviduct, the utero-vaginal junction (UVJ) (purple segment) - the site of primary sperm storage, was dissected and pinned open to expose the internal folds. A single fold was then dissected along its length and stained with Hoechst fluorescent dye. The fold was then laid flat and fixed onto a slide before being imaged along its entire length using fluorescent microscopy. SSTs are typically visible along the centre of UVJ folds. Schematic drawn in Adobe Illustrator (V.28.4.1)*

## 2.2 Dissection

Samples were cut longitudinally with micro-dissecting scissors, pinned open with butterfly specimen pins on Sylgard™ 184 gel (Sigma-Aldrich), and covered with phosphate buffered saline (PBS), to reveal the internal luminal mucosa and mucosal folds of the vagina (Figure 1; step 2). For each sample, 2 folds were dissected. Folds were examined under a Leica MZ75 dissecting microscope and dissected by cutting the entire length of one side of the 'valley' (as opposed to the crest) of a single fold along the entire length of the sample (Figure 1; step 3) (Briskie and Birkhead, 1993). Care was taken to remove only the mucosa, peeling it away from the underlying connective tissue (the lamina propria), but avoiding cuts to the underlying tissue. Before cutting along the remaining side, the fold was gently prised open along the length. Opening the fold out whilst it was still attached to the sample along one side had the benefit of providing leverage for gently teasing apart the 'crest' of the fold. Flattening the crest was necessary to allow the fold to lie smoothly once mounted, but was much more difficult to achieve once the fold was fully dissected. If necessary, additional cuts were made to remove connecting tissue whilst ensuring the integrity of the fold was maintained and the SSTs were not damaged. The entire length of the remaining attached side was then cut longitudinally, and the fold gently removed from the underlying tissue. The fold was then incubated for 5-10 minutes with 10-20µl of Hoechst 33342 dye (the exact volume depended on how much was needed to fully submerge the fold). Hoechst was used to help visualise sperm in storage, which aided in identifying SSTs, and had the added benefit of clarifying the edges of the SSTs against the surrounding tissue. The fold was then placed onto a slide (lamina propria side down) and gently opened to lie flat. Once flattened along the entire length, a coverslip was added with Fluoromount-G™ Aqueous Mounting Medium (Sigma-Aldrich) and left to dry. Dried samples were sealed with transparent nail varnish and kept in the dark until imaging (Figure 1; step 4).

## 2.3 Morphological observations from dissection

We observed a remarkable amount of variation in the thickness, texture, integrity, and overall appearance of vaginal tissue between species. The visual presentation of the folds of the vagina varied between species, with some species having large and very distinct folds whilst in others the individual folds were less pronounced. Some species displayed obvious

pigmentation in the vagina, and in many cases, tubules were visible by eye at this stage (Figure 1; step 5). There was always an obvious difference between vaginal and uterine tissue, though in some species this difference was more pronounced than others. Uterine tissue was always to some degree thicker, darker in appearance and with larger/taller folds relative to the vagina.

Samples also clearly varied in their response to preservation in formalin: While some samples remained very well preserved, others had become fragile and difficult to dissect. In the case of 2 species, Javan peahen (*Pavo muticus*), and Grey partridge (*Perdix perdix*), samples were so fragile they were impossible to dissect. Consequently, we removed these species from the sample pool, reducing the sample size to 26 species (52 folds). This difference in response to preservation, ease of dissection and mounting suggests there are some innate differences in tissue structure between species; however, most samples remained in suitable condition for dissection, despite there being an approximate 7-year gap between tissue fixation and sample dissection.

## 2.4 Imaging

Slides were examined under fluorescent and brightfield light on a Leica DMLB compound microscope with an LEJ ebq100 fluorescence source. Images were captured with Infinity Analyze software via a Lumenera Infinity3 USB camera. Images were taken along the entire length of each fold, following the centre line of the fold (Figure 1; step 5), at 50X magnification, with one image directly bordering the next with no overlap and no missing sections. Depending on the individual sample, images were taken either using brightfield, darkfield or fluorescent illumination, or a combination, in whichever way produced the highest quality image for that sample. In many cases, images of one field of view were taken repeatedly through multiple planes to avoid missing hidden structures where possible. Image scale bars were produced in ImageJ (Schneider et al., 2012) and contrast and brightness were adjusted in Adobe Photoshop (version 25.5) to aid visualisation where necessary. For some structures of note, additional images were taken at either 100 or 200X magnification.

## 2.5 Tubule categorisation

On microscopic examination of each image, careful notes of visible structures were made that were subsequently assessed to create distinct categories that could be used to define consistent features (Figure 1; step 6). An initial criterion for categorising structure type was then created, which was used to categorise the visible structures within each image across all samples. A second observer then used the categorisation criteria to categorise a subset of images: one image per sample (two samples per species; 52 images in total) was chosen for re-categorisation, at random (using a random number generator), from a pool of images that only contained visible structures (i.e., excluding images in which no features were observed). Repeatability of categorisation was then calculated in R (R Core Team, 2023) using the package rptR (Stoffel et al., 2017). A binary family was used for each model, with tubule category as the response variable and sample ID as the random effect. For categories with lower repeatability, adjustments to the categorisation criteria were made to improve reproducibility. We then used these revised categorisation criteria to create a flow-chart (also reproduced as a dichotomous key in the supplementary material) that could be used and built upon by future researchers to categorise the structures they observe in their own samples, with the aim of encouraging a consistent and definitive use of terminology throughout the literature. We acknowledge that a greater number of distinct structures are likely to exist across species and encourage future work to critically examine the structures observed and consider whether they fit into the existing categories presented here, or whether new categorisations are necessary.

To aid in categorising tubule location, we considered the samples as being divided into 3 sections, each containing an equal number of images: C (the most distal section, i.e., closest to the cloaca and bordering the rest of the vagina), M (the central third of the sample), and U (the most proximal section that borders the uterus) (Briskie, 1996). If the sample contained an uneven number of images making equal thirds impossible, the M section was expanded to accommodate the additional images, with the C and U sections always containing the same number of images and so being proportionally the same size. Note that we always consider the first image in C to be the start of the UVJ region of the vagina and will contain the first appearance of tubules.

# 3 Results

## 3.1 Tubule location within the UVJ

Tubule location varied between species, however in the majority of species (20; 76.9% of species), tubules were found in all three regions of the sample (C, M and U) in at least one of the sample folds. In 13 species (50% of species), tubules were only found in the C and M (but not the U) regions in at least one of the sample folds, but of these, 6 species never had tubules in the U region of either fold: harlequin quail (*Coturnix delegorguei*), Japanese quail, mountain quail, Swinhoe's pheasant (*Lophura swinhoii*), silver pheasant (*Lophura nycthemera*) and wild turkey. There was only 1 species (California quail) in which tubules were seen only in the C region (and not the M and U), but this was only the case for one fold of the sample. Taken together, tubules were commonly found across the entire length of the sample, but in half of all species, at least one fold sample did not have tubules that directly bordered the uterus.

## 3.2 Presentation of fold tissue

Variation in the texture of vaginal and uterine tissue between species was apparent upon microscopic examination: In some cases, tissue was thin, and tubules were clear, and in others the tissue was thick and layered, with tubules sometimes being embedded making them more difficult to identify, even when examining through multiple planes of focus. Sperm was not always observed in storage in every sample, possibly due to lower retention of sperm, fewer copulations, or fewer sperm being transferred during copulation to begin with. In samples that contained stored sperm, the distribution of sperm was uneven throughout the samples, with most tubules being free of sperm.

## 3.3 Channels

We repeatedly observed 'channel-like' structures (here-on referred to as channels) that presented as grooves along a single fold, and often extended along a large portion of the fold (See Figure 2A where no channels are present, relative to Figure 2B-F where channels can be seen). Although occasionally shorter and more 'funnel-like' in appearance (Figure 2E and F, seen in; black francolin (*Francolinus francolinus*), grey junglefowl (*Gallus sonneratii)*, golden

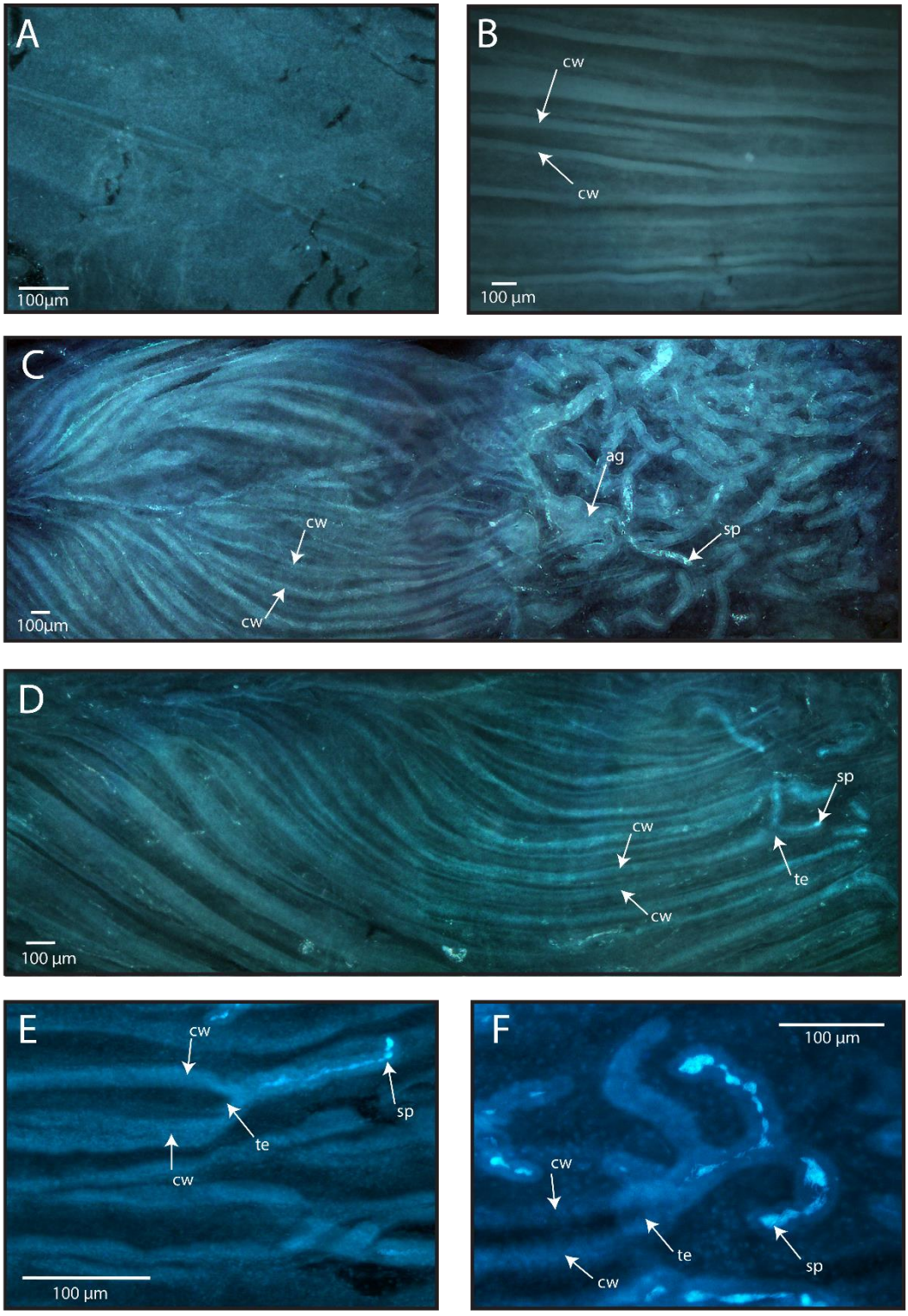


***Figure 2 A:*** *An example of the typical presentation of UVJ tissue that does not contain channels or SSTs, from European quail (Coturnix coturnix) at 50X magnification.* ***B:*** *Channel-like structures of the UVJ tissue from helmeted guineafowl (Numidea meleagris) at 50X magnification.* ***C and D:*** *Channel-like structures of the UVJ tissue in grey francolin (Francolinus pondicerianus) at 50X magnification. Each image crosses 3 fields of view. Channels are very long and end in a region populated by tubules. In* ***C****, you can see a large globular (agglomerate) branched tubule (ag) and channels that appear to end directly in a tubule with obvious stored*

*sperm (sp).* ***D:*** *Particularly clear example of a long channel ending in a forked tubule.* ***E:*** *Slightly different presentation of channel tissue in mountain quail (Oreortyx pictus) at 100X magnification. Channels are shorter in length, funnel-like and end directly in tubules.* ***F :*** *Example of channels apparently ending in a highly branched tubule in mountain quail at 200X magnification. Stored sperm are visible in more than one branch. Arrows point to representative (but not all) examples of channel walls (cw), tubule entrances (te), stored sperm (lighter/ brighter blue) (sp), and agglomerate tubules (ag) (where relevant). Images are all orientated with the cloaca closest to the left side and the uterus closest to the right.*

pheasant (*Chrysolophus pictus)*, Lady Amherst's pheasant (*Chrysolophus amherstiae)*, mountain quail, red legged partridge (*Alectoris rufa*), roul roul partridge (*Rollulus rouloul)* and Temminck's tragopan), these structures were distinct in presentation from tubules but contained clear thickened tissue on either side (channel walls) with a 'lumen-like' depression along the centre. Channels were sometimes seen beginning at the start of the sample (the C region) and ending at the presence of tubules (Figure 2C) and they may either extend strictly in parallel, or merge and split into a series of interconnected pathways. They often (but not always) traversed more than one field of view (Figure 2C and D). In some cases, channels appeared further along the sample, and were occasionally seen to end directly in a tubule (Figure 2C-F). Whilst in many cases channels appear as 'open-top' grooves (e.g., Figure 2B, E and F), it was not always clear from 2D imaging whether in some cases they may be enclosed tubes (e.g., 2C and D). Ultimately, future work exploring the 3D ultrastructure of channels will be needed to determine this.

Channels were observed to some degree in most species examined (24 species (92.3% of all species) and 46 folds (88.5% of fold samples)) (Figure 3). There were only 2 species where channels were observed in one fold but not the other (black francolin and Madagascar partridge (*Margaroperdix madagarensis*), and only 2 species where no channels were observed in either fold of the sample (European quail and Chinese quail *(Excalfactoria chinensis*). The categorisation of channel tissue was significantly repeatable between observers ($R_{Linkscale}$ = 0.99, CI = 0.99, 1.00, P < 0.0001).

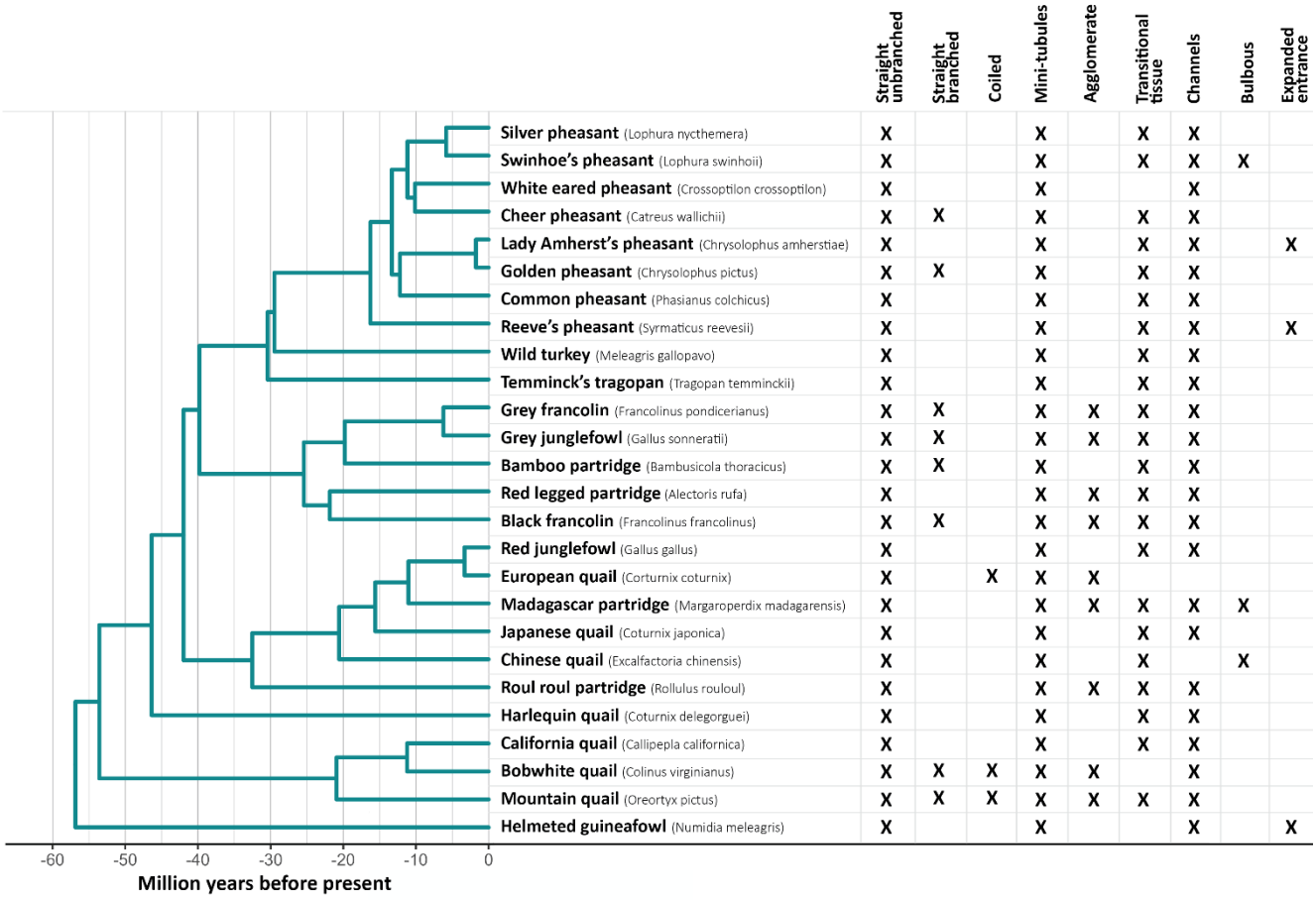


***Figure 3*** *Phylogeny for the 26 species examined, and the tubule and tissue types observed within those species. Cells marked with 'X' indicate that this species exhibited this tubule or tissue type to some degree in at least one fold of the sample. Galliformes phylogeny with time-calibrated branch lengths was obtained from* Stein et al., 2015*, which was trimmed using the R package treeplyr* (Harmon, 2023).

## 3.4 Transitional tissue

Across most species, we observed structures that we could neither conclusively define as tubule tissue, nor vaginal or uterine tissue. These structures always directly preceded the uterus, and often looked similar in morphology to tubules seen further down the sample, but in this case were either poorly defined, and/or much smaller and increasing in density until merging into uterine tissue. We refer to these structures as 'transitional tissue', as they appear transitional between regular tubules and uterine tissue. In many cases, when viewed at high magnification, very small tubule-like entrances could be seen (see Figure 4 C & D for a comparison of transitional tissue at low and high magnification). Transitional tissue was not considered to be tubule tissue as we did not deem it likely to be functioning in sperm storage.

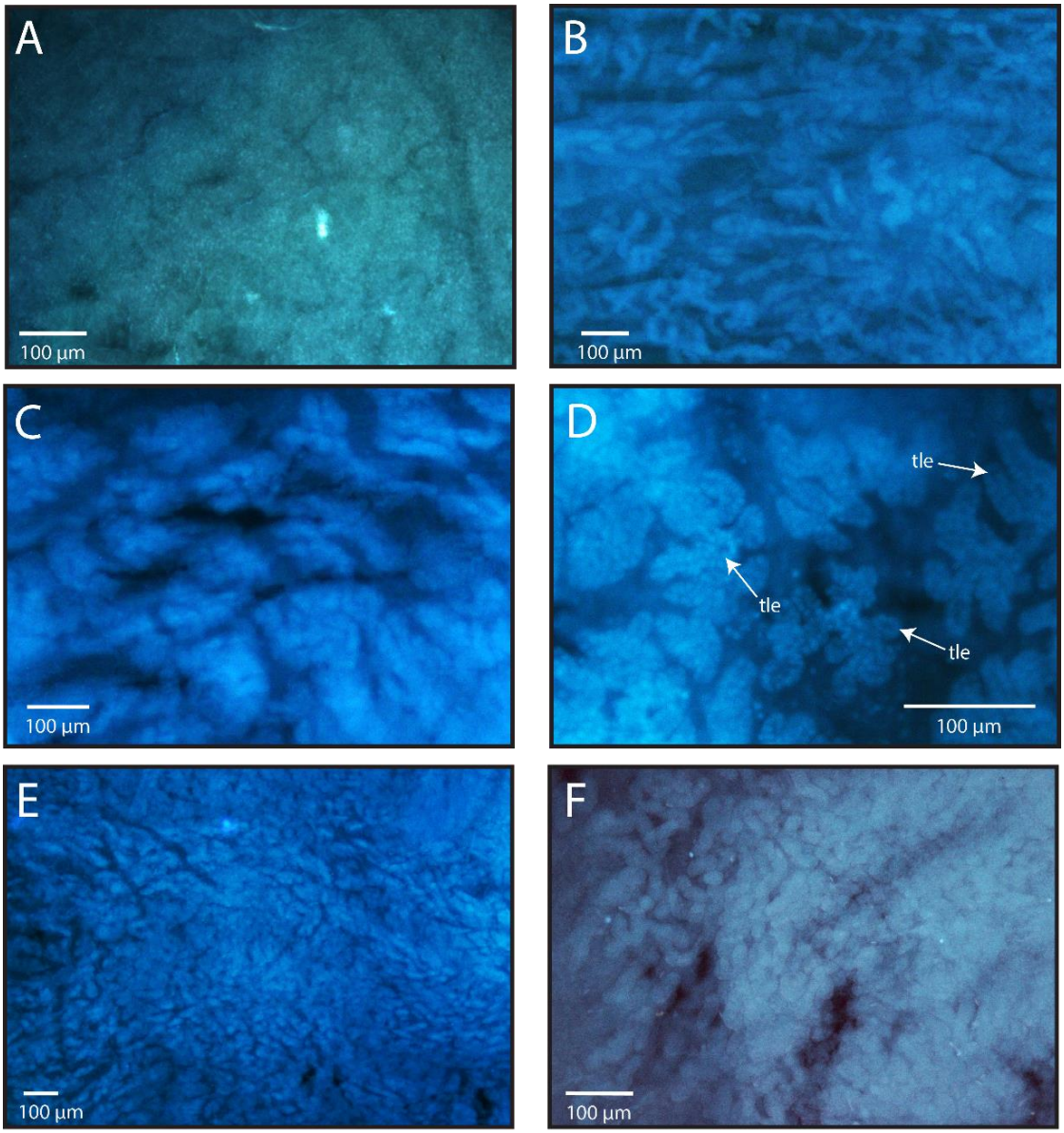


***Figure 4 A:*** *Example of typical (non-transitional) tissue at the uterus end of the vagina in black francolin (Francolinus francolinus) at 100X magnification.* ***B:*** *Transitional tissue in Japanese quail (Coturnix japonica) at 50X magnification. Tissue appears like poorly defined tubule tissue, much smaller than tubules and in places difficult to distinguish from surrounding tissue* ***C:*** *Mountain quail (Oreortyx pictus) at 50X magnification. Distinct clusters of agglomerate-like tissue that is distinct from uterine tissue but with a poorly defined 'fluffy' appearance. When viewed under high magnification, looks like smaller less well-defined agglomerate tissue (see* ***D*** *for an example).* ***D:*** *Transitional tissue from Mountain quail at 200X magnification.* ***E:*** *Transitional tissue from Harlequin quail (Coturnix delegorguei) at 50X magnification. Tissue appears like extremely densely packed tiny tubules, distinct from storage tubules in being overall significantly smaller, but with tubule like entrances only seen when examined under high magnification.* ***F:*** *Transitional tissue in Temminck's tragopan (Tragopan temminckii) at 100X magnification, showing a clear transition from tubule-like (but poorly defined) tissue in the vagina (to the left) to dense uterine tissue (on the right). Arrows point to representative (but not all) examples of*

*tubule-like entrances (tle). Images are all orientated with the cloaca closest to the left side and the uterus closest to the right.*

Transitional tissue was never seen to contain stored sperm and was observed to some degree in 22 species (84.6% of species) and 40 folds (76.9% of fold samples) with only 4 species not displaying transitional tissue in either fold of the sample (bobwhite quail (*Colinus virginianus*), European quail, helmeted guineafowl (*Numidia meleagris*) and white eared pheasant (*Crossoptilon crossoptilon*)) (Figure 3). The distinction between tubules approaching the U region of the UVJ and early transitional tissue was sometimes difficult and required a degree of subjectivity, however the categorisation of transitional tissue was significantly repeatable between observers ($R_{Linkscale}$ = 0.97, CI = 0.98, 1.00, P < 0.0001).

## 3.5 Tubule categorisation

In addition to transitional tissue and channel tissue, we found five distinct categories of tubules.

1) Straight unbranched tubules – the simplest structure, with a clear single lumen. May bend but they do not coil or branch. At least some proportion of tubules within every sample was straight unbranched, making it the most common type of tubule (Figure 3). The categorisation of straight unbranched tubules was highly repeatable between observers ($R_{Linkscale}$ = 0.80, CI = 0.40, 0.99, P = < 0.0001). Examples can be seen in Figures 2, 5, and 6.

2) Straight branched tubules – generally possessing between 1 and 3 long branches (though occasionally more), without coils or twists. As with straight unbranched tubules, branches may bend. These are commonly described in the literature and were present to some degree in 8 species (30.8% of species). For each species that contained straight branched tubules, they were common and found in both folds of the sample (Figure 3). The categorisation of straight branched tubules was highly repeatable between observers ($R_{Linkscale}$ = 0.93, CI = 0.95, 1.00, P = < 0.0001). Examples can be seen in Figures 2, 5 and 7.

3) Coiled tubules – highly coiled or twisted, in some cases small, 'blobby' and unbranched, whilst in others they appeared longer and branched. Coiled tubules were

uncommon, appearing in just 3 species (11.5%) (Figure 3). For each species that contained coiled tubules, they were common and found in both folds of the sample. In some cases, coiling was so extreme the tubule appeared 'corkscrewed' in shape. The categorisation of coiled tubules was highly repeatable between observers ($R_{Linkscale}$ = 0.79, CI = 0.89, 1.00, P = 0.002). Examples can be seen in Figure 5.

4) Mini-tubules – can be defined as being <30% the size of the largest tubule in the sample, and despite being very small relative to the longest tubules, often contained stored sperm. Due to the large number and consistency in length of mini-tubules, we are confident that we were observing their full length in most cases. Occasionally, mini-tubules appeared as barely longer than the tubule entrance. Mini-tubules were very common, appearing to some degree in every species and in 50 folds (96.2% of fold samples) (Figure 3). Mini-tubules could appear along the entire length of the sample but were particularly common towards the U region as the length of tubules generally declined closer to the uterus. Despite being small in length, the lumen and epithelial wall were similar in thickness to other tubules within the sample, and the tubule entrance was similar in size to other tubules in the sample. This distinguishes them from tubule-like transitional tissue which are significantly smaller (e.g. figure 4E). The categorisation of mini-tubules was highly repeatable between observers ($R_{Linkscale}$ = 0.99, CI = 0.98, 1.00, P = < 0.0001). Examples can be seen in Figure 5.

5) Agglomerate tubules – tubules were highly branched and appeared almost globular or star-like in shape, sometimes appearing as dense clusters of tubules with multiple entrances. It was often unclear whether these clusters shared a lumen or were in fact distinct clusters of small agglomerate tubules. Transitional tissue may often appear agglomerate but has a less distinct form and appears only towards the U region of the sample (e.g. Figure 2C-D). Agglomerate tubules were found in 9 species (34.6% of species) and 15 folds (28.8% of fold samples) (Figure 3). There were 3 species in which agglomerate tubules were found in one fold of the sample but not the other (Madagascar partridge, mountain quail and red legged partridge). The categorisation of agglomerate tubules was highly repeatable between observers ($R_{Linkscale}$ = 0.93, CI = 0.95, 1.00, P = < 0.0001). Examples can be seen in Figures 5 and 7.

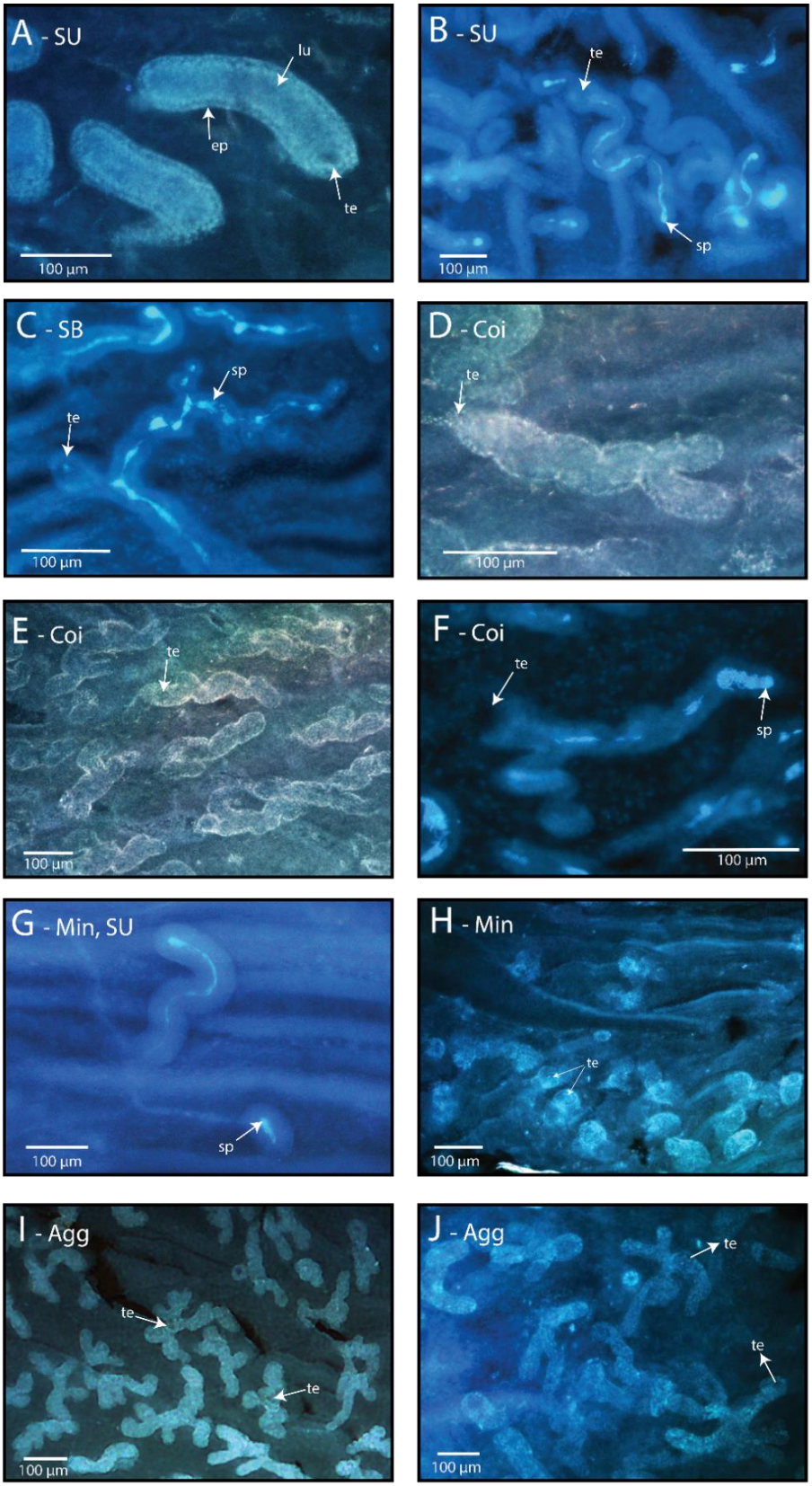


***Figure 5 A:*** *Example of straight unbranched tubules in bamboo partridge (Bambusicola thoracicus) at 200X magnification;* ***B:*** *Example of straight unbranched tubules in common pheasant (Phasianus colchicus) at 100X magnification;* ***C:*** *Example of straight branched tubules in mountain quail (Oreortyx pictus) at 200X*

*magnification;* ***D****: Example of a branched tubule that is also coiled, in bobwhite quail (Colinus virginianus) at 200X magnification. It is common for coiled tubules to also be branched;* ***E:*** *An example of a large number of coiled tubules in bobwhite quail at 100X magnification;* ***F:*** *An example of a branched tubule where one branch is coiled but not the other, in mountain quail at 200X magnification;* ***G:*** *An example of a mini-tubule and a regular sized tubule in the same field of view in wild turkey (Meleagris gallopavo) at 100X magnification. The mini-tubule has visible stored sperm;* ***H:*** *An example of many mini-tubules in close proximity to one another in bobwhite quail at 100X magnification. Some tubules are so short in length they appear as little more than an entrance;* ***I:*** *An example of agglomerate tubules in black francolin (Francolinus francolinus) at 50X magnification. Tubules are so highly branched they appear as globular rather than tubular structures;* ***J:*** *An example of agglomerate tubules in grey junglefowl (Gallus sonneratii) at 100X magnification. Tubules are so highly branched they form some unique and interesting shapes. Arrows point to representative (but not all) examples of the tubule lumen (lu), the tubule epithelium (ep), tubule entrances (te), and stored sperm (lighter/brighter blue) (sp). Images are all orientated with the cloaca closest to the left side and the uterus closest to the right. Image title abbreviations are as follows: Straight unbranched – SU; straight branched – SB; coiled – Coi; Mini-tubules – Min; Agglomerate tubules – Agg.*

In addition to the 5 categories above, we noticed several samples contained tubules with interesting features that were not consistent or common enough to be considered separate categories, but are of note and worth discussing: first, we observed some tubules with unusually large/wide or 'expanded' entrances (Figure 6A) in helmeted guineafowl, Lady

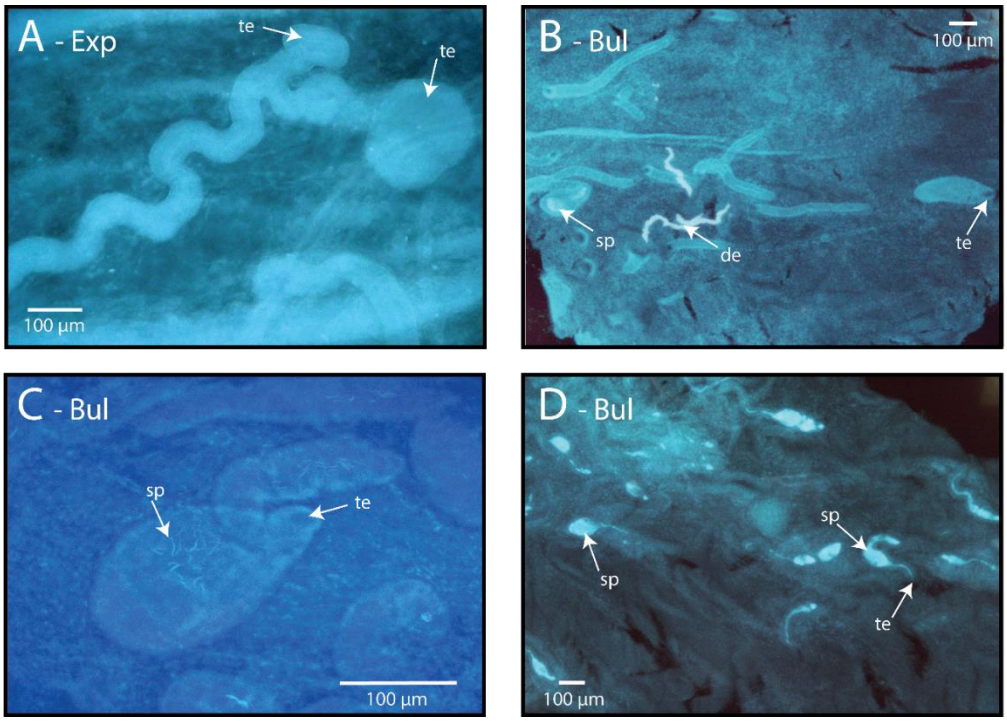


***Figure 6 A:*** *Example of tubule/s with 'expanded' entrances, in helmeted guinea fowl (Numidia meleagris) at 100X magnification, with tubule/s that appear to have very wide entrances. It is not clear whether this is two*

*overlapping tubules or a single tubule with multiple entrances.* ***B:*** *Examples of 'bulbous' types in Chinese quail (Excalfactoria chinensis) at 50X magnification, with 2 tubules that appear engorged relative to the other tubules in the sample. One tubule has obvious stored sperm.* ***C:*** *Examples of 'bulbous' types in Swinhoe's pheasant (Lophura swinhoii) at 200X magnification, with a smaller number of stored sperm.* ***D:*** *Examples of 'bulbous' types in Madagascar partridge (Margaroperdix madagarensis) at 50X magnification with clear stored sperm. Arrows point to representative (but not all) examples of tubule entrances (te), stored sperm (lighter/brighter blue) (sp) and some non-tissue debris that has picked up the dye (de). Images are all orientated with the cloaca closest to the left side and the uterus closest to the right. Image title abbreviations are as follows: Bulbous – Bul; expanded – Exp.*

Amherst's pheasant and Reeve's pheasant (*Syrmaticus reevesii*); and second, we observed some tubules that were bulbous or inflated along their length (but had normal sized entrances) compared to the rest of the tubules in the C region of Chinese quail, Madagascar partridge and Swinhoe's pheasant samples (Figure 6B-D)(Figure 3).

## 3.6 Categorisation flow chart

We created a flowchart for categorising tubule type based on our observations across species (Figure 7; and reproduced as a dichotomous key in the supplementary material). We found this process appropriate for categorising the tubules found in the species examined here, with the quality of images produced from our methodology, but recognise that there are likely to be more categories of tubules across other species/taxa and encourage researchers exploring tubule diversity to expand on our categories as necessary. Figure 3 provides a summary of the categories assigned to each species.

# 4 Discussion

Avian sperm storage tubules (SSTs) are commonly described as 'simple tubular invaginations', located within a small strip of vagina tissue bordering the uterus, known as the UVJ (Bakst, 2011; Sasanami, 2017). They are widely assumed to look- and function in – the same way across all birds, according to the most frequently examined species: the domestic chicken, turkey, Japanese quail, and zebra finches. Here, we provide evidence that Galliformes – a large group of ground-dwelling birds – exhibit striking and surprising variation in SST morphology across species. We propose the variety of tubules we observed across 26 species can be partitioned into 5 distinct categories: 1) Straight unbranched tubules; 2) straight

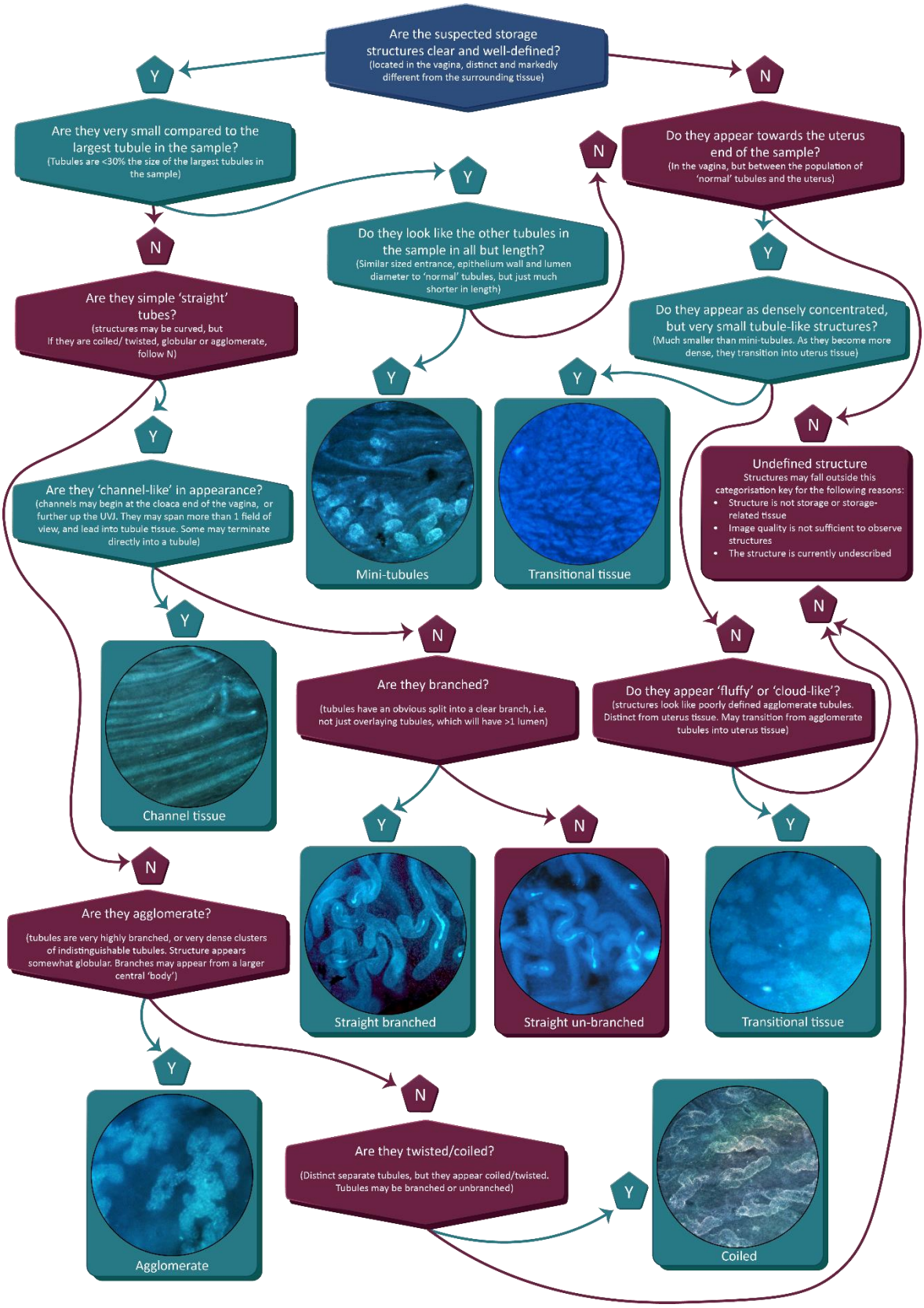


***Figure 7*** *Categorisation flowchart for determining tubule type in Galliformes. Images within this flowchart have been cropped and expanded for easy visualisation, but are taken from the following species: mini-tubules – bobwhite quail (Colinus virginianus) at 100X magnification; transitional tissue (top) – Harlequin quail (Coturnix*

*delegorguei) at 50X magnification; channel tissue – grey francolin (Francolinus pondicerianus) at 100X magnification; straight branched – Reeves pheasant (Syrmaticus reevesii) at 100X magnification; straight unbranched – common pheasant (Phasianus colchicus) at 100X magnification; transitional tissue (bottom) – black francolin (Francolinus francolinus) at 50X magnification; agglomerate – black francolin at 100X magnification; coiled – bobwhite quail at 100X magnification. This flowchart has also been provided in a dichotomous key format in the supplementary material, which may prove useful for practical applications.*

branched tubules; 3) coiled tubules; 4) mini-tubules and 5) agglomerate tubules. We also show that above and beyond the apparent diversity in SST morphology, the surrounding tissue of the UVJ region appears to vary between species in some respects and includes at least 2 additional features: 1) channels and 2) transitional tissue, the function (if any) of which is currently unknown but warrants further investigation. Additionally, whether tubule function varies by category is unknown, but may have important consequences for our understanding of sperm storage and post-copulatory sexual selection in general.

In most samples, tubules were found throughout the length of the UVJ. However, in half of all species, tubules did not always extend to the distal region of the sample that borders the uterus. The region directly preceding the uterus was often populated by what we refer to here as 'transitional tissue'. Transitional tissue could not be defined as tubule tissue, surrounding vaginal tissue, or uterine tissue. It often appeared to contain smaller or poorly defined forms of the tubules seen further down the sample (Figure 4). While many of the structures observed in transitional tissue appeared to have entrances (when viewed under high magnification), due to their small size and the fact they were never observed to contain stored sperm, it seems unlikely they function as sperm storage structures. Previous work in yellow-headed blackbirds has shown that SSTs vary in length and likelihood to store sperm across regions of the UVJ (Briskie, 1996); it's possible that the transitional tissue observed here represents the smaller and less functional tubules found in that study. Another possibility is that transitional tissue represents a transitional/developmental state between the surrounding tissue and fully formed and functional storage structures. We currently do not know the processes of SST ontogeny, or fully understand how tubule morphology changes in response to the regression and subsequent regrowth of the oviduct in seasonally

reproducing birds. Further work is needed to elucidate the precise ultrastructure and functional significance of transitional tissue.

Across the 26 Galliformes species examined here, we observed channel-like depressions within the surrounding tissue of the UVJ in most samples. Channels varied in appearance between species, but were generally either very long, extending multiple fields of view, or were short and funnel-like. Long channels (e.g. Figures 2B-D) often ended in a region populated by SSTs (e.g. Figure 2C), or in some cases ended directly in a tubule (Figure 2D). Shorter funnel-like channels (e.g. Figure 2E-F) were most often observed at the start of the sample (i.e. the C end of the UVJ), and also frequently ended directly in a tubule.

It is difficult to discern the precise orientation of structures from a 2D image, and we cannot rule out the possibility that in some cases tubules may be stacked directly on top of channels rather than being connected, however this seems unlikely to account for all observations, particularly for funnel-like channels that were frequent and consistent in appearance. Further investigation is certainly warranted to confirm the presence and 3D ultrastructure of channels, and channel-tubule connections, and to explore their functional significance. However, it seems likely that these channels are somehow involved in sperm transport or storage (at least to some degree). One convincing possibility is that channels simply provide a more direct route to the region of the vagina populated by tubules, or enhance swimming efficiency (Magdanz et al., 2015). The precise mechanism of sperm transport through the vagina is not known: in addition to sperm motility (Allen & Grigg 1957), it has been suggested that some additional mechanisms may exist including ciliary movements of the surface epithelium of the vagina, contractions of the oviduct (possibly in combination with ciliary movement), and/or chemotactic guidance (Orr and Brennan, 2015; Sasanami, 2017). It is possible that in species that have them, channels aid in the rapid passage of sperm, and/or provide some protection from the environment of the vagina (e.g. the anti-sperm immune response, vaginal sperm ejection, and mechanical flushes), while shorter funnel-like channels may simply direct sperm into tubules once they reach the site of storage. Alternatively, close association with the vaginal mucosal epithelium might increase sperm contact with immune cells (Yoshimura et al., 1997), providing an opportunity for cryptic female choice to be intensified.

Sperm swimming mechanics are known to be strongly influenced by their interaction with surfaces (Denissenko et al., 2012). In mammals, sperm are thought to travel through micro-channels in the reproductive tract, whereby motile sperm preferentially swim near the boundary of channel walls and this may act as a guiding mechanism towards the egg (Denissenko et al., 2012; Magdanz et al., 2015). Furthermore, there is some evidence that suggests that boundary-following behaviour may be associated with higher sperm DNA integrity in humans (Eamer et al., 2016), and that channels may elicit intensified competition between sperm (Zaferani et al., 2019). Whether the channels we observe here are analogous to the micro-channels of the mammalian female reproductive tract are unclear, but this possibility has potential implications for our understanding of sperm transport and post-copulatory sexual selection within the avian vagina and warrants further investigation.

On the other hand, if tubules are directly connected to a channel, then sperm boundary following behaviour (Denissenko et al., 2012) may make it more difficult for sperm to exit storage without having to first travel back down towards the channel entrance. This would also be the case if long channels exist as enclosed tubes rather than open grooves (which is yet to be determined). This possibility raises the intriguing prospect that SSTs may not always function to store the fertilising subset of sperm, but instead may act as sperm 'bins' that inhibit– rather than facilitate – sperm participation in fertilisation. Sperm stored in SSTs are usually assumed to make up the fertilising subset of sperm because past studies in turkeys have shown that the number of sperm residing in SSTs is strongly correlated with the number that reach the ovum (Bakst, 2011; Brillard and Bakst, 1990). However, evidence that stored sperm always form the fertilising subset is lacking for other species, and so it may be worth revisiting this assumption. There is also evidence from other taxa that females can directly differentially remove sperm from storage, for example through differential ejection (aka sperm 'dumping'), as seen in *Drosophila* (Snook and Hosken, 2004).

While the vagina is expected to be the major site of sperm selection in birds (Bakst, 2011; Orr and Brennan, 2015; Steele and Wishart, 1996), sperm storage tubules are now also predicted to play an important role in sperm selection by way of non-random acceptance or release of sperm. For example, sperm filling rate into SSTs has been found to be unevenly distributed across the UVJ (Briskie, 1996; Ito et al., 2011; Sasanami et al., 2015); and in zebra finches, SSTs have been found to vary widely in diameter, possess constricted entrances that may act

as a controlled barrier to sperm entry (Mendonca et al., 2019), and differentially store sperm from different inseminations (Hemmings and Birkhead, 2017). Sperm dumping and selective sperm displacement in other taxa (Barnett et al., 1995; Gasparini et al., 2018; Lüpold et al., 2012; Snook and Hosken, 2004) also supports the role of storage structures in sperm selection. In bats, species that store sperm also appear to produce more sperm, and testis size is correlated with sperm storage duration suggesting that longer sperm storage is associated with increased sperm competition (Orr and Brennan, 2015; Orr and Zuk, 2013). The large variation in SST morphology observed in the current study may lend support to the hypothesis that SSTs exhibit functional differences that could influence the outcome of sperm competition. In accordance with this, we also observed a non-uniform distribution of stored sperm among tubules, suggesting individual SSTs may vary in their ability to accept, store and release sperm. Sperm were also commonly observed distributed along the entire length of the tubules, rather than being congregated just within the blind-end, however we did not notice any patterns in sperm distribution across species or SST category.

Theoretically, tubule shape and length may influence the speed or order in which sperm are released from storage. Briskie (1996) found that in yellow-headed blackbirds, uterine-end SSTs were smaller and later to mature but released a greater number of sperm during the egg-laying period, suggesting that smaller tubules may accept sperm later, but release them more quickly, than longer tubules. Accordingly, sperm stored in the shorter mini-tubules we observe here (e.g. Figure 4G), may exit storage more quickly than sperm stored at the end of longer or more highly branched tubules (e.g., Figure 5C). Branch length and distance from the SST entrance will presumably influence how easily/fast sperm are released, potentially providing a mechanism of control over the order of sperm release from storage. Variation in tubule shape within samples may therefore also help to reduce mating order effects, by creating variation in ‘the playing field’, in which sperm from different males are stored in a variety of tubules of varying length, branch number, and complexity, and with a varying advantage/disadvantage over the timing of release (Hemmings and Birkhead, 2017).

We observed that agglomerate tubules are often extremely highly branched, complex, and diverse in morphology (e.g. Figure 5I and J). How this structural diversity might translate to variation in SST function is unclear and introduces several questions. For example, do all branches function in the same way or are there selective features that allow branches to vary

in their ability to accept sperm? Does mating order influence which branch sperm are accepted into? Do the number of branches of a tubule influence the ease with which sperm can exit the tubule upon release? Given the degree of variation in SST structures observed here, and the highly diverse nature of sperm cells (Pitnick et al., 2009), a logical line of questioning would be to assess whether SST morphology and complexity correlate with sperm morphology, sperm storage duration, and/or sexual selection intensity (which is typically correlated with mating system) across species. One possibility is that highly branched or complex tubule structures introduce variation in the ease with which sperm can enter or reach the most protective regions of the storage tubule. For example, if more vigorous sperm are better able to reach more distal branches, they may be better protected from further selective processes such as sperm ejection or the anti-sperm response within the vagina. Exploring differential storage of sperm of varying quality across a variety of tubule types may shed light on whether tubule morphology is linked to sperm selection.

In the case of coiled tubules (e.g., Figure 5D-F), the tightness of coils or their length may provide an even more extreme degree of manipulation over the ability of sperm to enter storage, or the timing and speed of sperm release. The structure of coiled tubules has some interesting parallels with the coiled vaginas of some species of waterfowl, where the coiled shape of the reproductive tract has a sexually antagonistic co-evolutionary relationship with the coiled penis of the male (which is coiled in the opposite direction to the vagina, making intromission more difficult) (Brennan et al., 2010). In addition to their spiral structure, the vaginas of these species also have multiple blind-ended ‘pouches’, in which the penis can be directed to act as a mechanism of female control over sperm use following forced copulations (Brennan et al., 2010). Figure 2F presents a striking example of a tubule in which one branch (notably, the branch closest to the tubule entrance) is coiled whilst the other is not. One possibility is that coiled tubules function in a similar way to the blind-ended and spiral-shaped vaginas of ducks, possibly providing a sperm ‘bin’ by which a proportion of sperm are prevented from being released, or released quickly enough, to participate in sperm competition.

Another comparison can be drawn with the uterine muscular coiling (UMC) of viperid snakes. UMC involves a contraction of the innermost layers of the UVJ, which causes the tissue to form a coiled shape (not dissimilar to the coiled appearance of tubules seen in Figure 5D-F)

(Muniz-Da-Silva et al., 2020). It is thought that UMC may function as a mechanism of sperm storage by maintaining the position and viability of sperm. There is evidence that SSTs in turkeys are innervated, and individual SSTs house F-actin rich terminal webs in the epithelium that exhibit a coiled appearance, suggesting they are capable of contraction (Freedman et al., 2001). Nerve fibres have also been observed in close association with SSTs in the alpine accentor (*Prunella collaris*) (Chiba and Nakamura, 2001), although contractile elements were not found in this case. In support of the contractile potential of SSTs, a contraction-like change to SST morphology was observed in Japanese quail following an injection of progesterone (which is thought to be one factor that triggers sperm release from tubules) (Ito et al., 2011), suggesting that individual SSTs are capable of contraction and relaxation under presumably fine temporal control. It may therefore be possible that tubule coiling is a plastic mechanism (under neural or hormonal control) for maintaining and protecting sperm in storage. Interestingly, it is reported that UMC in snakes becomes less visible in tissue preserved in 10% formalin relative to fresh tissue. It may therefore be worth examining the morphology of SSTs across birds using fresh, rather than preserved tissue, and across different stages of reproduction (e.g., before, during and after sperm acceptance and release). Exploring SST morphology in fresh tissue will also ensure that any features lost as a result of the preservation process are uncovered. Coiled tubules have in fact been observed at least once before, in a study examining the morphology of SSTs in the American kestrel (*Falco sparverius*) (Bakst and Bird, 1987). Their shape was only alluded to briefly and their significance was not discussed at the time nor (to our knowledge) since. We believe our images provide the first evidence for such structures in Galliformes. While we have hypothesised several possible functional explanations for coiled SSTs, further work is needed to uncover the significance of these structures for our understanding of SST function and post-copulatory sperm storage and selection in general.

Finally, we observed two additional features that were uncommon and inconsistent within samples, suggesting they were not appropriate for inclusion as separate categories. 1) Expanded entrances- these unusual tubules were observed to have abnormally wide and funnel-like entrances. SSTs have been shown to have constricted gate-like entrances (Mendonca et al., 2019), and so it could be that these tubules were simply in the processes of relaxing following sperm release or prior to sperm acceptance. Future work exploring SST

morphological changes through time is a challenging but necessary step to understanding the process of sperm acceptance and release. 2) Bulbous tubules- whether these are typical structures for these species is unclear, but these tubules were seen containing stored sperm and are clearly functional. Further work exploring intraspecific variation in tubule morphology will be needed to explore this. If these structures do appear to be consistent features within and across species, re-evaluation of their inclusion as a separate tubule category may be needed.

# 5 Conclusions

Sperm storage tubules in birds are often described as 'simple tubular invaginations'. Whilst that may be true of the tubules typically seen in well studied birds like chickens and turkeys, we find that across other Galliformes, tubule structure is far more variable, diverse, and complex than previous assumed. The variation in tubule and surrounding tissue structure we observe here may have important implications for our understanding of sperm storage tubule function in general, with broader consequences for our understanding of post-copulatory sperm selection. Further research is needed to quantify this variation across Galliformes, explore variation across other species of birds, and determine the functional significance of the structures we observe here. An obvious first step will be to explore whether storage tubule types are associated with sperm morphology, sperm storage capacity and sperm competition intensity. It may also be useful for future work to examine different sperm storage types using scanning and transmission electron microscopy, including the use of 3D imaging techniques, which will help confirm their ultrastructure, internal morphology, and relationships with surrounding tissues. Ultimately, these findings contribute to the growing body of evidence across taxa that female sperm storage structures can be complex, highly specialised and variable (Beese and Baur, 2006; Berger et al., 2011; Holt and Fazeli, 2016; Hopkins et al., 2020; Lüpold et al., 2013; Orr and Brennan, 2015; Ward, 2000).

# Data accessibility

No quantitative data were generated.

## Author contributions

Katherine Assersohn: Conceptualization; Data Curation; Formal Analysis; Investigation; Methodology; Project Administration; Software; Validation; Visualization; Writing – Original Draft Preparation; Writing – Review & Editing. Paul Richards: Conceptualization; Methodology; Writing – Review & Editing; Nicola Hemmings: Conceptualization; Data Curation; Funding Acquisition; Investigation; Methodology; Project Administration; Resources; Supervision; Validation; Writing – Review & Editing.

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## Competing interests

The authors declare there are no conflicts of interest.

## Ethics statement

All tissue samples were taken from animals that were provided to us having already been euthanised for other purposes.

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## References

Assersohn, K., Brekke, P., Hemmings, N., 2021. Physiological factors influencing female fertility in birds. R. Soc. open sci. 8, 202274. https://doi.org/10.1098/rsos.202274

Bakst, M.R., 2011. Role of the oviduct in maintaining sustained fertility in hens. Journal of Animal Science 89, 1323–1329. https://doi.org/10.2527/jas.2010-3663

Bakst, M.R., 1994. Fate of fluorescent stained sperm following insemination- new light on oviducal sperm transport and storage in the turkey. Biology of Reproduction 50, 987–992. https://doi.org/10.1095/biolreprod50.5.987

Bakst, M.R., Bauchan, G., 2015. Apical blebs on sperm storage tubule epithelial cell microvilli: Their release and interaction with resident sperm in the turkey hen oviduct. Theriogenology 83, 1438–1444. https://doi.org/10.1016/j.theriogenology.2015.01.016

Bakst, M.R., Bird, D.M., 1987. Localization of oviductal sperm-storage tubules in the American kestrel (Falco sparverius). The Auk.

Barnett, M., Telford, S.R., Tibbles, B.J., 1995. Female mediation of sperm competition in the millipede AIIoporusuncinatus (Diplopoda: Spirostreptidae). Behavioral Ecology and Sociobiology 36, 413–419.

Beese, K., Baur, B., 2006. Expandable spermatheca influences sperm storage in the simultaneously hermaphroditic snail *Arianta arbustorum*. Invertebrate Reproduction and Development. https://doi.org/10.1080/07924259.2006.9652198

Berger, D., Bauerfeind, S.S., Blanckenhorn, W.U., Schäfer, M.A., 2011. High temperatures reveal cryptic genetic variation in a polymorphic female sperm storage organ: evolvability of female reproductive morphology. Evolution 65, 2830–2842. https://doi.org/10.1111/j.1558-5646.2011.01392.x

Birkhead, T.R., 1992. Sperm storage and the fertile period in the Bengalese finch. The Auk 109, 620–625.

Birkhead, T.R., del Nevo, A.J., 1987. Egg formation and the pre-laying period of the Common guillemot *(Uria aalge)*. Journal of Zoology 211, 83–88. https://doi.org/10.1111/j.1469-7998.1987.tb07454.x

Birkhead, T.R., Møller, A.P., 1990. Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study 45, 363–372. https://doi.org/doi.org/10.1111/j.1095-8312.1992.tb00649.x

Bobr, L., Lorenz, F.W., Ogasawara, F.X., 1964a. Distribution of spermatozoa in the oviduct and fertility in domestic birds I. Residence sites of spermatozoa in fowl oviducts 8, 39–47.

Bobr, L., Ogasawara, F.X., Lorenz, F.W., 1964b. Distribution of spermatozoa in the oviduct and fertility in domestic birds II. Transport of spermatozoa in the fowl oviduct 8, 49–58.

Brennan, P.L.R., Clark, C.J., Prum, R.O., 2010. Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia. Proc. R. Soc. B. 277, 1309–1314. https://doi.org/10.1098/rspb.2009.2139

Brillard, J.P., Bakst, M.R., 1990. Quantification of spermatozoa in the sperm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biology of Reproduction 43, 271–275. https://doi.org/10.1095/biolreprod43.2.271

Briskie, J., 1996. Spatiotemporal patterns of sperm storage and last-male sperm precedence in birds. Functional Ecology. https://doi.org/10.2307/2390286

Briskie, J.V., Birkhead, T.R., 1993. A review of the methods used to study the anatomy of avian sperm storage. Ornis Scandinavica, b. https://doi.org/10.2307/3676795

Briskie, J.V., Montgomerie, R., 1993. Patterns of Sperm Storage in Relation to Sperm Competition in Passerine Birds. The Condor 95, 442–454. https://doi.org/10.2307/1369366

Chiba, A., Nakamura, M., 2001. Microscopic structure of the sperm storage tubules in the polygynandrous alpine accentor, *Prunella collaris* (Aves). Acta Zoologica. https://doi.org/10.1046/j.1463-6395.2001.00091.x

Cramer, E.R.A., Yilma, Z.B., Lifjeld, J.T., 2023. Selection on sperm size in response to promiscuity and variation in female sperm storage organs. J of Evolutionary Biology 36, 131–143. https://doi.org/10.1111/jeb.14120

Denissenko, P., Kantsler, V., Smith, D.J., Kirkman-Brown, J., 2012. Human spermatozoa migration in microchannels reveals boundary-following navigation. Proc. Natl. Acad. Sci. U.S.A. 109, 8007–8010. https://doi.org/10.1073/pnas.1202934109

Eamer, L., Vollmer, M., Nosrati, R., San Gabriel, M.C., Zeidan, K., Zini, A., Sinton, D., 2016. Turning the corner in fertility: high DNA integrity of boundary-following sperm. Lab Chip 16, 2418–2422. https://doi.org/10.1039/C6LC00490C

Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Freedman, S., Akuffo, V., Bakst, M., 2001. Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo*). Reproduction. https://doi.org/10.1530/rep.0.1210809

Gasparini, C., Daymond, E., Evans, J.P., 2018. Extreme fertilization bias towards freshly inseminated sperm in a species exhibiting prolonged female sperm storage. R. Soc. open sci. 5, 172195. https://doi.org/10.1098/rsos.172195

Harmon, L., 2023. treeplyr: "dplyr" Functionality for Matched Tree and Data Objects.

Hatch, S.A., 1983. Mechanism and Ecological Significance of Sperm Storage in the Northern Fulmar with Reference to Its Occurrence in Other Birds. The Auk 100, 593–600.

Hemmings, N., Birkhead, T., 2017. Differential sperm storage by female zebra finches Taeniopygia guttata. Proceedings. Biological sciences 284.

Hemmings, N., Birkhead, T.R., 2020. Extraordinary sperm to egg ratios in seabirds. The Auk 137, ukaa052. https://doi.org/10.1093/auk/ukaa052

Hemmings, N., Birkhead, T.R., 2015. Polyspermy in birds: sperm numbers and embryo survival. Proceedings of the Royal Society B: Biological Sciences 282, 20151682. https://doi.org/10.1098/rspb.2015.1682

Hemmings, N., Birkhead, T.R., Brillard, J.P., Froment, P., Briere, S., 2015. Timing associated with oviductal sperm storage and release after artificial insemination in domestic hens. Theriogenology 83, 1174–1178. https://doi.org/10.1016/j.theriogenology.2014.12.022

Higginson, D.M., Miller, K.B., Segraves, K.A., Pitnick, S., 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. U.S.A. 109, 4538–4543. https://doi.org/10.1073/pnas.1111474109

Hiyama, G., Matsuzaki, M., Mizushima, S., Dohra, H., Ikegami, K., Yoshimura, T., Shiba, K., Inaba, K., Sasanami, T., 2014. Sperm activation by heat shock protein 70 supports the migration of sperm released from sperm storage tubules in Japanese quail (Coturnix japonica). Reproduction 147, 167–178. https://doi.org/10.1530/REP-13-0439

Holm, L., Ekwall, H., Wishart, G.J., Ridderstråle, Y., Ridderstrale, Y., 2000. Localization of calcium and zinc in the sperm storage tubules of chicken, quail and turkey using X-ray microanalysis. Journal of reproduction and fertility 118, 331–336. https://doi.org/10.1530/reprod/118.2.331

Holt, W.V., 2011. Mechanisms of Sperm Storage in the Female Reproductive Tract: an Interspecies Comparison. Reproduction in Domestic Animals 46, 68–74. https://doi.org/10.1111/j.1439-0531.2011.01862.x

Holt, W.V., Fazeli, A., 2016. Sperm Storage in the Female Reproductive Tract. Annual Review Of Animal Biosciences, Vol 4, Annual Review of Animal Biosciences. https://doi.org/10.1146/annurev-animal-021815-111350

Hopkins, B.R., Sepil, I., Wigby, S., 2020. Structural variation in Drosophila melanogaster spermathecal ducts and its association with sperm competition dynamics. Royal Society Open Science 7. https://doi.org/10.1098/rsos.200130

Huang, A., Isobe, N., Obitsu, T., Yoshimura, Y., 2016. Expression of lipases and lipid receptors in sperm storage tubules and possible role of fatty acids in sperm survival in the hen oviduct. Theriogenology 85, 1334–1342. https://doi.org/10.1016/j.theriogenology.2015.12.020

Huang, A., Isobe, N., Yoshimura, Y., 2017. Changes in localization and density of CD63-positive exosome-like substances in the hen oviduct with artificial insemination and their effect on sperm viability. Theriogenology 101, 135–143. https://doi.org/10.1016/j.theriogenology.2017.06.028

Ito, T., Yoshizaki, N., Tokumoto, T., Ono, H., Yoshimura, T., Tsukada, A., Kansaku, N., Sasanami, T., 2011. Progesterone is as sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology 152, 3952–3962. https://doi.org/10.1210/en.2011-0237

Khillare, G.S., Sastry, K.V.H., Agrawal, R., Prakash, A., Mohan, J., Singh, R.P., 2018. Molecular and biochemical events in sperm storage tubules of oviduct in birds. World's Poultry Science Journal 74, 35–44. https://doi.org/10.1017/S004393391700085X

Kustra, M.C., Alonzo, S.H., 2023. The coevolutionary dynamics of cryptic female choice. Evolution Letters 7, 191–202. https://doi.org/10.1093/evlett/qrad025

Levine, B.A., Schuett, G.W., Booth, W., 2021. Exceptional long-term sperm storage by a female vertebrate. PLoS ONE 16, e0252049. https://doi.org/10.1371/journal.pone.0252049

Lüpold, S., Manier, M.K., Berben, K.S., Smith, K.J., Daley, B.D., Buckley, S.H., Belote, J.M., Pitnick, S., 2012. How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in Drosophila melanogaster. Current Biology 22, 1667–1672. https://doi.org/10.1016/j.cub.2012.06.059

Lüpold, S., Pitnick, S., Berben, K.S., Blengini, C.S., Belote, J.M., Manier, M.K., 2013. Female mediation of competitive fertilization success in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 110, 10693–10698. https://doi.org/10.1073/pnas.1300954110

Magdanz, V., Koch, B., Sanchez, S., Schmidt, O.G., 2015. Sperm Dynamics in Tubular Confinement. Small 11, 781–785. https://doi.org/10.1002/smll.201401881

Matsuzaki, M., Mizushima, S., Hiyama, G., Hirohashi, N., Shiba, K., 2015. Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. Scientific Reports 5, 1–12. https://doi.org/10.1038/srep17643

Mendonca, T., Cadby, A.J., Hemmings, N., 2019. Sperm gatekeeping: 3D imaging reveals a constricted entrance to zebra finch sperm storage tubules. Biophysical Journal 117, 2180–2187. https://doi.org/10.1016/j.bpj.2019.10.038

Miller, G.T., Pitnick, S., 2002. Sperm-Female Coevolution in *Drosophila*. Science 298, 1230–1233. https://doi.org/10.1126/science.1076968

Muniz-Da-Silva, D.F., Passos, J., Siegel, D.S., Almeida-Santos, S.M., 2020. Caudal oviduct coiling in a viperid snake, *Crotalus durissus*. Acta Zoologica 101, 69–77. https://doi.org/10.1111/azo.12271

Ogasawara, F.X., Lorenz, F.W., Bobr, L.W., 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds III. Intra-uterine insemination of semen from low-fecundity cocks. Journal of Reproductive Fertility 11, 33–41.

Orr, T.J., Brennan, P.L.R., 2015. Sperm storage: distinguishing selective processes and evaluating criteria. Trends in Ecology & Evolution 30, 261–272. https://doi.org/10.1016/j.tree.2015.03.006

Orr, T.J., Zuk, M., 2013. Does delayed fertilization facilitate sperm competition in bats? Behav Ecol Sociobiol 67, 1903–1913. https://doi.org/10.1007/s00265-013-1598-2

Orr, T.J., Zuk, M., 2012. Sperm storage. Current Biology 22, R8–R10. https://doi.org/10.1016/j.cub.2011.11.003

Payne, L.F., 1914. Preliminary report of vitality and activity of sperm cells and artificial insemination of the chicken. Oklahoma agricultural and mechanical college.

Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm Biology: an evolutionary perspective. Academic Press, pp. 69–149.

R Core Team, 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Sasanami, T., 2017. Avian Reproduction: From Behaviour to Molecules. Springer Singapore. https://doi.org/10.1007/978-981-10-3975-1

Sasanami, T., Izumi, S., Sakurai, N., Hirata, T., Mizushima, S., Matsuzaki, M., Hiyama, G., Yorinaga, E., Yoshimura, T., Ukena, K., Tsutsui, K., 2015. A unique mechanism of successful fertilization in a domestic bird. Scientific Reports 5. https://doi.org/10.1038/srep07700

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9, 671–675. https://doi.org/doi:10.1038/nmeth.2089

Shankar, G., Gagan, T.A., Kumari, T.R.S., Marathe, G.K., 2022. Sperm storage by females across the animal phyla: A survey on the occurrence and biomolecules involved in sperm storage. Journal of Experimental Zoology Part B- Molecular and Developmental Evolution. https://doi.org/10.1002/jez.b.23189

Snook, R.R., Hosken, D.J., 2004. Sperm death and dumping in Drosophila. Nature 428, 939–941. https://doi.org/10.1038/nature02455

Steele, M., Wishart, G., 1996. Demonstration that the removal of sialic acid from the surface of chicken spermatozoa impedes their transvaginal migration. Theriogenology. https://doi.org/10.1016/S0093-691X(96)00268-3

Stein, R.W., Brown, J.W., Mooers, A.Ø., 2015. A molecular genetic time scale demonstrates Cretaceous origins and multiple diversification rate shifts within the order Galliformes (Aves). Molecular Phylogenetics and Evolution 92, 155–164. https://doi.org/10.1016/j.ympev.2015.06.005

Stoffel, M.A., Nakagawa, S., Schielzeth, H., 2017. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods in Ecology and Evolution 8, 1639–1644. https://doi.org/10.1111/2041-210X.12797

Van Drimmelen, G.C., 1946. “Spermnests” in the oviduct of the domestic hen. J. South African Vet. Med. Assoc. 17, 42–52.

Verma, O., Cherms, F., 1965. Appearance of sperm and their persistency in storage tubules of turkey hens after a single insemination. Poultry Science. https://doi.org/10.3382/ps.0440609

Verma, O.P., Cherms, F.L., 1964. Observations on the Oviduct of Turkeys. Avian Diseases 8, 19. https://doi.org/10.2307/1587814

Wanless, S., Harris, M.P., 1986. Time spent at the colony by male and female Guillemots *Uria aalge* and Razorbills *Alca torda*. Bird Study 33, 168–176. https://doi.org/10.1080/00063658609476916

Ward, P.I., 2000. Cryptic female choice in the yellow dung fly Scathophaga stercoraria (L.). Evolution 54, 1680–1686. https://doi.org/10.1111/j.0014-3820.2000.tb00712.x

Yoshimura, Y., Okamoto, T., Tamura, T., 1997. Localisation of MHC class II, lymphocytes and immunoglobulins in the oviduct of laying and moulting hens. British Poultry Science 38, 590–596. https://doi.org/10.1080/00071669708418041

Zaferani, M., Palermo, G.D., Abbaspourrad, A., 2019. Strictures of a microchannel impose fierce competition to select for highly motile sperm. Sci. Adv. 5, eaav2111. https://doi.org/10.1126/sciadv.aav2111

# Chapter 5:

## A comparative analysis of oviductal and sperm storage traits in relation to post-copulatory sexual selection intensity in Galliformes

*Golden pheasant (Chrysolophus pictus) pair*

# A comparative analysis of oviductal and sperm storage traits in relation to post-copulatory sexual selection intensity in Galliformes

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## Abstract

Post-copulatory sexual selection, comprised of sperm competition and cryptic female choice, is a powerful evolutionary force that can drive the rapid diversification of reproductive traits across taxa. In birds, the female reproductive tract provides the arena for post-copulatory processes, but we lack a comprehensive understanding of the female factors influencing sexually selected traits. Here, we use a comparative approach to exploring the relationships between female reproductive tract morphology, sperm competition intensity, and sperm traits across Galliformes. While accounting for phylogenetic and allometric relationships, we find that species that experience greater sperm competition intensity have relatively longer vaginas. This finding suggests that important co-evolutionary dynamics exist between male and female reproductive physiology. Contrary to predictions, we also find no evidence of a relationship between sperm length and sperm storage tubule morphology across Galliformes. Overall, our findings suggest that the vagina has a significant influence on the processes of post-copulatory sexual selection, but the factors driving variation in sperm storage tubule morphology and capacity remain elusive and require further study.

Keywords: female fertility, sexual selection, sperm competition, sperm morphology, sperm storage tubule

# 1 Introduction

Post-copulatory sexual selection can drive the rapid evolution of reproductive traits including morphological, physiological and behavioural adaptations (Birkhead and Pizzari, 2002). When females mate with multiple males, sexual selection can continue to operate after insemination through both sperm competition - where ejaculates from different males compete for fertilisation of the ova (Parker, 1970); and cryptic female choice - where females bias paternity towards sperm from preferred males (Eberhard, 1996). The female reproductive tract provides the arena for post-copulatory processes, but it is within the vagina specifically that sperm selection is expected to be strongest (Bakst et al., 1994; Birkhead and Brillard, 2007; Steele and Wishart, 1992). In birds, sperm selection in the vagina is so effective, that less than 1% of ejaculated sperm make it through to the next region of the oviduct (Bakst et al., 1994; Birkhead and Brillard, 2007). This rapid post-copulatory sperm loss is at least in part owing to the hostile nature of the vagina: mechanical flushes and muscular contractions can physically eject sperm (Bakst et al., 1994; Matsuzaki et al., 2015; Pizzari and Birkhead, 2000); numerous compounds within vaginal fluid act to depress sperm motility and viability (Huang et al., 2016); immunological activity within the vagina increases in the presence of sperm (Bakst, 2011; Bakst et al., 1994; Higaki et al., 1995; Yoshimura et al., 1997); and in some species, the vagina is anatomically adapted to make sperm acceptance and transport more difficult (e.g., blind-ended 'pouches' and vaginal coiling, as observed in waterfowl) (Brennan et al., 2010).

The existence of physiological adaptations to remove, incapacitate or impede sperm presents a challenging environment in which males/sperm better able to overcome these obstacles can achieve greater fertilisation success. These selective pressures should manifest in the co-evolution of male and female adaptations for control over paternity (Birkhead and Pizzari, 2002; Brennan et al., 2007). This relationship has been most well-studied in males. For example, when sperm competition intensity is high, selection favours males with an increased investment in testis mass, because larger testes (relative to their body mass) are associated with an increased rate of sperm production (Lüpold et al., 2009; Ramm and Stockley, 2010). This relationship is so well established that testis mass is commonly used as a proxy for sexual selection intensity across taxa. In some taxa (e.g., mammals and insects), there is an increasing body of evidence that post-copulatory selection can result in sexually

antagonistic co-evolution between male reproductive traits and female reproductive tract anatomy (Arnqvist and Rowe, 2002; Brennan and Prum, 2015; Orr and Brennan, 2015), but few studies have explored this in birds (but see Brennan et al., 2007).

While females that mate with multiple males are expected to develop mechanisms of sperm selection within the reproductive tract, they must also balance this against the need for sufficient availability of sperm at the time of ovulation (Assersohn et al., 2021; Hemmings et al., 2015). Unlike most mammals – where insemination must be precisely timed with the release of ova – female birds can utilise sperm from a single copulation to produce fertile eggs over prolonged periods through the storage of sperm (Das et al., 2008). If sperm successfully traverse the initial region of the avian vagina, they then encounter the utero-vaginal junction, a region typically populated by sperm storage tubules (SSTs) (Sasanami et al., 2013). SSTs are essential for ensuring sufficient sperm are available for every ovulation, but there is increasing evidence that they may also provide an additional opportunity for female control over sperm use through selective sperm storage (Firman et al., 2017). For example, in Japanese quail (*Coturnix Japnica*), the storage of sperm is unevenly distributed among SSTs, with individual tubules varying in their sperm filling rate (Ito et al., 2011; Sasanami et al., 2015); in chickens, (*Gallus domesticus*), sperm are unable to enter SSTs if they lack surface membrane proteins (Steele and Wishart, 1996); and perhaps most compellingly, zebra finch SSTs vary widely in diameter, possess constricted 'gate-like' and innervated entrances (Mendonca et al., 2019), and differentially store sperm from different inseminations in different tubules (Hemmings and Birkhead, 2017).

While we still do not fully understand the mechanisms by which SSTs are able to accept, maintain and release sperm, they are increasingly shown to be highly specialised and under fine temporal and possibly nervous control (Khillare et al., 2018). Recent evidence now also suggests that SSTs are highly variable in morphology between species, with at least 5 different SST morphological types having been identified so far including complex coiled, branched, and agglomerate structures (Assersohn et al., *in press*; Chapter 4). Sperm are the most diverse cell types in the animal kingdom (Pitnick et al., 2009), it therefore stands to reason that SSTs may experience selection for adaptive (or antagonistic) morphological co-evolution with the sperm cells they store. While there is some evidence that SST length correlates with sperm length across passerines (Birkhead and Moller, 1990), we generally know little about

the relationship between SST morphological diversity, sperm traits, and post-copulatory sexual selection. Since SSTs play an essential role in the fate of sperm post-copulation, understanding SST function is vital to our understanding of sexual selection in birds. Additionally, whilst it has been acknowledged that SSTs may provide an opportunity for cryptic female choice, the relative contribution of SSTs to the total selective potential of the vagina has rarely been explored.

Here, we conduct a comparative analysis (controlling for both phylogeny and body mass) across the Galliformes – a diverse group of heavy-bodied land fowl (including chickens, turkeys and quail), to explore the relationships between sexual selection intensity, vaginal and SST traits, and sperm morphology. First, since the vagina is known to provide a selective environment for sperm, we test the hypothesis that sexual selection intensity (measured as relative testis mass) correlates with relative vagina length. Second, we explore the hypotheses that interspecific variation in SST traits is associated with (i) sexual selection intensity and (ii) sperm morphology.

# 2 Methods

## 2.1 Oviduct and testis dissections

Oviduct samples used in this study were the same as those described in Chapter 4, and a full description of how they were sourced, dissected, prepared, and imaged is provided there (see also Figure 1 therein). As in Chapter 4, the final sample size for female oviduct tissue was 26 species (one individual per female per species).

Testis samples used in this study were from males obtained from the same source, also collected in 2016. Males were housed with females prior to dissection and copulations were not restricted. Males were all confirmed to have well developed testes with sperm present in their seminal glomera at the time of dissection. Testes and seminal glomera were removed and cleaned in PBS, and total testis wet mass was measured on digital scales. Sperm samples were obtained by squeezing the distal end of the seminal glomerus. 2µl of sperm were fixed in 50µl of 5% formalin, and later labelled with fluorescent cell labels (Hoechst 33342 and MitoTracker FM Green), before being photographed under a Leica DMLB fluorescence microscope with a darkfield filter. Sperm photos were exported to ImageJ and measured to

0.01µm. We obtained additional testis mass and sperm length data for the common pheasant (*Phasianus olchicus*) and Swinhoe's pheasant (*Lophura swinhoii*) from Liao et al., (2019), but lacked male trait data for 4 species that we had female trait data for: golden pheasant (*Chrysolophus pictus*), Reeve's pheasant (*Syrmaticus reevesii*), roul roul partridge (*Rollulus rouloul*), and white eared pheasant (*Crossoptilon crossoptilon*). Consequently, these species were removed from analyses that required testis mass or sperm length data, resulting in a final sample size of 22 males.

## 2.2 Sperm storage structure analysis

Folds of vaginal tissue were examined under a fluorescence microscope, and the entire region containing the SSTs was imaged as described in Chapter 4. In each field of view per sample, 5 SSTs were selected and measured using Image J. Tubules often overlapped meaning we could not accurately identify every individual tubule, making counts of tubules, and true random sampling unviable. Consequently, choice of tubules for measurement was made by haphazard sampling. Where possible (depending on the distribution of tubules), this was done by splitting the image into quadrants, choosing one tubule in each quadrant, and a tubule from the centre of the sample. Tubules were only chosen for measurement if the entire length from entrance to end was clearly discernible. While this may introduce a bias towards smaller tubules (because longer tubules are more likely to overlap), this was difficult to avoid in extremely dense samples. If fewer than 5 SSTs were present in the sample, then the total number of visible SSTs were measured. We considered branched tubules (i.e. tubules with one entrance but more than one 'blind end'; see Chapter 4) to be one tubule of greater total length, rather than multiple shorter tubules. For a branched tubule, total length was therefore calculated as the sum of all branch lengths.

SST capacity was then calculated for each fold using particle analysis in ImageJ. The area of each tubule was traced using a Wacom pen and tablet (Wacom Co., Ltd.), after which the image was converted into black and white 8-bit. The image threshold was then adjusted to leave only the white filled outline of tubules against a black background. Particle analysis counts the total number of pixels contained within a selection and provides a measure of area in the given scale. The area of tubule tissue ($\mu m^2$) was then calculated in this way for each image, providing a total area of tubule tissue coverage for each fold.

The location of SSTs varied between species, and while we consider every sample to contain the majority – and certainly the peak region – of SSTs, in some cases (6 samples) it appeared as if the tail end of the SST population may continue beyond the point of dissection at the vagina end (see Chapter 4 for further details). Additionally, tubule morphology varied across the length of the sample (tubule size generally decreases towards the uterus), and sperm was never observed to be stored towards the uterus end of the fold, suggesting variation in the functional significance of SST structures along the sample. To account for these factors, we restricted our analyses of SST tissue area and length to the image within each fold that contained the highest density of tubule tissue, working under the assumption that the region of peak SST density is most likely to represent functional storage structures and therefore be the most comparable region of the UVJ across species.

SST length measurements and area calculations, and SST morphological categorisations, were repeated by a second observer on one randomly chosen image (from the pool of images containing SSTs) for each fold sample. SST length measurements were repeated for the same subset of tubules measured originally, but also for an additional subset, to ensure that the method of haphazard SST choice was also repeatable. Individual repeatability in SST length and area was also calculated for the image of highest tubule density across both folds of the same sample and was found to be highly repeatable (see results). Consequently, we considered it appropriate to calculate the mean SST length and area across both folds, providing us with one consolidating mean value for SST length and area per species.

## 2.3 Tubule morphological categorisations

Tubules were categorised based on their morphological appearance according to the methods in Chapter 4. The presence or absence (1/0) of a given tubule type was determined by whether a fold of the sample contained (1) or didn't contain (0) tubules of that type in any of the images along the fold. We considered tubules to be one of 3 types: straight unbranched (simple, may bend but do not coil or branch), straight branched (generally possess between 1 and 3 long branches), or 'complex'. Complex tubules may be either branched, coiled, or agglomerate in appearance, according to the categorisation criteria in Chapter 4.

## 2.4 Statistical analysis

All analyses were run in R V 4.3.1 (R Core Team, 2023). Measurement repeatability, associated standard error, and 95% confidence intervals were calculated using the R package rptR (Stoffel et al., 2017). Sample ID was included as a random effect, with a Gaussian family and log link (SST length and area), or binary family and logit link (SST morphological categorisations), and 1000 parametric bootstraps. Measurements were considered highly repeatable at $R > 0.7$ with a *P-value* < 0.05 (Harper, 1994; Nakagawa and Schielzeth, 2010).

The shared evolutionary history of related species introduces non-independence between observations that must be controlled for in comparative analyses. We accounted for phylogenetic dependency using phylogenetic generalised least squares regression analyses (PGLS) and phylogenetic logistic regression (PLR), which incorporates the expected covariance between taxa using branch length data within a phylogeny. We used phylogenetic comparative techniques to explore the relationships between female reproductive traits (vagina length and sperm storage traits including SST area, length, and morphology), sperm length, and sexual selection intensity. Relative testis mass is widely accepted as a reliable predictor of sperm competition intensity, but since we find relative vagina length to be highly correlated with relative testis mass (see results), we used relative vagina length as a more relevant proxy for sexual selection intensity inside the female reproductive tract and removed testis mass from subsequent analyses.

We obtained a Galliformes phylogeny with time-calibrated branch lengths from Stein et al., (2015), which was trimmed and combined with our data using the R packages *treeplyr* (Harmon, 2023) and *geiger* (Pennell et al., 2014) (Figure 1). PGLS models incorporating continuous variables were performed using the R package *caper* (Orme et al., 2023), with the branch length transformation lambda (λ) estimated using maximum likelihood. Pagel's λ values range from 0-1, with 0 indicating that trait (primarily the response variable) similarity is independent of phylogeny, while 1 indicates strong phylogenetic signal.

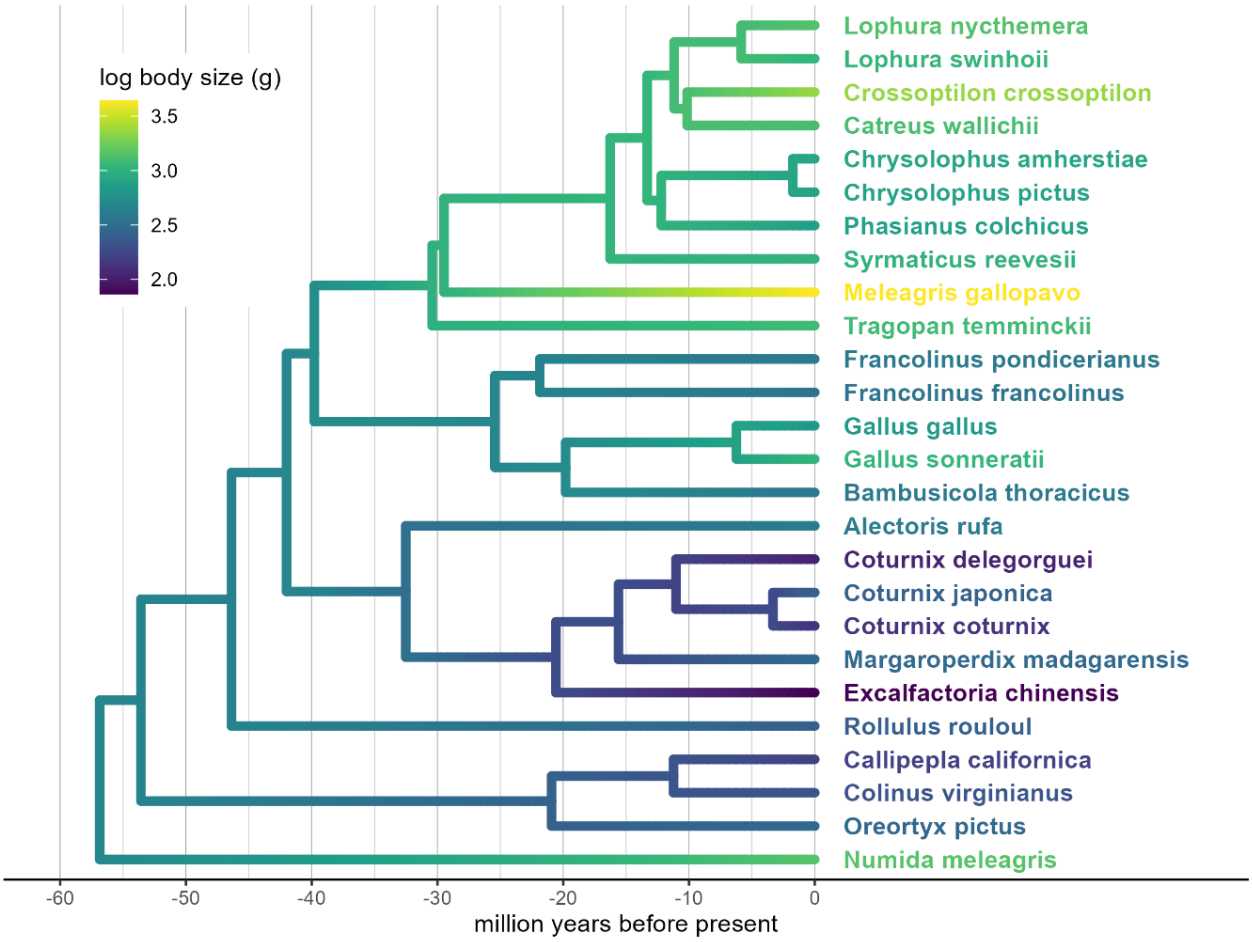


**Figure 1.** Galliformes phylogeny for the 26 species analysed in our dataset, with the estimated ancestral state for body size (log) incorporated. Ancestral state estimates were generated using the R package *phytools* (Revell, 2024) with the Brownian motion model. Note that for analyses including either sperm length or testis mass data, this dataset was reduced to 22 species (i.e., excluding *Chrysolophus pictus*, *Syrmaticus reevesii, Rollulus rouloul* and *Crossoptilon crossoptilon,* for which we lacked testis mass and sperm length data).

We evaluated models using a combination of diagnostic plot functions in *caper*, adjusted $R^2$ values and *p-values* < 0.05. For the analyses incorporating tubule morphological categorisations, we used the R package *phylolm* (Ho and Ane, 2014) to perform a phylogenetically controlled logistic regression (i.e. for non-Gaussian data) (PLR) using the function *phyloglm*. For all models, continuous variables were log transformed, and female body mass (which had a strong phylogenetic signal ($\lambda = 0.99$, $p = < 0.0001$)), was included in every model as a fixed effect to account for allometric relationships (figure 1).

# 3 Results

## General Observations

Tubules were highly variable in morphology along the length of the fold, and between species. Sperm were commonly observed stored within tubules towards the cloacal end of the fold, and generally within the region of highest tubule density, but stored sperm were unequally distributed among the population of tubules. Tubules usually decreased in size along the length of the fold towards the uterus end, by which point stored sperm were never observed, suggesting variation in SST function along the length of the fold. The position of tubules along the fold varied between species. In some cases, tubules were spread over a larger area and ended at the boundary of the uterus, whilst others did not approach the boundary of the uterus.

## Repeatability

We found that SST length measurements were highly repeatable both within the same subset and across a different subset of tubules, indicating that haphazard SST choice was a reliable indicator of average SST length within an image. Repeatability was also high for SST area calculations, and the categorisation of straight unbranched tubules, straight branched tubules, and tubule complexity (Table 1). Both average SST length and average SST area were repeatable across both folds within an individual (Table 1).

**Table 1**: Repeatability estimates (R- given on the link scale), with associated standard error (SE), confidence intervals (CI) and *P-values*.

| | R | SE | CI | P |
|---|---|---|---|---|
| Measurement repeatability for SST area | 0.75 | 0.07 | 0.59 - 0.84 | < 0.0001 |
| Measurement repeatability for SST length (same subsample) | 0.98 | 0.01 | 0.97 – 0.99 | < 0.0001 |
| Measurement repeatability for SST length (different subsample) | 0.81 | 0.05 | 0.70 - 0.89 | < 0.0001 |
| Categorisation repeatability for straight unbranched tubules | 0.80 | 0.20 | 0.37 - 0.99 | < 0.0001 |
| Categorisation repeatability for straight branched tubules | 0.93 | 0.15 | 0.94 - 1.00 | < 0.0001 |
| Categorisation repeatability for tubule complexity | 0.95 | 0.01 | 0.95 - 1.00 | < 0.0001 |
| Within individual repeatability in SST area | 0.83 | 0.07 | 0.67 - 0.92 | < 0.0001 |
| Within individual repeatability in SST length | 0.84 | 0.07 | 0.66 - 0.93 | < 0.0001 |

## Phylogenetic comparative analyses

Whilst accounting for phylogenetic and allometric relationships, vagina length was highly positively correlated with testis mass, such that in species where males had relatively larger testes for their body mass, females also had relatively long vaginas for their body mass ($\lambda = 0$; $R^2_{adj} = 0.8$, $F = 44$, $df = 2,19$, $P = 0.0036$) (Figure 2). Given this, we included vagina length as a proxy for sexual selection intensity in future models (where relevant).

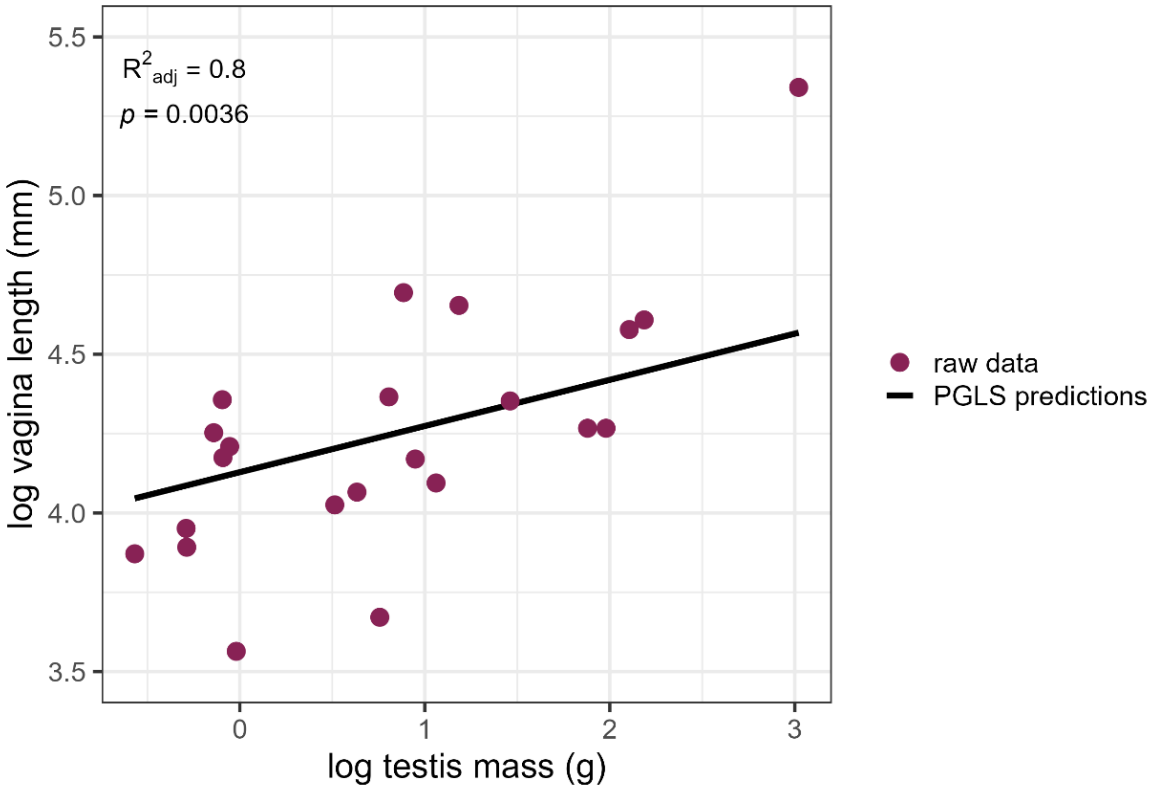


**Figure 2:** The relationship between vagina length and testis mass, across 22 species of Galliformes. Dots are the raw data points (each representing distinct species), and the solid line gives the predictions from the PGLS model, which corrects for both phylogeny and body mass. For the sake of plotting, predictions were calculated on data with body mass held constant at the mean to remove variation as a result of allometric relationships. Adjusted $R^2$ and p-values from the PGLS model are provided.

SST tissue area was positively correlated with SST length, albeit with a moderate amount of unexplained variation ($\lambda = 0$, $R^2_{adj} = 0.51$, $F = 13.2$, $df = 2,23$, $P = 0.0274$) (Figure 3). To avoid issues of multicollinearity, we therefore chose to include only SST length in our models, since it was the more repeatable measure of the two.

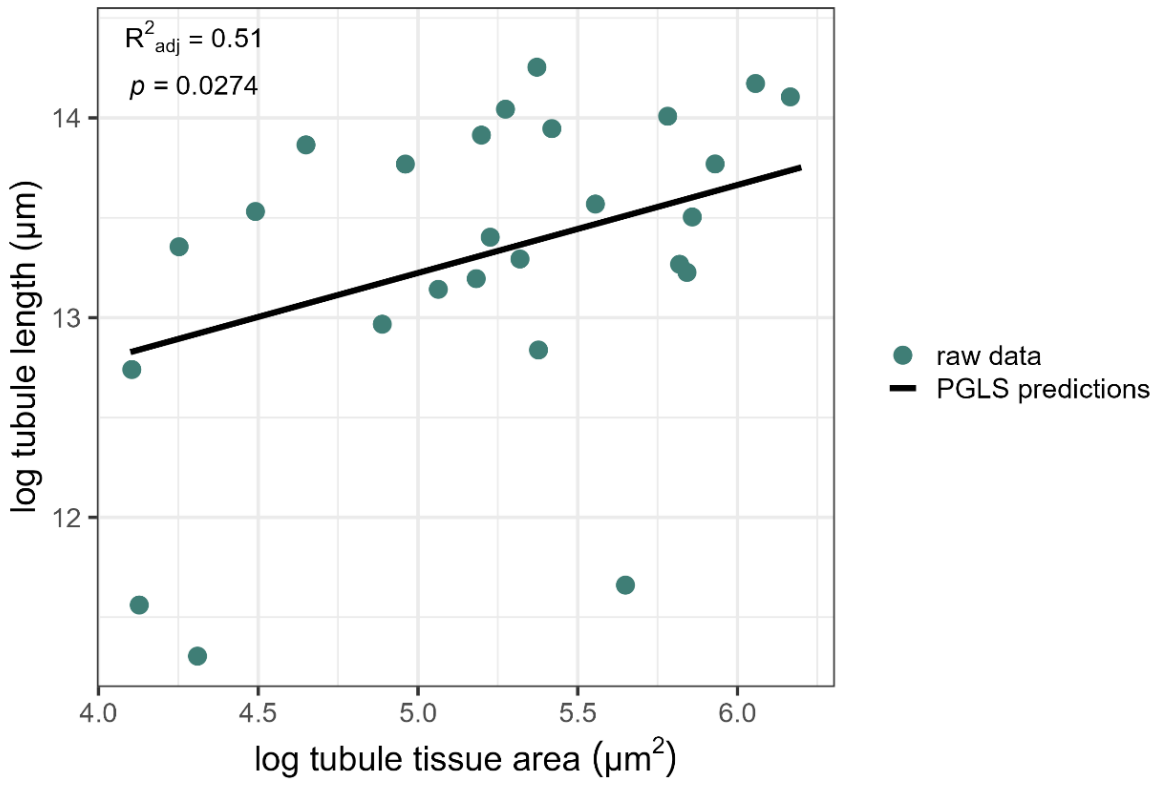


**Figure 3:** The relationship between average tubule length and species average tubule tissue (within the region of highest tubule density), across 26 species of Galliformes. Dots are the raw data points (each representing distinct species), and the solid line gives the predictions from the PGLS model. The PGLS model corrects for both phylogeny and body mass, but for the sake of plotting, predictions were calculated on data with body mass held constant at the mean to remove variation as a result of allometric relationships. Adjusted $R^2$ and p-values from the PGLS model are provided.

Relative vagina length (i.e., sexual selection intensity) was not significantly correlated with either tubule length ($\lambda = 0$, $R^2_{adj}$ =-0.07, F = 0.26, df = 2, 19, P = 0.999) (figure 4A), the degree of SST branching ($\alpha = 0.02$, Z = 0.001, P = 0.999) (Figure 4B), or tubule complexity ($\alpha = 0.06$, Z =-0.814, P = 0.416) (Figure 4C). Similarly, sperm length was not significantly correlated with either SST length ($\lambda = 0$, $R^2_{adj}$ =-0.01, F = 0.93, df = 2, 19, P = 0.266) (Figure 5A), the degree of SST branching ($\alpha = 0.02$, Z =-1.01, P = 0.313) (Figure 5B), or tubule complexity ($\alpha = 0.91$, Z =-1.24, P = 0.214) (Figure 5C).

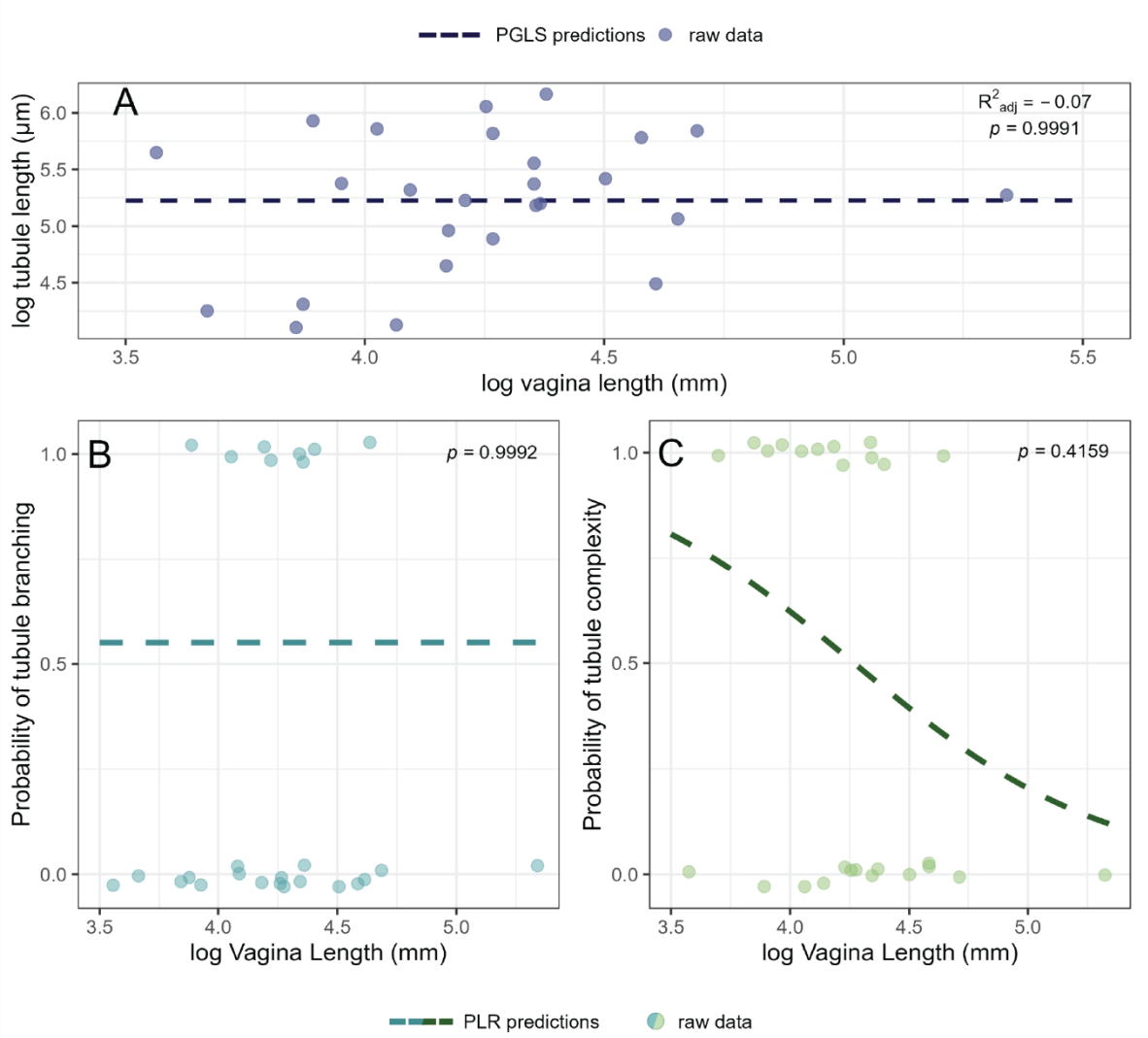


**Figure 4 A:** The relationship between vagina length and average tubule length (within the region of highest tubule density). Dots are the raw data points (each representing distinct species), and the dashed line gives the (non-significant) predictions from the PGLS model, correcting for phylogeny and body mass. For the sake of plotting, predictions were calculated on data with body mass held constant at the mean to remove variation as a result of allometric relationships. P and adjusted $R^2$ values from the model are presented; **B and C:** Variation in the probability that a species will have branched (B) or complex (C) tubules (across the entire UVJ) in relation to vagina length. Dots are the raw data points, and the dashed line gives the (non-significant) predictions (logistic function) from the PLR models, correcting for phylogeny and body mass. For the sake of plotting, predictions were calculated on data with body mass held constant at the mean. P values from the PLR models are presented. All points in B and C are jittered slightly to avoid overlaying points and aid visualisation.

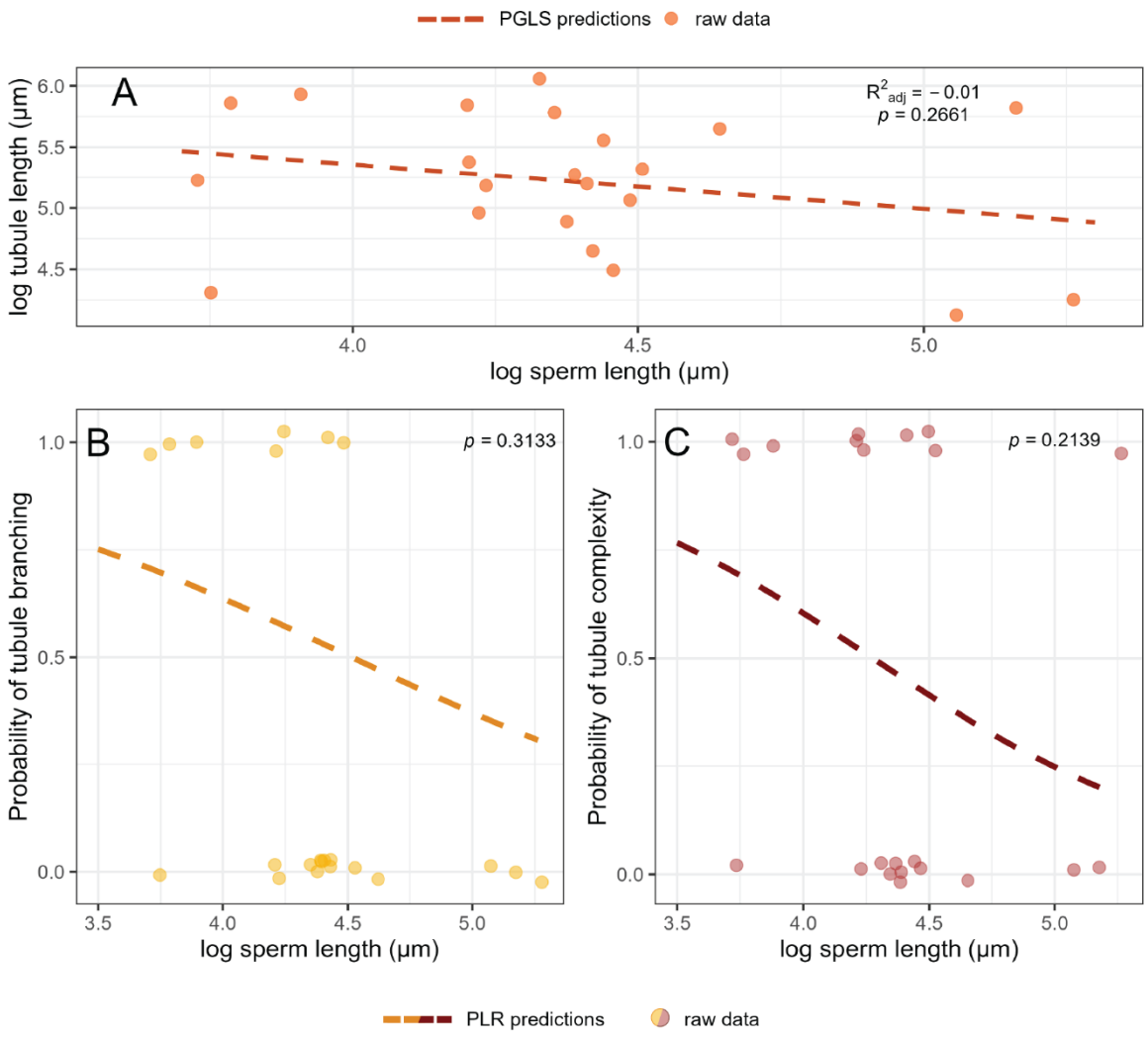


**Figure 5 A:** The relationship between sperm length and average tubule length (within the region of highest tubule density). Dots are the raw data points (each represent distinct species), and the dashed line gives the predictions from the PGLS model, correcting for phylogeny and body mass. For the sake of plotting, predictions were calculated on data with body mass held constant at the mean to remove variation as a result of allometric relationships. P and adjusted $R^2$ values from the model are presented; **B and C:** Variation in the probability that a species will have branched (B) or complex (C) tubules (across the entire UVJ) in relation to vagina length. Dots are the raw data points, and the dashed line gives the predictions (logistic function) from the PLR models, correcting for phylogeny and body mass. For the sake of plotting, predictions were calculated on data with body mass held constant at the mean. P values from the PLR models are presented. All points in B and C are jittered slightly to avoid overlaying points and aid visualisation.

# 4 Discussion

Here, we have conducted a novel exploration of the relationships between sexual selection intensity, vaginal and SST traits, and sperm morphology. We explored several distinct hypotheses: relatively long vaginas provide a more selective environment for sperm, so vagina length should be positively associated with the number of sperm males produce; and SST morphology will be associated with both sexual selection intensity, and sperm length, indicating a functional role for SSTs in post-copulatory sperm selection. Overall, we found that vagina length, but not SST morphology, was an important indicator of sexual selection intensity across species. We also found no significant relationships between SST traits and sperm traits. We discuss these findings in more detail below, suggest evolutionary explanations for our findings, and recommend areas for future research.

After accounting for variation associated with body mass and phylogenetic relationships, vagina length and testis mass were highly correlated. Relative testis mass is typically strongly associated with the amount of sperm producing tissue in the testes (Lupold et al., 2009), a metric which increases when sperm competition intensity is high. Our finding that relative vagina length increases with increasing relative testis mass implies a similar association exists between vagina length and sperm competition intensity. The vagina is typically hostile to sperm, eliciting numerous processes that act to remove or incapacitate sperm or impede their progress through the vagina. This process is so effective that on average only 1% of inseminated sperm make it into storage (Bakst et al., 1994; Birkhead and Brillard, 2007). From the male perspective, increased vagina length generates greater exposure of sperm to such selective processes. This should, in turn, result in increased selective pressure for sperm traits that counteract sperm loss, such as higher sperm concentration, speed, or resilience. From the female perspective, an increase in sperm concentration should select for an enhanced effectiveness of processes that remove excess sperm, allowing only the highest quality sperm to reach the site of storage. In both cases, the expected outcome is that relative vagina length should be positively associated with sperm competition intensity, as we show here. To our knowledge, this relationship has not been explored in birds before, but in eutherian mammals, there is evidence that males increase the number of sperm produced when females have long oviducts for their body mass (Gomendio and Roldan, 1993).

This finding potentially has several important implications: 1) relative vagina length and relative testis mass likely co-evolve; 2) sperm selection within the vagina is a powerful selective force, and since reproductive tract length is related to sperm production in some mammals as well, this suggests this relationship may be a cross-taxa female adaptation for enhanced post-copulatory control; 3) relative vagina length can be used as a proxy for sperm competition intensity (as it has been in this study); and 4) where obtaining testis tissue is difficult, or the only reason for acquiring male tissue, female reproductive tract traits may be used as an alternative.

Historically, the coevolutionary dynamics between male and female reproductive structures have largely been ignored across taxa. Most research of genital evolution has focused on the large diversity in male genitalia and sperm traits whilst overlooking the diversity in female reproductive systems (Ah-King et al., 2014). These results support the growing evidence that female genital tracts can also be variable, complex, and that they may covary with male genital traits, in both birds and other taxa (e.g., Brennan et al., 2007; Brennan and Prum, 2015; Dallai et al., 2021; Greenwood et al., 2022; Rönn et al., 2007). This further highlights the importance of considering both male and female reproductive physiology, and their co-evolutionary dynamics (which are likely to be complex) when exploring factors affecting post-copulatory sexual selection.

In addition to investigating the relationship between vagina length and sexual selection intensity, we also considered the potential drivers of variation in female sperm storage traits. We found that, after accounting for variation associated with body mass and phylogenetic relationships, neither sperm competition intensity (measured as vagina length) or sperm length was associated with SST length, the degree of SST branching, or SST morphological complexity across species. Since SST length and storage capacity (SST tissue area) were significantly positively correlated across species (which was not surprising given that longer tubules necessarily contain more tissue per unit$^2$), we assume a lack of an association with SST area as well.

The lack of a relationship between sperm competition intensity and SST morphology is surprising but may suggest that if SSTs are involved in sperm selection, they do so in a way that is unrelated to their size and capacity. For example, there is evidence that SSTs contain gate-like entrances which are capable of plastic contraction (Freedman et al., 2001;

Hemmings and Birkhead, 2017; Mendonca et al., 2019). It is unknown whether SST entrances themselves play a role in the non-random acceptance/storage of sperm, but this could present a logical explanation for past evidence suggesting SSTs may be selective (Hemmings and Birkhead, 2017; Ito et al., 2011; Sasanami et al., 2015; Steele and Wishart, 1996). If SSTs can also contract or relax along their entire length, then SST morphology could theoretically change through time. This raises the possibility that non-random sperm storage could be a responsive and plastic trait. The nature of our methods, which only view SSTs at a static point in time, restrict us from observing any dynamic SST function. However, this could provide one explanation for the large variation observed in SST morphology between species (Assersohn et al., *in press;* Chapter 4). In particular, the coiled and peculiar 'expanded and bulbous' tubules observed in Assersohn et al., (2024), lend credence to the theory that SSTs are capable of contraction and relaxation along their length, but whether this contraction is under plastic control remains to be seen. In addition to the evidence that SSTs can contract at their entrances (Mendonca et al., 2019), there is some evidence that individual tubules are innervated, with nerve fibres having been observed adjacent to SSTs in turkeys (Freedman et al., 2001). The development of novel methods for observing SST function, particularly in response to ejaculations from different males, will be important for exploring the role of SSTs in post-copulatory sexual selection in the future.

Other features of the vagina that could play a role in the non-random selection of sperm include variation in the structure of the underlying tissue. For example, Assersohn et al., (*in press*; Chapter 4) also found that the tissue of the UVJ commonly contained groove-like 'channels' that appeared to end at the region populated by SSTs, and in many cases directly ended in a tubule. These relationships may be explored further if the differential location of stored sperm (from different males) is examined in relation to SST morphology, the location of channels, and variation in the structure or function of SST entrances.

Alternatively, the lack of a relationship between SST morphology and sperm competition intensity may simply indicate that sperm storage is not a key site of sperm selection, contrary to predictions (Birkhead, 2000; Briskie and Montgomerie, 1993; Eberhard, 1996; Hellriegel and Ward, 1998). As we have shown here, sperm selection is likely to be strongly influenced by vagina length, so it is possible that by the time they reach the SSTs, sperm have already undergone rigorous selection and represent the vast majority of the 'fertilising set' – a subset

of the inseminated sperm population of the required 'quality' for fertilisation. The idea that the ejaculated subset of sperm is partitioned into a fertilising and a non-fertilising subset has been suggested in the past. For example, in rabbits, most ejaculated sperm will bind with immunoglobulins in the vagina, becoming incapacitated and phagocytosed, leaving behind a subset of sperm that then go on to fertilise the ovum (Cohen and Tyler, 1980). Experimental evidence in poultry suggests a similar function in the avian vagina: there is a strong correlation between the number of sperm stored in SSTs and those that reach the ovum (Brillard and Bakst, 1990); dead sperm inseminated beyond the vagina reach the site of fertilisation in as great numbers as living sperm, but will not reach the site of fertilisation if inseminated into the vagina (Allen and Grigg, 1957); and it is widely accepted that morphologically abnormal sperm are unlikely to enter SSTs (although it is worth noting that this assumption is based on fairly limited evidence; Allen and Grigg, 1957; Bakst et al., 1994; Ogasawara et al., 1966), suggesting they may be prevented from reaching/entering SSTs at some point in the vagina.

Understanding the mechanisms of sperm selection within the vagina may be further complicated if vagina length interacts with SST function. Theoretically, selection at the level of the vagina and selection at the level of SSTs are not mutually exclusive processes. Our findings here do not rule out the possibility that relatively longer vaginas (i.e., greater sperm competition intensity) are also associated with SSTs that provide either additional challenges or enhanced benefits for sperm. For example, even if SSTs do contain the 'fertilising set' of sperm, this does not rule out a role for differential acceptance at the point of sperm entry, or differential timing of sperm release from storage. These mechanisms could introduce variation in the order in which sperm reach the ova (therefore introducing a degree of control over paternity) but wouldn't necessarily prevent them from reaching the site of fertilisation entirely. In species with a greater degree of sperm competition (and presumably therefore with relatively longer vaginas), any potential interactions between sperm quality and SST function are likely to be intensified. Whether or not differential storage occurs as a mechanism facilitating the coordinated timing of sperm release based on sperm quality is an intriguing possibility, and one that will be challenging but nevertheless worthwhile exploring. Variation in individual tubule acceptance and release rates may also be influenced by SST morphology, possibly explaining the vast amount of variation in SST morphology previously

observed in these species (Assersohn et al., *in press;* Chapter 4), and the variation in storage among tubules observed here.

In addition to not finding a link between SST traits and vagina length, we also found no evidence for a relationship between SST morphology and sperm length, which is surprising. Previous work in passerines found sperm length was positively correlated with SST length, and negatively correlated with SST numbers across 20 species (Briskie and Montgomerie, 1992). The authors suggested that there may be a co-evolutionary relationship between sperm length and SST length, such that protection of sperm by the SST is maximised when sperm 'fit' well in the tubule. However, we do not believe this is likely in Galliformes, given that we observed SSTs to be generally many times larger than sperm, even when tubules were very small. Furthermore, our results are not entirely comparable with that previous work, since Briskie and Montgomerie (1992) considered each individual branch of an SST as a separate tubule, whereas we consider branched tubules as one long tubule of greater total length. We believe our approach is more functionally appropriate, since while a branched tubule has multiple blind ends it still only has one entrance, meaning the function (acceptance and release of sperm) between branches is non-independent. Galliformes also vary significantly in physiology and body size, possibly introducing a larger proportion of variation in reproductive structures than exists across passerines. Our contrasting findings may also indicate that birds differ across groups in the co-evolutionary dynamics of post-copulatory sexual selection. Future work exploring these relationships across other bird groups will be important for teasing these findings apart.

One potential driver of variation in SST morphology and tissue area, could be the length of time sperm are required to reside in storage. Sperm storage duration varies considerably between species. For example, sperm are stored for 5-10 days in Japanese quail, 14-21 days in chickens, but up to 15 weeks in turkeys (Birkhead and Moller, 1990; Sasanami, 2017). The causes of such variation are unknown, but it stands to reason that females that store sperm for a longer period might require: i) tubules with more protective qualities (such as increased length or shape complexity) to prevent sperm loss, or ii) greater storage capacity because passive sperm loss might require a greater number of sperm to begin with. However, there is some evidence that shorter sperm may have enhanced survival in storage, and that sperm loss through extended storage may be counteracted by an increase in sperm production by

the male (Immler et al., 2007; Liao et al., 2019). This is demonstrated by the fact increased sperm storage duration in pheasants is associated with an increase in sperm production (Liao et al., 2019), and a decrease in sperm length (Immler et al., 2007; Liao et al., 2019), but with no trade-off between sperm length and number, and no association between sperm morphometry and sperm competition risk. If shorter sperm are also associated with greater sperm storage duration across all Galliformes, then the fact we found that sperm length was not associated with SST traits indirectly suggests that SST morphology may also not be associated with sperm storage duration. However, these relationships are complex, and given the diversity in physiology, sperm storage duration, SST morphology and sperm competition intensity observed across Galliformes species, it would be worth directly exploring the relationships between sperm storage duration and SST morphology across a wider number of species within this group. Particularly given that SST complexity – a trait not previously incorporated into studies investigating the mechanisms of post-copulatory sexual selection – is highly variable between species and seems likely to have implications for sperm storage.

One limitation of our approach is that we lack information on intraspecific variation. For some species (but not all, and not enough for inclusion in analysis), we did have multiple individuals that we were able to sample. Anecdotally, while there did appear to be some variation in tubule length and density between samples from different individuals, these differences were small. While we are unable to quantify intraspecific variation here, it is worth noting that our current knowledge of intra-specific variation in SST density and morphology is limited across all species, as it is with inter-specific variation. However, intra-specific variation in sperm storage traits may play an important role in post-copulatory selection, and this warrants greater future research effort. It is also worth acknowledging that our limited sample sizes, particularly for analyses including testis mass and sperm length data (22 species, relative to 26 for the other analyses) can mean individual data points hold more influence over our results and increase uncertainty. Large datasets in comparative analyses are difficult to obtain, but it may be worth future work exploring these relationships across a greater number of species to confirm our findings here and increase our understanding of these complex relationships.

Overall, our results suggest that firstly, the vagina has a much stronger influence on the co-evolutionary dynamics of post-copulatory sexual selection than has previously been assumed,

and that sperm competition intensity appears to be primarily associated with vagina length, rather than SST length or morphological complexity in Galliformes. Furthermore, we found no evidence for a co-evolutionary relationship between sperm morphology and SST morphology in Galliformes. Further comparative analyses, using consistent and comparable methodology, (particularly for branch length calculations), in addition to the development of novel methodology for examining SST function in vivo, will be important for teasing apart the differences across species and groups, and the role of SSTs in post-copulatory sexual selection. The mechanisms of sperm selection within the female reproductive tract are clearly complex, and likely driven by a host of possibly interactive processes that are difficult to explore and examine owing to their cryptic nature within the female reproductive tract. We nevertheless urge that future work considers female processes and physiology when exploring the mechanisms of post-copulatory sexual selection. We have suggested several explanations and alternative theories for our findings, including the possibility that SST morphological complexity is associated with other mechanisms of post-copulatory sexual selection including sperm storage duration, and functional variation in sperm acceptance and release from storage. These represent exciting avenues for further research.

# 5 References

Ah-King, M., Barron, A.B., Herberstein, M.E., 2014. Genital Evolution: Why Are Females Still Understudied? PLoS Biol. 12, e1001851. https://doi.org/10.1371/journal.pbio.1001851

Allen, T., Grigg, G., 1957. Sperm transport in the fowl. Aust. J. Agric. Res. 8, 788–789. https://doi.org/10.1071/AR9570788

Arnqvist, G., Rowe, L., 2002. Antagonistic coevolution between the sexes in a group of insects. Nature 415, 787–789. https://doi.org/10.1038/415787a

Assersohn, K., Brekke, P., Hemmings, N., 2021. Physiological factors influencing female fertility in birds. R. Soc. Open Sci. 8, 202274. https://doi.org/10.1098/rsos.202274

Assersohn, K., Richards, J.P., Hemmings, N., 2024. The surprising complexity and diversity of sperm storage structures across birds (in review).

Bakst, M.R., 2011. Role of the oviduct in maintaining sustained fertility in hens. J. Anim. Sci. 89, 1323–1329. https://doi.org/10.2527/jas.2010-3663

Bakst, M.R., Wishart, G., Brillard, J.-P., 1994. Oviducal sperm selection, transport, and storage in poultry. Poult. Sci. Rev. 5, 117–143.

Birkhead, T.R., Brillard, J.P., 2007. Reproductive isolation in birds: postcopulatory prezygotic barriers. Trends Ecol. Evol. 22, 266–272. https://doi.org/10.1016/j.tree.2007.02.004

Birkhead, T.R., Moller, A.P., 1990. Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study 45, 363–372. https://doi.org/doi.org/10.1111/j.1095-8312.1992.tb00649.x

Birkhead, T.R., 2000. Defining and Demonstrating Postcopulatory Female Choice-Again. Evolution 54, 1057–1060.

Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. Nat. Rev. Genet. 3, 262–273.

Brennan, P.L.R., Clark, C.J., Prum, R.O., 2010. Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia. Proc. R. Soc. B Biol. Sci. 277, 1309–1314. https://doi.org/10.1098/rspb.2009.2139

Brennan, P.L.R., Prum, R.O., 2015. Mechanisms and Evidence of Genital Coevolution: The Roles of Natural Selection, Mate Choice, and Sexual Conflict. Cold Spring Harb. Perspect. Biol. 7, a017749. https://doi.org/10.1101/cshperspect.a017749

Brennan, P.L.R., Prum, R.O., McCracken, K.G., Sorenson, M.D., Wilson, R.E., Birkhead, T.R., 2007. Coevolution of Male and Female Genital Morphology in Waterfowl. PLoS ONE 2, e418. https://doi.org/10.1371/journal.pone.0000418

Briskie, J.V., Montgomerie, R., 1993. Patterns of Sperm Storage in Relation to Sperm Competition in Passerine Birds. The Condor 95, 442–454. https://doi.org/10.2307/1369366

Brillard, J.P., Bakst, M.R., 1990. Quantification of spermatozoa in the sperm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod. 43, 271–275. https://doi.org/10.1095/biolreprod43.2.271

Briskie, J., Montgomerie, R., 1992. Sperm size and sperm competition in birds. Proc. R. Soc. B-Biol. Sci. https://doi.org/10.1098/rspb.1992.0013

Cohen, J., Tyler, K.R., 1980. Sperm populations in the female genital tract of the rabbit. Reproduction 60, 213–218. https://doi.org/10.1530/jrf.0.0600213

Dallai, R., Fanciulli, P.P., Lupetti, P., Mercati, D., 2021. The ultrastructure of sperm and female sperm storage organs in the water strider Gerris lacustris L. (Heteroptera) and a

possible example of genital coevolution. ARTHROPOD Struct. Dev. https://doi.org/10.1016/j.asd.2021.101043

Das, S.C., Isobe, N., Yoshimura, Y., 2008. Mechanism of prolonged sperm storage and sperm survivability in hen oviduct: A review. Am. J. Reprod. Immunol. 60, 477–481. https://doi.org/10.1111/j.1600-0897.2008.00651.x

Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating Female Control: 20 Years of Cryptic Female Choice. Trends Ecol. Evol. 32, 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Freedman, S., Akuffo, V., Bakst, M., 2001. Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo*). REPRODUCTION. https://doi.org/10.1530/rep.0.1210809

Gomendio, M., Roldan, E.R.S., 1993. Coevolution between male ejaculates and female reproductive biology in eutherian mammals. Proc. R. Soc. Lond. B Biol. Sci. 252, 7–12. https://doi.org/10.1098/rspb.1993.0039

Greenwood, J.F., Lara Granados, G., Secor, S.M., Todd, B.D., Showalter, I., Hedrick, B.P., Brennan, P.L.R., 2022. Divergent Genital Morphologies and Female–Male Covariation in Watersnakes. Integr. Comp. Biol. 62, 569–580. https://doi.org/10.1093/icb/icac020

Harmon, L., 2023. treeplyr: "dplyr" Functionality for Matched Tree and Data Objects.

Harper, D.G.C., 1994. Some comments on the repeatability of measurements. Ringing Migr. 15, 84–90. https://doi.org/10.1080/03078698.1994.9674078

Hellriegel, B., Ward, P.I., 1998. Complex Female Reproductive Tract Morphology: Its Possible Use in Postcopulatory Female Choice. J. Theor. Biol. 190, 179–186. https://doi.org/10.1006/jtbi.1997.0546

Hemmings, N., Birkhead, T., 2017. Differential sperm storage by female zebra finches Taeniopygia guttata. Proc. Biol. Sci. 284.

Hemmings, N., Birkhead, T.R., 2015. Polyspermy in birds: sperm numbers and embryo survival. Proc. R. Soc. B Biol. Sci. 282, 20151682. https://doi.org/10.1098/rspb.2015.1682

Hemmings, N., Birkhead, T.R., Brillard, J.P., Froment, P., Briere, S., 2015. Timing associated with oviductal sperm storage and release after artificial insemination in domestic

hens. Theriogenology 83, 1174–1178. https://doi.org/10.1016/j.theriogenology.2014.12.022

Higaki, K., Yoshimura, Y., Tamura, T.O., 1995. Localization of spermatozoa and leukocytes in vagina and utero vaginal junction after copulation in Japanese quail (Coturnix coturnix japonica). Jpn. Poult. Sci. 32, 387–393. https://doi.org/10.2141/jpsa.32.387

Ho, L.S.T., Ane, C., 2014. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. Syst. Biol. 63, 397–408.

Huang, A., Isobe, N., Obitsu, T., Yoshimura, Y., 2016. Expression of lipases and lipid receptors in sperm storage tubules and possible role of fatty acids in sperm survival in the hen oviduct. Theriogenology 85, 1334–1342. https://doi.org/10.1016/j.theriogenology.2015.12.020

Immler, S., Saint-Jalme, M., Lesobre, L., Sorci, G., Roman, Y., Birkhead, T.R., 2007. The evolution of sperm morphometry in pheasants. J. Evol. Biol. 20, 1008–1014. https://doi.org/10.1111/j.1420-9101.2007.01302.x

Ito, T., Yoshizaki, N., Tokumoto, T., Ono, H., Yoshimura, T., Tsukada, A., Kansaku, N., Sasanami, T., 2011. Progesterone is as sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology 152, 3952–3962. https://doi.org/10.1210/en.2011-0237

Khillare, G.S., Sastry, K.V.H., Agrawal, R., Prakash, A., Mohan, J., Singh, R.P., 2018. Molecular and biochemical events in sperm storage tubules of oviduct in birds. Worlds Poult. Sci. J. 74, 35–44. https://doi.org/10.1017/S004393391700085X

Liao, W.B., Zhong, M.J., Lüpold, S., 2019. Sperm quality and quantity evolve through different selective processes in the Phasianidae. Sci. Rep. 9, 19278. https://doi.org/10.1038/s41598-019-55822-3

Lupold, S., Calhim, S., Immler, S., Birkhead, T.R., 2009. Sperm morphology and sperm velocity in passerine birds. Proc. R. Soc. B-Biol. Sci. 276, 1175–1181. https://doi.org/10.1098/rspb.2008.1645

Lüpold, S., Linz, G.M., Rivers, J.W., Westneat, D.F., Birkhead, T.R., 2009. Sperm Competition Selects Beyond Relative Testes Size in Birds. Evolution 63, 391–402. https://doi.org/10.1111/j.1558-5646.2008.00571.x

Matsuzaki, M., Mizushima, S., Hiyama, G., Hirohashi, N., Shiba, K., 2015. Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. Sci. Rep. 5, 1–12. https://doi.org/10.1038/srep17643

Mendonca, T., Cadby, A.J., Hemmings, N., 2019. Sperm gatekeeping: 3D imaging reveals a constricted entrance to zebra finch sperm storage tubules. Biophys. J. 117, 2180–2187. https://doi.org/10.1016/j.bpj.2019.10.038

Nakagawa, S., Schielzeth, H., 2010. Repeatability for Gaussian and non-Gaussian data: A practical guide for biologists. Biol. Rev. 85, 935–956. https://doi.org/10.1111/j.1469-185X.2010.00141.x

Ogasawara, F.X., Lorenz, F.W., Bobr, L.W., 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds III. Intra-uterine insemination of semen from low-fecundity cocks. J. Reprod. Fertil. 11, 33–41.

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., Pearse, W., 2023. caper: Comparative Analyses of Phylogenetics and Evolution in R.

Orr, T.J., Brennan, P.L.R., 2015. Sperm storage: distinguishing selective processes and evaluating criteria. Trends Ecol. Evol. 30, 261–272. https://doi.org/10.1016/j.tree.2015.03.006

Parker, G., 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. Camb. Philos. Soc. 45, 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Pennell, M.W., Eastman, J.M., Slater, G.J., Brown, J.W., Uyeda, J.C., Fitzjohn, R.G., Alfaro, L.J., Harmon, L.J., 2014. geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. Bioinformatics 30, 2216–2218.

Pitnick, S., Hosken, D.J., Birkhead, T.R., 2009. Sperm Biology: an evolutionary perspective. Academic Press, pp. 69–149.

Pizzari, T., Birkhead, T.R., 2000. Female feral fowl eject sperm of subdominant males. Nature 405, 787–789. https://doi.org/10.1038/35015558

R Core Team, 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ramm, S.A., Stockley, P., 2010. Sperm competition and sperm length influence the rate of mammalian spermatogenesis. Biol. Lett. 6, 219–221. https://doi.org/10.1098/rsbl.2009.0635

Revell, L.. J., 2024. phytools 2.0: an updated R ecosystem for phylogenetic comparative methods (and other things). PeerJ 12, e16505. https://doi.org/10.7717/peerj.16505

Rönn, J., Katvala, M., Arnqvist, G., 2007. Coevolution between harmful male genitalia and female resistance in seed beetles. Pnas 104, 10921–10925. https://doi.org/10.1073/pnas.0701170104

Sasanami, T., Izumi, S., Sakurai, N., Hirata, T., Mizushima, S., Matsuzaki, M., Hiyama, G., Yorinaga, E., Yoshimura, T., Ukena, K., Tsutsui, K., 2015. A unique mechanism of successful fertilization in a domestic bird. Sci. Rep. 5. https://doi.org/10.1038/srep07700

Sasanami, T., Matsuzaki, M., Mizushima, S., Hiyama, G., 2013. Sperm storage in the female reproductive tract in birds. J. Reprod. Dev. 59, 334–338. https://doi.org/10.1146/annurev-animal-021815-111350

Savage, J.L., Crane, J.M.S., Kākāpō Recovery Team, Hemmings, N., 2022. Low hatching success in the critically endangered kākāpō is driven by early embryo mortality not infertility. Anim. Conserv. 25, 352–360. https://doi.org/10.1111/acv.12746

Steele, M., Wishart, G., 1996. Demonstration that the removal of sialic acid from the surface of chicken spermatozoa impedes their transvaginal migration. THERIOGENOLOGY. https://doi.org/10.1016/S0093-691X(96)00268-3

Steele, M.G., Wishart, G.J., 1992. Evidence for a species-specific barrier to sperm transport within the vagina of the chicken hen. Theriogenology. https://doi.org/10.1016/0093-691X(92)90124-A

Stein, R.W., Brown, J.W., Mooers, A.Ø., 2015. A molecular genetic time scale demonstrates Cretaceous origins and multiple diversification rate shifts within the order Galliformes (Aves). Mol. Phylogenet. Evol. 92, 155–164. https://doi.org/10.1016/j.ympev.2015.06.005

Stoffel, M.A., Nakagawa, S., Schielzeth, H., 2017. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods Ecol. Evol. 8, 1639–1644. https://doi.org/10.1111/2041-210X.12797

Yoshimura, Y., Okamoto, T., Tamura, T., 1997. Localisation of MHC class II, lymphocytes and immunoglobulins in the oviduct of laying and moulting hens. Br. Poult. Sci. 38, 590–596. https://doi.org/10.1080/00071669708418041

# Chapter 6:

# General discussion

*Collection of watercolour paintings associated with each chapter, brought together using Adobe Photoshop (version25.5).*

# 1 Overview

All organisms are under selection to maximise individual lifetime reproductive success, and yet rates of infertility often remain remarkably variable. In this thesis, I aimed to uncover and explore some of the factors that drive variation in fertility, particularly in females, using birds as a model system. In Chapter 2, I performed a systematic review of the field of avian fertility, finding that female fertility traits are comparatively understudied relative to males. This finding influenced my decision to focus primarily on female fertility traits throughout my PhD. I also identified barriers to increasing knowledge in the field and suggested ways in which these could be overcome in future research. Since I found broadly that both the genetic factors and female sperm selection are particularly understudied, Chapters 3 – 5 consequently focused on exploring these gaps in greater detail.

Since each chapter presented in this thesis already includes a detailed stand-alone discussion of the content therein, I will refrain from repeating that content here. Instead, this final chapter will summarise and discuss the broader primary insights of my PhD work, collating key knowledge from the collective body of work presented here and then using this to propose important areas of focus for future research in the field of avian fertility. Finally, I will set the thesis in context with concluding remarks on the wider impacts of this work.

# 2 New Insights

## 2.1 Wild/non-commercial species are understudied

A key finding from Chapter 2 was that nearly 80% of all research (between 1985 and 2019) within the field of avian fertility has focused on captive populations (relative to wild), the vast majority of which has exclusively focused on the domestic chicken alone. This finding is not necessarily surprising; the poultry science literature is expansive, providing important insights into the reproductive productivity of commercial birds. Poultry provides one of the world's most important sources of protein, particularly in developing countries, and understanding the factors effecting reproduction in poultry has clear benefits for global food security (Vaarst et al., 2015). However, from the perspective of avian reproductive science, the bias towards commercial birds has several implications. While captive birds may be more likely to suffer

from compromised fertility due to poor husbandry, stress, and dietary issues (relative to wild birds), domestic poultry have been typically exposed to strong and prolonged artificial selection for high fertility and are generally kept in stable environmental conditions. Commercial species are also relatively abnormal in terms of reproductive physiology: domestic hens can produce eggs almost continuously, laying between 250 and 300 eggs a year, around 10x what might be expected of most wild seasonally breeding birds. Consequently, we know little of natural rates of infertility across bird species, and the genetic and environmental factors that contribute towards it.

Despite the expectation that fertility rates in commercial birds should not represent a natural degree of reproductive trait variation (owing to strong artificial selection for high fertility), domestic chickens and turkeys still experience rates of hatching failure not dissimilar to the global average (around 15% in chickens and turkeys (Beaumont et al., 1997), and around 17% across all birds (Marshall et al., 2023). This suggests that a considerable degree of early reproductive failure is a pervasive and probably ubiquitous feature of birds. The factors contributing to this background level of hatching failure are poorly understood, but increasing our understanding of fertility across a greater diversity of species will be an important step to improving our knowledge of base-line reproductive issues in birds.

Chapter 4 provides an important perspective on this issue: our previous knowledge of female storage organs in birds comes predominantly from a small number of model species (i.e., primarily chicken, turkey, Japanese quail and zebra finch). Most of the research on female sperm storage structures in birds has typically assumed all species have SSTs that look and function as they do in these model species. When looking across a greater diversity of species, I found that birds exhibit extraordinary interspecific variation in the morphology and complexity of their sperm storage structures. Clearly, basing our assumptions about avian fertility on knowledge from model species alone has masked important trait variation, and this is likely to also be true of other reproductive traits. In this case, it has also masked potentially important insights into the processes of post-copulatory sexual selection.

## 2.2 Fertilisation failure of eggs is often poorly defined

In the process of reviewing the literature for Chapter 2, I found it commonplace for studies to refer to all forms of reproductive failure as infertility. Consequently, it is difficult to interpret

information from the existing literature on the mechanisms of reproductive failure. True infertility (which I define in this thesis as the failure to achieve successful syngamy – i.e., fertilisation failure) is mechanistically distinct from embryo mortality (where the embryo fails to develop post-syngamy). Failure of studies to acknowledge this distinction makes it difficult to calculate accurate estimates of infertility and embryo death, and therefore to determine the underlying causes of each form of reproductive failure. In a separate review paper, published during my PhD, we report that the highest incidences of hatching failure occur during early and late stages of embryo development (Appendix 1; Assersohn et al., 2021). Early stages of embryo death (occurring within 3 days of development) are not visible by eye, and so fertility estimates in studies using traditional candling methods are typically overestimated. Indeed, many eggs initially deemed to be infertile upon macroscopic examination are re-defined as fertile (but where the embryo died very early) when examined using microscopic techniques (Hemmings and Evans, 2020).

Generally, the consensus across the existing literature is that infertility is more commonplace in captive populations, with infertility in wild populations having been traditionally overestimated. For example, in the endangered whooping crane (*Grus americana*), fertility rates in captive populations are extremely low (0.37) (thought to be linked to female age and inbreeding) but reported to be significantly higher in wild populations (0.73) (Brown et al., 2019). Higher rates of fertility in wild relative to captive populations have also been observed in other bird species (Assersohn et al., 2021b). However, the lack of a clear and precise definition of infertility may mean this conclusion is somewhat premature.

## 2.3. Egg production rates are an underappreciated variable in female fertility

In avian reproductive science, infertility is viewed as the failure to produce fertilised eggs, and measures of infertility in both domestic and wild populations are almost always provided as such. This view of infertility makes one key assumption: that a female can produce a fertilisable ovum in the first place. Given this, standard measures of infertility only include instances where an already ovulated ovum is not fertilised by sperm. The only ways in which female processes could drive this type of infertility are if a) the ovum has an abnormality or genetic issue preventing penetration by sperm or syngamy, or b) the female has a

physiological issue preventing inseminated sperm from traversing the reproductive tract successfully. Fertilisation failure of an ovulated ovum could also occur due to a problem with the sperm provided by the male(s), but in wild populations extra-pair copulations are common, making fertilisation failure resulting from a lack of sperm an unlikely scenario for most females. Indeed, measures of infertility in wild populations (that do distinguish between infertility and early embryo mortality) are increasingly finding that infertile laid eggs are rare (Hemmings et al., 2012; Hemmings and Evans, 2020; Schut et al., 2014), probably because the above-mentioned issues that can prevent a successfully ovulated ovum from being fertilised are relatively uncommon in wild populations. Illness or disease resulting in a failure to produce eggs or ovulate correctly may also be correlated with a risk of mortality, which increases the likelihood these types of infertility will remain un-recorded. Relative to wild populations, infertile egg production is probably more common (and more commonly recorded) in captive populations, which does seem to be evidenced by the increased recorded rates of fertilisation failure in captivity (Hemmings et al., 2012). Captive populations are less likely to offer sufficient suitable options for extra pair copulations and may be associated with higher degrees of inbreeding and husbandry related issues. They are also more likely to already be managed in captivity because of an existing fertility issue (Assersohn et al., 2021a, 2021b (Chapter 2 and Appendix 1)).

From the female perspective, the production of a fertilisable ovum requires a significant energetic investment. Issues occurring during egg formation or ovulation may be an important form of infertility but are rarely included in fertility estimates, and so their prevalence remains largely unknown. In males, the equivalent process of sperm production is comparatively well studied, and issues occurring during spermatogenesis or ejaculation are expected to be an important form of male infertility. The lack of broad-scale acknowledgement and understanding of egg production or ovulatory issues in female birds is a major barrier to identifying the incidence and causes of female infertility across species and populations. Ovulation/egg production related infertility can therefore be considered an invisible fraction in infertility estimates, potentially driving a wide-spread under/mis-estimation of infertility rates across birds. It may therefore be more accurate to amend my earlier statement ‘infertility is more commonplace in captive populations’, to: ‘the laying of

infertile eggs is more commonplace in captive populations', as this better represents what is (usually) being measured in fertility estimates.

## 2.4 There is a disparity in research between males and females

In Chapter 2, I show that around twice as many papers have focused on male relative to female fertility traits in birds, and this gap appears to be increasing over time. Since the publication of this finding (Assersohn et al., 2021), more recent work has also uncovered a similar pattern of male-bias in research effort across the entire field of sexual selection (Ah-King, 2022). Even though females are known to exert significant control over the outcome of fertilisation, we know far less about female-mediated processes of post-copulatory sexual selection compared to those in males. The causes of this imbalance in research effort are difficult to discern, but one possible factor is the relative ease of study of male compared to female reproductive traits. In addition, the relative conspicuousness of male sexually selected traits compared to those of females, and the historic (and often inaccurate) assumption that males are the more active participants in reproduction, both likely contribute to the male focus seen across the field of sexual selection. My focus on female reproductive traits throughout this thesis can be considered in part an attempt to address this disparity in knowledge between males and females in avian reproductive science. It is important to recognise, however, that female traits do not exist in isolation, but are instead part of a dynamic interaction between both male and female factors. Therefore, I acknowledged and accounted for the interaction between male and female traits where relevant.

## 2.5 Strong effects in one sex are not always associated with a correlated response in the other

Many studies that have examined female reproductive traits have done so by incorporating both male and female traits and targeting their correlated evolutionary responses. This is because adaptations in one sex are expected to trigger an evolutionary response in the other (provided there is an inter-sexual genetic correlation), and since male reproductive traits are often well studied, they provide a tractable basis for gaining insight into female traits. Males and females share the majority of their genome, and most traits are encoded by the same loci in both sexes (Bonduriansky and Chenoweth, 2009). Male and female reproductive traits may

be positively correlated when fitness optima between the sexes are aligned (e.g. genital tract morphologies that are often adapted for a degree of mechanical confluence) (Greenwood et al., 2022). Males and females often have divergent evolutionary interests, and this can result in a genetic 'tug-of-war', where alleles experience opposing selection in males and females that can present as negative intersexual correlations for fitness traits (Brommer et al., 2007; Chapman et al., 2003). Conflict can occur between different loci (i.e., inter-locus sexual antagonism), or within the same locus (i.e., intra-locus sexual antagonism) (Ebel and Phillips, 2016). This type of conflict can maintain genetic variation and drive the coevolution in reproductive traits and behaviours between males and females (Arnqvist and Rowe, 2002). For example, in Chapter 5, I found that relative vagina length was strongly positively correlated with relative testis mass in Galliformes. This type of correlated response may have evolved through a co-evolutionary arms race over post-copulatory control, whereby the challenging environment presented within the vagina may have triggered selection for an increase in sperm production (associated with relatively larger testes), which in turn is likely to generate selection for females to counteract this with an increase in vagina length.

Conversely, in Chapter 3, I adopted the targeted approach outlined above to explore the effects of a Z-linked supergene, with known important effects in males, on females (for which its effects were unknown). Variants of the supergene have strong and quite extreme effects on male sperm traits: up to 90% of the variation in sperm morphology and swimming speed was explained by the Z chromosome supergene (Kim et al., 2017). We explored whether the effects of the inversion have a co-evolutionary effect for females. However we found no discernible correlated responses across several important reproductive traits including egg production in females, or for hatching success of eggs. We suggested several possible explanations for this finding, including resolved prior antagonism, a lack of genes responsible for egg production within the inversion, and/or that egg production is a relatively conserved trait exhibiting little genetic variation. Sex-dependent regulation in gene expression is increasingly found to be common and may be able to recover independent fitness optima between the sexes. For example, up to 57% of *Drosophila melanogaster* genes show sex-biased expression, most of which are involved in reproduction (Ellegren and Parsch, 2007).

Strong selection in one sex need not always correspond to strong selection in the other. Furthermore, it cannot always be assumed that male traits will have a correlated female

equivalent, or that shared traits share the same function. The evolution of ornamental traits in both males and females presents an overt example. Male ornamental traits are almost always attributed to male-male competition under sexual selection. Contrastingly, while ornaments in females may have evolved as a correlated response, either due to an artefact of the shared genetic architecture between the sexes, or because females are under equivalent selective pressure to males (i.e. female-female competition for access to males) (Dale et al., 2015; Tobias et al., 2012), female ornamentation may also evolve under female-specific selection pressures (Ah-King, 2022; Tobias et al., 2012). For example, reindeer antlers have evolved in males as a sexually selected trait for competition over females, but are used by females in competition for access to resources (Ah-King, 2022). Similarly, the extravagant tail display of the peacock is considered the archetype of a sexually selected trait, and while peahens also fan their tails, this phenomenon – which is not well studied, is anecdotally thought to function instead as a defensive display (O'Donnel, 2024). Exploring female traits through the lens of what is known in males, may be a valid jumping off point for traits expected to have been selected under correlative evolution, but it is also important to consider female-specific functions that may not have a male-equivalent process, or may have been selected for alternative functions.

In Chapter 5, we show that contrary to expectations, sperm morphology is not correlated with the morphology of female sperm storage organs. SST morphology could be related to other sperm traits we could not account for, but it may also be worth expanding our exploration of SST function to include additional female-specific roles not related to their ability to maintain sperm. For example, SSTs are known to produce numerous compounds important for sperm survival, but whether they are also involved in the production of other reproductive fluid including compounds related to immunological activity, and/or are involved in functions related to the passage of the egg through the vagina, does not appear to have been explored.

## 2.6. Female post-copulatory reproductive traits may be an underappreciated agent of sexual selection

If polyandrous females can bias paternity towards preferred males, then sexual selection may continue post-copulation in a process known as cryptic female choice (CFC) (Eberhard, 1996;

Thornhill, 1983). Demonstrating CFC is challenging owing to the complex but subtle nature of female post-copulatory processes (Birkhead, 2000, 1998; Eberhard, 2000; Kempenaers et al., 2000; Pitnick and Brown, 2000; Ward, 2000). One predicted mechanism of CFC is the ability to differentially store sperm within sperm storage tubules (SSTs). There is some limited evidence for preferential sperm storage: in *Drosophila melanogaster*, female seminal receptacles favour males with long sperm (Miller and Pitnick, 2002); SSTs are thought to probably limit the acceptance of damaged sperm (Allen and Grigg, 1957; Bakst et al., 1994; Ogasawara et al., 1966); and zebra finches store sperm from different inseminations in different tubules (Hemmings and Birkhead, 2017). The mechanisms of differential sperm storage are unknown, although our lack of knowledge of the base-line functions of SSTs (i.e. how precisely SSTs accept, maintain and release sperm), as well as their basic morphology across species, is an even more fundamental barrier to our understanding of postcopulatory selection in birds.

Sperm storage tubules (SSTs) have typically been described in the literature as 'simple' structures, taking the form of blind-ended tubular invaginations (Hiyama et al., 2014; Sasanami et al., 2013). The extent of variation usually ascribed to tubule structure is akin to 'branched or not'. Despite only observing tubules in a small number of species, with most studies only focusing on a handful of model birds (i.e. domestic chickens, turkeys, Japanese quail and zebra finches), it is usually expected that all birds have SSTs and that they will look and function in the same way according to observations in model species. I believe the definition of tubules as 'simple tubular invaginations' is misleading. These structures, at the very least, perform very complex functions – for example, the ability to store and maintain foreign material for many days or even weeks within the reproductive tract (a typically very hostile and immunologically active region of the body), whilst retaining their viability, only to release them at precisely the right time, is remarkable. In Chapter 4, I show that the assumption of a simple morphological structure is also not true for all species. Indeed, I found that sperm storage tubules are far more morphologically complex than has previously been assumed, with both tubule structure, and the surrounding tissue, varying remarkably across Galliformes. I suggested several functional explanations for this variation and developed a framework for future researchers to explore this variation across other species. This included

producing a flowchart for categorising SST morphology, which I fully expect will need to be expanded upon as further variation is discovered across other species.

In Chapter 5, I take this further by quantitatively exploring the functional significance of this variation. Surprisingly, I found that SST morphology (at least in terms of length, branching and complexity) was not associated with sperm competition intensity or sperm traits. While this finding does not rule out a role of SSTs in post-copulatory sexual selection, it does suggest that other, probably more subtle variation in SST function may be more important. For example, one possibility is that SST morphology changes through time in response to male quality, or that selection occurs at the point of SST acceptance i.e. under the control of SST entrances.

An alternative explanation is that SSTs themselves are not selective, but rather the sperm that enter storage have already gone through rigorous selection during their journey through the vagina. In support of this, we found that vagina length was strongly positively correlated with testis mass, such that species with relatively longer vaginas also had relatively larger testes. This is in alignment with the evidence that only 1% of inseminated sperm make it through the vagina and into storage (though it doesn't discount a role for selection at the point of sperm acceptance into storage) (Bakst, 2011). I discussed several important implications this finding has for our understanding of post-copulatory sexual selection in Chapter 5. Importantly, the fact that vagina length and relative testis mass likely co-evolve suggests that the development of larger testes in the male is not simply just the result of polyandry. Females drive competition between sperm by mating with multiple males, which results in selection for an increase in sperm production. But mating comes with its own risks for females, including an increase in the likelihood of transmitted infections (Brillard, 2003; Westneat and Rambo, 2000). If sperm concentration becomes high enough to negate the benefits of polyandry for females, how can females regain post-copulatory control? Increasing the length of time sperm are exposed to selection within the vagina is one convincing possibility and paints a picture of a more complex co-evolutionary relationship between genital/reproductive tract morphology, sperm competition and female selective control. Additionally (or alternatively), since the anti-sperm environment of the female reproductive tract functions in part as a defensive mechanism against infection, an increase in vagina length in combination with an increase in mate number may also act as a mechanism of infection resistance.

It has been assumed that female genital tract morphology is relatively non-variable and is consequently not well studied across taxa (Ah-King et al., 2014; Brennan and Prum, 2015). Comparatively, there is an extensive literature base exploring male genital form variation. The finding presented in Chapter 5 that vagina length is associated with sperm competition intensity contributes to the growing body of research revealing the variation in female reproductive tract forms and their co-evolutionary relationships with male genital forms, both within and between species (Brennan and Prum, 2015). If the relationship between vagina length and testis mass I present here holds across all birds, then relative vagina length can be used as an appropriate proxy for sperm competition intensity, which has the benefit of reducing the requirement for male tissue in addition to female tissue in studies exploring female post-copulatory processes. This could be especially useful for studies of threatened populations where tissue samples are valuable and only acquired from natural deaths.

# 3 Future directions

## 3.1 Measuring female fertility

A key issue raised in this thesis has been the lack of accurate reporting of infertility in birds. To tackle this issue, first and foremost infertility should be defined and used correctly, and particularly any study exploring reproductive failure in birds should acknowledge the distinct difference between infertility and embryo mortality. To reiterate, infertility can be defined as the failure of successful syngamy (the fusion of male and female pronuclei with the ovum). Therefore, any reproductive attempt that fails to result in the production of an ovum that has undergone syngamy can be considered as infertility. If syngamy has occurred, any arrested development prior to hatching is embryo mortality, even if it occurs after just a few cell divisions. If a researcher cannot distinguish between infertility and embryo mortality by using the appropriate microscopic methods (see Birkhead et al., 2008)), then this should be stated clearly in the methodology, and the terms infertility and embryo mortality avoided. This will aid future attempts to monitor the causes of hatching failure and their incidence across the literature.

Another issue raised was the widespread failure across the literature to acknowledge failed egg production as a mechanism of female infertility. To begin to address this gap in our

measurement of infertility, future work must consider failed breeding attempts. I acknowledge this will be much more difficult in wild than captive populations. Hormone monitoring presents one viable method for determining the timing of ovulation events, although few studies have explored this option, probably because of concerns over stress caused by routine blood collection, and the labour-intensive nature of such monitoring. However, non-invasive techniques for monitoring sex hormone concentrations are now a possibility, with some studies finding that faecal analysis can provide a viable alternative to blood collection. For example, a recent study in cockatoos (*Cacatua alba)* found that urofecal progesterone and estradiol-17β can be monitored through enzyme immunoassays, providing a reliable indication of follicle growth and ovulation (Kusuda et al., 2023). Additionally, this study also found that feather moult varied with hormone levels, with moults halting during periods of high ovarian activity. This suggests that, particularly in species with monitored nest boxes, regular faecal and feather collections may provide a useful indication of ovarian activity. Future development of similar techniques, in combination with more regular recording of mating attempts and subsequent egg production, may provide a useful and non-invasive option for monitoring the fertility status of female birds in wild populations.

One alternative (or supplement) to this type of behavioural monitoring is to increase the routine collection of year-on-year census data for non-breeding individuals. Some types of census data may be relatively easy to collect and could provide quite broad but valuable insights into the fertility status of females within the population, including: the number of females of reproductive age that are participating in reproductive attempts (for example, seen mating at least once, partaking in nest-building behaviour or have laid at least one clutch/egg within a season); whether these females are successful in their attempts (i.e., whether a female seen mating or nest-building is also seen laying); and whether these females are repeating their attempts annually or are more sporadic breeders. These types of data could be collected through intensive breeding monitoring in small populations or scaled up to larger populations by incorporating passive integrated transponder (PIT) tag data. Long-term breeding monitoring is rare, but there are some exceptions, such as the the hihi (*Notiomystis cincta*) on Tiritiri Matangi Island (Armstrong and Ewen, 2013), kākāpō (*Strigops habroptilus*) primarily on Whenua Hou/Codfish Island and Anchor Island (Savage et al., 2022), and the Isle of May Long-term study (IMLOTS; which monitors UK seabird populations) (Newell, 2022).

These types of studies may provide a valuable opportunity for insight into the numbers of non-breeding females within a population and allow us to gain a better understanding of the factors driving this type of infertility.

It is also important to consider clutch size when determining fertility in female birds. A failure to produce any eggs is an extreme or even absolute form of infertility, but smaller than normal clutches (relative to the population or species average) can also be considered a form of infertility. Failure during follicle development for some but not all the follicular hierarchy may result in a small clutch. Clutch size is known to decrease with age, and indeed we found that the probability of producing single egged- clutches increased with age in zebra finches (Chapter 3). While we acknowledge that the age these effects became apparent in our population are unlikely to be reached in a natural population, many wild birds do experience a fertility decline with age (Lemaître et al., 2015; Morland et al., 2023; Nussey et al., 2013), and reductions in clutch size may be indicative of fertility problems in younger birds too.

I therefore suggest that future work focus on a combination of the following: i) increasing the number of long-term population studies that monitor breeding attempts, including the activity of non-breeding females within the population, which may be done through a combination of visual observation, camera monitoring and PIT-tagging; ii) for long-term population studies that already have good population census data, consider performing analyses of the dynamics of non-breeding females within the population, particularly where male fertility status can be accounted for, and combine this information with clutch size data, including (where possible) incidences where mating was known to occur, but no eggs were laid; iii) developing non-invasive techniques for recording hormone fluctuations as an indication of ovarian activity in wild populations.

## 3.2 Generating hypotheses in female-focused avian reproductive research

By re-examining common lines of thinking that are often driven by what is known in males and model species, I have been able to identify where the primary gaps in our understanding lie. I have generated some unique insights and hypotheses regarding these. Chapter 1 was instrumental in shaping my thinking: I considered every stage during the reproductive cycle that fertility can be compromised from the perspective of the female and noted several very

important oversights in doing so. For example, I found that sperm production was a common (if not the most common) metric examined when investigating male infertility but was particularly surprised to find that gamete production in females was not typically considered in most measures of female fertility. Similarly, the process of fertilisation is usually considered from the perspective of the sperm and their ability to penetrate the ovum, without typically considering the same process from the perspective of the egg: If sperm vary in their 'fertilising ability', might ova also vary in their ability to be fertilised? Similarly, a line of questioning that sparked the development of Chapters 4 and 5, was that sperm are the most diverse cell type on Earth, but we do not know how the structures adapted to store them (i.e., SSTs) vary between species.

It is also important to consider female specific processes separately: Male and female processes are not always correlated, and a factor that influences male fertility does not necessarily translate to an effect for females (e.g., Chapter 3; and as discussed above in section 2.5). For example, female fertility is heavily dependent on the proper functioning of the endocrine system. Infertility or reduced fertility can therefore be heavily influenced by exposure to environmental contaminants such as endocrine disrupting compounds (EDCs). Few long-term studies have monitored the effects of EDCs on reproduction in wild birds, but experimental evidence suggests that even environmentally relevant doses of certain compounds can have significant implications for female fertility, including egg production (Assersohn et al., 2021a, 2021b (Chapter 2 and Appendix 1)). Improving our understanding of the sensitivity of the female endocrine system to disruption will be vital for developing interventions, which may be particularly relevant for threatened populations that suffer from often high and unexplained levels of reproductive failure. Other female-specific factors that require further investigation include the prevalence and implications of diseases and disorders affecting female fertility, and the effects of a changing climate on egg production and the seasonal regrowth and regression of the reproductive tract (Halupka et al., 2023; Lara and Rostagno, 2013).

Throughout this thesis, I have attempted to critically consider reproduction and sexual selection from the perspective of females and non-model species and explored both i) how our current knowledge in males and model species can be applied to these groups and ii) whether there are specific features of these groups that require separate lines of enquiry.

Ultimately, it is important for work exploring the factors driving reproductive outcomes to consider both male and female factors, and their interactions, including developing questions and hypotheses with a female-specific focus that doesn't necessarily rely on what we know already know in males. To further improve our understanding of fertility in female birds, I encourage future work to develop questions and hypotheses using a similar approach.

## 3.3 Priority research gaps

Here, I provide a summary of the key research gaps that I have identified throughout this thesis. I believe these are likely to be the most important barriers to our understanding in this field and should be a priority for future research.

1. We must focus on improving our understanding of the factors affecting fertility in wild/non-commercial species, seasonally breeding birds, and opportunistic breeders. This will be particularly important for conservation breeding programmes. When populations are small and vulnerable, the loss of every egg poses a risk to species survival, and high rates of hatching failure must be acted upon swiftly. Implementing interventions based on inadequate knowledge may hinder, rather than help, conservation efforts (Assersohn et al., 2021; Appendix 1). This recommendation begins with accurate reporting and measuring of infertility: Infertility must be defined correctly and distinguished from embryo mortality; the fertility status of unhatched eggs should ideally be determined through proper microscopic methods (Birkhead et al., 2008); the activity of non-breeding females must be more routinely incorporated into long-term study monitoring; and when generating hypotheses as to the causes of infertility, both male and female processes should be considered independently and in combination.

2. The genetic basis of variation in female fertility is still poorly understood in birds, however it seems likely that there is genetic variation in female fertility traits, given that heritable variation in individual relative fitness (i.e. additive genetic variance) does exist in wild birds (Bonnet et al., 2022). Despite finding no effects of the Z-linked supergene for reproductive traits in Chapter 3, it remains the case that the genome is largely shared between the sexes and a reasonable line of questioning is to explore genetic factors that we already know influence male fertility and examine their effects in females. However, it will also be

important to consider the genetic basis of female-specific processes independently of their known effects in males. One logical place to start would be the W- chromosome, the only female-specific region of the avian genome. Compared to XY systems, we know far less about how sex-specific selection has shaped Z and W gene content (Xu and Zhou, 2020). Empirical data widely supports the idea that, despite many Y chromosomes containing few functional genes, Y-linked genes are masculinised, and often related to male fertility (Mank, 2012). However, evidence for the equivalent feminisation of the W is currently mixed (Moghadam et al., 2012; Xu et al., 2020; Xu and Zhou, 2020), and we know far less about the avian W than the mammalian Y, so further work is needed to gain a better understanding of the dynamics of female-specific selection on the sex chromosomes. That said, the likely polygenic nature of female fertility (Santure et al., 2013), combined with the expectation that the W chromosome will have few functional genes (Mank, 2012), provides support for the autosomal location of female fertility genes in birds. One alternative approach to GWAS, is to predict the combined effects of all genes (i.e., breeding values) influencing female fertility using genomic prediction/selection (Gienapp et al., 2019; Yang et al., 2021). This method can be helpful for increasing our understanding of the genetic architecture of highly polygenic traits and predicting how evolutionary forces might affect them, and can be a useful tool in both captive (VanRaden, 2020) and natural populations (Gienapp et al., 2019).

3. In Chapter 4, I demonstrated that sperm storage tubules in the female reproductive tract in birds can be far more variable and morphologically complex than has previously been appreciated. However, the causes and consequences of this variation are not clear. The focus of research on a small number of model species has masked important trait variation, and so a priority for future research will be to examine SST morphological variation across other species and groups, and to explore the factors that drive this variation. Several possible starting points include exploring; whether SST morphology is plastic and changes through time in response to male quality; whether selection occurs at the point of SST acceptance i.e. under the control of SST entrances; or whether SST morphology is associated with sperm storage duration. It may also be worth future work exploring the relationship between SST morphology and sperm competition intensity across a greater diversity of species. A better understanding of the role of SSTs in post-copulatory sexual

selection will also require further research into the specific mechanisms by which SSTs accept, store and release sperm. This may include also exploring the interaction of SSTs with the surrounding tissue of the vagina (e.g., channels and transitional tissue).

4. In Chapter 5, I found that relative vagina length was positively correlated with relative testis mass. This finding implies that vagina length and sperm production have co-evolved, and that vagina length plays an important role in generating, and responding to, post-copulatory sexual selection. To further understand this finding, and before being able to generalise across all species, it will first prove useful to examine this relationship across a greater number of species.

5. Other priority areas that I identified in Chapter 1 and was not able to explore within this thesis, but nevertheless remain important, include: the effects of pollution and a changing climate on fertility in females, particularly the impacts of heat stress and EDCs on reproductive tract function; whether ova vary in their quality and ability to accept sperm between females; the incidence and importance of hormonal and physiological reproductive disorders in wild birds; and the role of the female immune response within the oviduct (including the ovaries) for fertility, particularly the interaction between immune function and sperm storage.

## 3.4 Wider impacts

Improving our understanding of the factors that influence variation in fertility has broad impacts. Firstly, the importance of poultry as a commercial food source means that ensuring high reproductive productivity in commercial stocks will benefit global food security (Vaarst et al., 2015). Despite consistent selection for high rates of egg production with high fertility, hatching failure rates in domestic chickens and turkeys remain similar to the global average across all bird species and the causes of this baseline level of infertility are not well understood (Beaumont et al., 1997; Marshall et al., 2023). As the global demand for animal production is expected to increase in the coming decades, the effects of a changing climate are simultaneously expected to have significant negative impacts on livestock health and productivity (Nardone et al., 2010). Poultry is known to be particularly sensitive to heat stress, with the effects of increasing ambient temperature having a negative effect for most metrics of reproductive health (Kumar et al., 2021). Improving our understanding of the factors that

influence fertility in birds will be increasingly important for generating selective breeding programmes and husbandry management techniques for mitigating the effects of climate change on livestock performance.

Conservation strategies for threatened species depends upon increasing population growth through improved survival and productivity (Digby et al., 2023). Reproductive output represents one of the largest factors limiting conservation success, but management strategies often require quick action to mitigate egg loss (Comizzoli and Holt, 2019). For example, in the critically endangered kākāpō, conservation efforts have been significantly hampered by infrequent breeding, and low fertility and hatching success (Clout, 2006). Recently, a study investigating the causes of infertility in Kākāpō identified several important factors that have led to novel management recommendations (Digby et al., 2023). This highlights the importance of conducting species and population specific studies on the causes of reproductive failure. By also improving our general understanding of reproductive function and the factors that cause variable fertility rates across birds, this may guide species-specific studies, and trickle down into conservation management for improving survival outcomes in threatened species (Assersohn et al., 2021; Appendix 1).

# 4 Concluding remarks

Within the field of avian reproduction, female fertility remains understudied, and this has limited our knowledge of the incidence and factors driving variation in reproduction in birds. The focus on commercial and captive species in this field has also heavily skewed our knowledge towards species that are typically non-representative, and even among studies that do include wild-populations, fertility is often incorrectly defined or measured, and key female variables that might influence fertility are commonly ignored. There are, however, an increasing number of studies that are acknowledging these gaps, and there are several exciting avenues for future research in this field, which I have illustrated throughout this thesis. In particular, I have highlighted that the female reproductive tract can be physiologically variable and complex, and play a pivotal role in the processes of post-copulatory sexual selection and the co-evolution of male and female reproductive traits. I expect that future work will continue to uncover the remarkable functions of the avian female reproductive tract, providing insight into the role of females in sexual selection, and

generating knowledge of the female factors influencing reproductive outcomes. This knowledge will be valuable in the coming decades as we increasingly require the development of novel methods for managing the reproductive productivity of commercial species, and threatened populations, amidst a changing climate and global biodiversity crisis.

# 4 References

Ah-King, M., 2022. The history of sexual selection research provides insights as to why females are still understudied. Nat. Commun. 13, 6976. https://doi.org/10.1038/s41467-022-34770-z

Ah-King, M., Barron, A.B., Herberstein, M.E., 2014. Genital Evolution: Why Are Females Still Understudied? PLoS Biol. 12, e1001851. https://doi.org/10.1371/journal.pbio.1001851

Allen, T., Grigg, G., 1957. Sperm transport in the fowl. Aust. J. Agric. Res. 8, 788–789. https://doi.org/10.1071/AR9570788

Arnqvist, G., Rowe, L., 2002. Antagonistic coevolution between the sexes in a group of insects. Nature 415, 787–789. https://doi.org/10.1038/415787a

Assersohn, K., Brekke, P., Hemmings, N., 2021a. Physiological factors influencing female fertility in birds. R. Soc. Open Sci. 8, 202274. https://doi.org/10.1098/rsos.202274

Assersohn, K., Marshall, A.F., Morland, F., Brekke, P., Hemmings, N., 2021b. Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. Anim. Conserv. 1–12. https://doi.org/10.1111/acv.12674

Bakst, M.R., Wishart, G., Brillard, J.-P., 1994. Oviducal sperm selection, transport, and storage in poultry. Poult. Sci. Rev. 5, 117–143.

Bakst, M.R., 2011. Role of the oviduct in maintaining sustained fertility in hens. J. Anim. Sci. 89, 1323–1329. https://doi.org/10.2527/jas.2010-3663

Beaumont, C., Millet, N., Le Bihan-Duval, E., Kipi, A., Dupuy, V., 1997. Genetic parameters of survival to the different stages of embryonic death in laying hens. Poult. Sci. 76, 1193–1196. https://doi.org/10.1093/ps/76.9.1193

Birkhead, T.R., 2000. Defining and Demonstrating Postcopulatory Female Choice-Again. Evolution 54.

Birkhead, T.R., 1998. Cryptic female choice: Criteria for establishing female sperm choice. Evolution 52, 1212–1218. https://doi.org/10.1111/j.1558-5646.1998.tb01848.x

Birkhead, T.R., Hall, J., Schut, E., Hemmings, N., 2008. Unhatched eggs: Methods for discriminating between infertility and early embryo mortality. Ibis 150, 508–517. https://doi.org/10.1111/j.1474-919X.2008.00813.x

Bonduriansky, R., Chenoweth, S.F., 2009. Intralocus sexual conflict. Trends Ecol. Evol. 24, 280–288. https://doi.org/10.1016/j.tree.2008.12.005

Bonnet, T., Morrissey, M.B., De Villemereuil, P., Alberts, S.C., Arcese, P., Bailey, L.D., Boutin, S., Brekke, P., Brent, L.J.N., Camenisch, G., Charmantier, A., Clutton-Brock, T.H., Cockburn, A., Coltman, D.W., Courtiol, A., Davidian, E., Evans, S.R., Ewen, J.G., Festa-Bianchet, M., De Franceschi, C., Gustafsson, L., Höner, O.P., Houslay, T.M., Keller, L.F., Manser, M., McAdam, A.G., McLean, E., Nietlisbach, P., Osmond, H.L., Pemberton, J.M., Postma, E., Reid, J.M., Rutschmann, A., Santure, A.W., Sheldon, B.C., Slate, J., Teplitsky, C., Visser, M.E., Wachter, B., Kruuk, L.E.B., 2022. Genetic variance in fitness indicates rapid contemporary adaptive evolution in wild animals. Science 376, 1012–1016. https://doi.org/10.1126/science.abk0853

Brennan, P.L.R., Prum, R.O., 2015. Mechanisms and Evidence of Genital Coevolution: The Roles of Natural Selection, Mate Choice, and Sexual Conflict. Cold Spring Harb. Perspect. Biol. 7, a017749. https://doi.org/10.1101/cshperspect.a017749

Brillard, J., 2003. Practical aspects of fertility in poultry. WORLDS Poult. Sci. J. https://doi.org/10.1079/WPS20030027

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. https://doi.org/10.1371/journal.pone.0000744

Brown, M.E., Keefer, C.L., Songsasen, N., 2019. Factors affecting captive whooping crane egg fertility: A retrospective analysis. J. Wildl. Manag. 83, 1377–1386. https://doi.org/10.1002/jwmg.21717

Chapman, T., Arnqvist, G., Bangham, J., Rowe, 2003. Sexual conflict. Trends Ecol. Evol. 18, 41–47. https://doi.org/10.1016/S0169-5347(02)00004-6

Clout, M.N., 2006. A celebration of kakapo: progress in the conservation of an enigmatic parrot. notornis 53, 1–2.

Comizzoli, P., Holt, W.V., 2019. Breakthroughs and new horizons in reproductive biology of rare and endangered animal species. Biol. Reprod. 101, 514–525. https://doi.org/10.1093/biolre/ioz031

Dale, J., Dey, C.J., Delhey, K., Kempenaers, B., Valcu, M., 2015. The effects of life history and sexual selection on male and female plumage colouration. Nature 527, 367–370. https://doi.org/10.1038/nature15509

Digby, A., Eason, D., Catalina, A., Lierz, M., Galla, S., Urban, L., Le Lec, M.F., Guhlin, J., Steeves, T.E., Dearden, P.K., Joustra, T., Lees, C., Davis, T., Vercoe, D., Kākāpō Recovery Team, 2023. Hidden impacts of conservation management on fertility of the critically endangered kākāpō. PeerJ 11, e14675. https://doi.org/10.7717/peerj.14675

Ebel, E.R., Phillips, P.C., 2016. Intrinsic differences between males and females determine sex-specific consequences of inbreeding. BMC Evol. Biol. 16, 36. https://doi.org/10.1186/s12862-016-0604-5

Eberhard, W.G., 2000. Criteria for Demonstrating Postcopulatory Female Choice. Evolution 54, 1047–1050.

Eberhard, W.G., 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton, NJ.

Ellegren, H., Parsch, J., 2007. The evolution of sex-biased genes and sex-biased gene expression. Nat. Rev. Genet. 8, 689–698. https://doi.org/10.1038/nrg2167

Gienapp, P., Calus, M.P.L., Laine, V.N., Visser, M.E., 2019. Genomic selection on breeding time in a wild bird population. Evol. Lett. 3, 142–151. https://doi.org/10.1002/evl3.103

Greenwood, J.F., Lara Granados, G., Secor, S.M., Todd, B.D., Showalter, I., Hedrick, B.P., Brennan, P.L.R., 2022. Divergent Genital Morphologies and Female–Male Covariation in Watersnakes. Integr. Comp. Biol. 62, 569–580. https://doi.org/10.1093/icb/icac020

Halupka, L., Arlt, D., Tolvanen, J., Millon, A., Bize, P., Adamík, P., Albert, P., Arendt, W.J., Artemyev, A.V., Baglione, V., Bańbura, J., Bańbura, M., Barba, E., Barrett, R.T., Becker, P.H., Belskii, E., Bolton, M., Bowers, E.K., Bried, J., Brouwer, L., Bukacińska, M., Bukaciński, D., Bulluck, L., Carstens, K.F., Catry, I., Charter, M., Chernomorets, A., Covas, R., Czuchra, M., Dearborn, D.C., de Lope, F., Di Giacomo, A.S., Dombrovski, V.C., Drummond, H., Dunn, M.J., Eeva, T., Emmerson, L.M., Espmark, Y., Fargallo, J.A., Gashkov, S.I., Golubova, E.Yu., Griesser, M., Harris, M.P., Hoover, J.P., Jagiełło, Z., Karell, P., Kloskowski, J., Koenig, W.D., Kolunen, H., Korczak-Abshire, M., Korpimäki, E., Krams,

I., Krist, M., Krüger, S.C., Kuranov, B.D., Lambin, X., Lombardo, M.P., Lyakhov, A., Marzal, A., Møller, A.P., Neves, V.C., Nielsen, J.T., Numerov, A., Orłowska, B., Oro, D., Öst, M., Phillips, R.A., Pietiäinen, H., Polo, V., Porkert, J., Potti, J., Pöysä, H., Printemps, T., Prop, J., Quillfeldt, P., Ramos, J.A., Ravussin, P.-A., Rosenfield, R.N., Roulin, A., Rubenstein, D.R., Samusenko, I.E., Saunders, D.A., Schaub, M., Senar, J.C., Sergio, F., Solonen, T., Solovyeva, D.V., Stępniewski, J., Thompson, P.M., Tobolka, M., Török, J., van de Pol, M., Vernooij, L., Visser, M.E., Westneat, D.F., Wheelwright, N.T., Wiącek, J., Wiebe, K.L., Wood, A.G., Wuczyński, A., Wysocki, D., Zárybnická, M., Margalida, A., Halupka, K., 2023. The effect of climate change on avian offspring production: A global meta-analysis. Proc. Natl. Acad. Sci. 120, e2208389120. https://doi.org/10.1073/pnas.2208389120

Hemmings, N., Birkhead, T., 2017. Differential sperm storage by female zebra finches Taeniopygia guttata. Proc. Biol. Sci. 284.

Hemmings, N., Evans, S., 2020. Unhatched eggs represent the invisible fraction in two wild bird populations. Biol. Lett. 16, 20190763. https://doi.org/10.1098/rsbl.2019.0763

Hemmings, N., West, M., Birkhead, T.R., 2012. Causes of hatching failure in endangered birds. Biol. Lett. 8, 964–967. https://doi.org/10.1098/rsbl.2012.0655

Hiyama, G., Matsuzaki, M., Mizushima, S., Dohra, H., Ikegami, K., Yoshimura, T., Shiba, K., Inaba, K., Sasanami, T., 2014. Sperm activation by heat shock protein 70 supports the migration of sperm released from sperm storage tubules in Japanese quail (Coturnix japonica). Reproduction 147, 167–178. https://doi.org/10.1530/REP-13-0439

Kempenaers, B., Foerster, K., Questiau, S., Robertson, B.C., Vermeirssen, E.L.M., 2000. Distinguishing between female sperm choice versus male sperm competition: a comment on Birkhead. Evolution 54, 1050–1052. https://doi.org/10.1111/j.0014-3820.2000.tb00106.x

Kim, K.-W., Bennison, C., Hemmings, N., Brookes, L., Hurley, L.L., Griffith, S.C., Burke, T., Birkhead, T.R., Slate, J., 2017. A sex-linked supergene controls sperm morphology and swimming speed in a songbird. Nat. Ecol. Evol. 1, 1168–1176. https://doi.org/10.1038/s41559-017-0235-2

Kumar, M., Ratwan, P., Dahiya, S.P., Nehra, A.K., 2021. Climate change and heat stress: Impact on production, reproduction and growth performance of poultry and its mitigation

using genetic strategies. J. Therm. Biol. 97, 102867. https://doi.org/10.1016/j.jtherbio.2021.102867

Kusuda, S., Hashizume, R., Yoshikawa, A., Kimpara, H., Sanada, N., Sanada, Y., 2023. Reproductive status of a female white cockatoo (*Cacatua alba*) based on relationships among urofecal steroid hormone dynamics, molting, and body weight. J. Vet. Med. Sci. 85, 207–213. https://doi.org/10.1292/jvms.22-0331

Lara, L., Rostagno, M., 2013. Impact of heat stress on poultry production. Animals 3, 356–369. https://doi.org/10.3390/ani3020356

Lemaître, J.-F., Berger, V., Bonenfant, C., Douhard, M., Gamelon, M., Plard, F., Gaillard, J.-M., 2015. Early-late life trade-offs and the evolution of ageing in the wild. Proc. R. Soc. B Biol. Sci. 282, 20150209. https://doi.org/10.1098/rspb.2015.0209

Mank, J.E., 2012. Small but mighty: the evolutionary dynamics of W and Y sex chromosomes. Chromosome Res. 20, 21–33. https://doi.org/10.1007/s10577-011-9251-2

Marshall, A.F., Balloux, F., Hemmings, N., Brekke, P., 2023. Systematic review of avian hatching failure and implications for conservation. Biol. Rev. 807–832. https://doi.org/10.1111/brv.12931

Miller, G.T., Pitnick, S., 2002. Sperm-Female Coevolution in *Drosophila*. Science 298, 1230–1233. https://doi.org/10.1126/science.1076968

Moghadam, H.K., Pointer, M.A., Wright, A.E., Berlin, S., Mank, J.E., 2012. W chromosome expression responds to female-specific selection. Proc. Natl. Acad. Sci. 109, 8207–8211. https://doi.org/10.1073/pnas.1202721109

Morland, F., Ewen, J.G., Simons, M.J.P., Brekke, P., Hemmings, N., 2023. Early-life telomere length predicts life-history strategy and reproductive senescence in a threatened wild songbird. Mol. Ecol. 32, 4031–4043. https://doi.org/10.1111/mec.16981

Nardone, A., Ronchi, B., Lacetera, N., Ranieri, M.S., Bernabucci, U., 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130, 57–69. https://doi.org/10.1016/j.livsci.2010.02.011

Newell, M.A., Harris, M.P., Gunn, C.M., Daunt, F., 2022. The Isle of May long-term study (IMLOTS). NERC EDS Environ. Inf. Data Cent. https://doi.org/10.5285/3bf9b65d-727c-4b46-9db6-c0814dc5895f

Nussey, D.H., Froy, H., Lemaitre, J.-F., Gaillard, J.-M., Austad, S.N., 2013. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. Ageing Res. Rev. 12, 214–225. https://doi.org/10.1016/j.arr.2012.07.004

O'Donnel, P., 2024. Female Peacock (Male vs Female Comparison) [WWW Document]. BirdZilla. URL https://www.birdzilla.com/learn/female-peacock/ (accessed 5.10.24).

Ogasawara, F.X., Lorenz, F.W., Bobr, L.W., 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds III. Intra-uterine insemination of semen from low-fecundity cocks. J. Reprod. Fertil. 11, 33–41.

Pitnick, S., Brown, W., 2000. Criteria for Demonstrating Female Sperm Choice. Evolution 54, 1052–1056.

Santure, A.W., De Cauwer, I., Robinson, M.R., Poissant, J., Sheldon, B.C., Slate, J., 2013. Genomic dissection of variation in clutch size and egg mass in a wild great tit ( *Parus major* ) population. Mol. Ecol. 22, 3949–3962. https://doi.org/10.1111/mec.12376

Sasanami, T., Matsuzaki, M., Mizushima, S., Hiyama, G., 2013. Sperm storage in the female reproductive tract in birds. J. Reprod. Dev. 59, 334–338. https://doi.org/10.1146/annurev-animal-021815-111350

Schut, E., Vedder, O., Komdeur, J., Magrath, M.J.L., 2014. Sperm depletion does not account for undeveloped eggs in Blue Tits Cyanistes caeruleus. IBIS 156, 366–373. https://doi.org/10.1111/ibi.12123

Thornhill, R., 1983. Cryptic Female Choice and Its Implications in the Scorpionfly Harpobittacus nigriceps. Am. Nat. 122, 765–788. https://doi.org/10.1086/284170

Tobias, J.A., Montgomerie, R., Lyon, B.E., 2012. The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. Philos. Trans. R. Soc. B Biol. Sci. 367, 2274–2293. https://doi.org/10.1098/rstb.2011.0280

Vaarst, M., Steenfeldt, S., Horsted, K., 2015. Sustainable development perspectives of poultry production. Worlds Poult. Sci. J. 71, 609–620. https://doi.org/10.1017/S0043933915002433

VanRaden, P.M., 2020. Symposium review: How to implement genomic selection. J. Dairy Sci. 103, 5291–5301. https://doi.org/10.3168/jds.2019-17684

Ward, P.I., 2000. Cryptic female choice in the yellow dung fly Scathophaga stercoraria (L.). Evolution 54, 1680–1686. https://doi.org/10.1111/j.0014-3820.2000.tb00712.x

Westneat, D.F., Rambo, T.B., 2000. Copulation exposes female Red-winged Blackbirds to bacteria in male semen. J. AVIAN Biol. 31, 1–7. https://doi.org/10.1034/j.1600-048X.2000.310101.x

Xu, L., Irestedt, M., Zhou, Q., 2020. Sequence Transpositions Restore Genes on the Highly Degenerated W Chromosomes of Songbirds. Genes 11, 1267. https://doi.org/10.3390/genes11111267

Xu, L., Zhou, Q., 2020. The Female-Specific W Chromosomes of Birds Have Conserved Gene Contents but Are Not Feminized. Genes 11, 1126. https://doi.org/10.3390/genes11101126

Yang, R., Xu, Z., Wang, Q., Zhu, D., Bian, C., Ren, J., Huang, Z., Zhu, X., Tian, Z., Wang, Y., Jiang, Z., Zhao, Y., Zhang, D., Li, N., Hu, X., 2021. Genome-wide association study and genomic prediction for growth traits in yellow-plumage chicken using genotyping-by-sequencing. Genet. Sel. Evol. 53, 82. https://doi.org/10.1186/s12711-021-00672-9

# Appendix 1:

# Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation

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*This is an additional paper produced during the course of my PhD in collaboration with researchers from my and another institution, which although separate from the main body of my thesis, is relevant to the broad field of research. This published manuscript is included in its full published form here with no alterations. All authors contributed equally to writing the manuscript; K.A. produced figures 1 & 2 and wrote the sections on pollution and fertilisation failure.*

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REVIEW

# Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation

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## Abstract

Reproductive failure is ubiquitous. However, research on the mechanisms underpinning reproductive failure is still lacking in most species. This gap in our understanding has particularly strong repercussions for threatened species and it hinders our ability to establish effective interventions to improve survival. In this review, we focus on why eggs fail to hatch – one of the most critical and understudied aspects of bird reproduction. We identify the main drivers of hatching failure in threatened populations of birds and the key mechanisms that cause failure at different stages of development inside the egg. We then discuss the importance of management interventions aimed at reducing hatching failure in species of conservation concern. Our review highlights the need for a better understanding of the mechanistic basis of hatching failure in non-model bird species and identifies the methodological tools necessary to achieve this.

## Introduction

Around 40% of all bird species have declining populations and 13% are threatened with extinction (BirdLife International, 2020). One of the most common and important problems for threatened bird species is the failure of eggs to hatch. Many eggs are lost as a result of consumption, damage, or disturbance by humans and other animals, but even beyond these losses, some threatened bird populations experience up to 75% hatching failure as a result of indirect anthropogenic or other causes (Jamieson & Ryan, 2000; Ferreira *et al*., 2005). High rates of hatching failure not only influence individual reproductive success but can have strong repercussions for population growth and species recovery (e.g. Jamieson & Ryan, 2000; Ferreira *et al*., 2005; Brekke *et al*., 2010; White *et al*., 2015). However, the drivers of hatching failure are complex and poorly understood. In this review, we highlight the key factors associated with high levels of hatching failure beyond the impacts of predation, damage, desertion and exploitation. We then explore the underlying reproductive problems linked to hatching failure and how these are influenced by ecological and behavioural factors. We argue that a lack of understanding of the mechanistic basis of hatching failure can lead to flawed conclusions about how and why it occurs, with important implications for our understanding of avian ecology and conservation.

## Major drivers of hatching failure in threatened birds

### Inbreeding depression

Threatened bird populations are generally small and isolated, resulting in high levels of inbreeding and low genetic diversity (Keller & Waller, 2002). Threatened and invasive species that have undergone single or multiple bottleneck and founder events associated with low levels of genetic diversity and high inbreeding, have significantly higher levels of hatching failure (Briskie & Mackintosh, 2004; Heber &

Briskie, 2010). A large number of studies in laboratory systems and non-threatened species across multiple taxa also support that inbreeding depresses hatching success (e.g. Morrow, Arnqvist, & Pitcher, 2002; Spottiswoode & Møller, 2004; Slatyer *et al*., 2012), and a few suggest that genetic recovery (in populations of common species) can improve hatching rates (Ortego *et al*., 2010, Lindsay *et al*., 2020). Both parental and embryonic inbreeding depresses hatching success (Briskie & Mackintosh, 2004; Heber & Briskie, 2010) and egg viability (Brekke *et al*., 2010; Hemmings, Slate & Birkhead, 2012), but the effects of parental inbreeding on fertilization and embryo development are poorly understood. Although most studies show that inbreeding depresses hatching (e.g. White *et al*., 2015), some have found that parental inbreeding has no effect (e.g. Brekke *et al*., 2010) or, in a few exceptional circumstances, has a positive effect on hatching success (e.g. Weiser *et al*., 2016). Research on the effects of maternal inbreeding on fertility, egg traits and egg number in wild threatened populations is sorely lacking, despite considerable evidence of these effects in non-threatened species (e.g. Keller, 1998).

Inbreeding depression also varies with the development stage (Keller & Waller, 2002). Mutations in early acting genes that are functionally critical are generally thought to be lethal or at least highly detrimental (Keller & Waller, 2002), so the impact of inbreeding depression due to the expression of genetic load should be strongest at early stages of development (Brekke *et al*., 2010). However, our inability to correctly measure the impact of inbreeding at early stages of embryo development in birds (Hemmings, West & Birkhead, 2012) could have repercussions for the management and recovery of threatened species, as vital information on the magnitude and severity of inbreeding depression is unreliable (Grueber *et al*., 2015). This is particularly important in wild populations, where the effects of inbreeding can be exacerbated by changing environmental conditions (Keller & Waller, 2002).

## Climate change

Climate change effects on hatching success in small populations are complex and confounded by other factors, such as disturbance, habitat degradation, lack of habitat connectivity, food supply and synchrony in phenology (de Villeremuil *et al*., 2019). Environmental stress as a result of climate change has, however, been shown to influence a number of different reproductive traits across a wide range of species. Changes in lay-date as a response to climate change, for example, seem to be ubiquitous (Dunn, 2019), and such shifts may have a knock-on influence on hatching success. However, the evidence for this in threatened species is unclear. In the New Zealand Hihi (Stitchbird, *Notiomystis cincta*), for example, lay-date has not shifted to match changes in climate, showing little adaptive potential (de Villeremuil *et al*., 2019).

Experimental evidence has shown that fertility and egg viability decline with rising temperatures (Lara & Rostagno, 2013). For example, in the threatened Florida Scrub Jay (*Aphelocoma coerulescens*), females with larger clutches that experienced longer periods of pre-incubation exposure to ambient temperature had reduced hatching success (Aldredge, Leclair, & Bowman, 2012). Increased frequency of extreme weather events such as drought has also led to increased hatching failure in the Lesser Prairie Chicken (*Tympanuchus pallidicinctus*), as incubating females are unable to maintain microclimate conditions in the nest, exceeding lethal limits to embryo development (Grisham *et al*., 2016).

In species where anthropogenic incubation disturbance is frequent, the impact of environmental change may also be compounded. In ground-nesting seabirds that breed in highly vulnerable coastal regions, like in the critically endangered Tara iti (Fairy Tern, *Sternula nereis*) human disturbance and extreme weather events are the main drivers of hatching failure (Ferreira *et al*., 2005; Supplementary Material). Rising temperatures have also impacted hatching success and population sex ratios in megapodes, a family of birds in which half of species are at risk of extinction (IUCN, 2020). Megapodes rely on environmental sources of heat for incubation, and high incubation temperatures lead to male-biased mortality in the Australian Brush-turkey (*Alectura lathami*) (Eiby, Wilmer, & Booth, 2008).

## Pollution

Pollution is known to interfere with reproductive function and egg viability in birds, and has been associated with widespread adult mortalities, species declines and extinctions (Giesy *et al*., 2003). Over 90,000 chemicals have been released into the environment by humans, and the vast majority of these have not been tested for their effects on humans or wildlife (Patisaul, Fenton, & Aylor, 2018). Pollutants currently known to affect bird reproduction include persistent organic pollutants (particularly chlorinated hydrocarbons such as DDT, PCBs and BFRs), non-halogenated pesticides (e.g. organophosphorus) and metal toxins (e.g. lead, mercury, cadmium, selenium) (Fry, 1995; Giesy *et al*., 2003).

Several hundred anthropogenic pollutants are known to be Endocrine-Disrupting Compounds (EDCs) – substances that interfere with normal hormone function (Borgeest *et al*., 2002; Patisaul, Fenton, & Aylor, 2018). Known EDCs include many organic pollutants and metal toxins, as well as phytoestrogens, PAHs, alkylphenols and phthalate esters (Borgeest *et al*., 2002; Giesy *et al*., 2003). Many EDCs are highly toxic to birds and can have severe effects on fertility, embryo viability and mating behaviour (Fry, 1995; Giesy *et al*., 2003). Embryonic exposure to pollution can occur through maternal deposition into the yolk, with significant implications for egg quality and embryo development (Ottinger *et al*., 2005). When EDCs are passed on to developing embryos, they can reduce egg quality (e.g. through eggshell thinning), disrupt development, cause abnormalities of the reproductive tract, and result in sterility or even embryo death (Leighton, 1993; Fry, 1995). The effects of the organochlorine insecticide DDT and its primary metabolite DDE is a widely known example. DDTs led to the demise of many birds of prey in the 20$^{th}$ century, primarily due to

eggshell thinning and embryo malformations that resulted from exposure. Despite their ban in the 1980s, DDT (and similar pesticides such as MXC) still affects wild bird reproduction today (Borgeest *et al.*, 2002; Helander *et al.*, 2002, Burnett *et al.*, 2013, Hernández *et al.*, 2018, van Oosten, 2019). Understanding the consequences of EDCs on avian reproductive physiology and fertility is crucial for conservation efforts; however, the mechanisms underpinning the effects these chemicals have on birds are not fully understood (Giesy *et al.*, 2003). We also have little to no knowledge of how the majority of anthropogenic pollutants affect wildlife (Patisaul, Fenton, & Aylor, 2018), and few long-term studies have monitored the effects of EDCs on fertility in wild birds (Bernanke & Köhler, 2009).

Emerging environmental contaminants that are likely to impact avian reproduction and hatching success are those from human and veterinary health care pharmaceuticals (Espín *et al.*, 2018). The last two decades have seen a rise in the effects of veterinary pharmaceuticals on avian scavenger populations (Cuthbert *et al.*, 2014). Avian scavengers frequently eat medicated dead livestock, either opportunistically or when it is provided during supplementary feeding for conservation purposes (Cuthbert *et al.*, 2014; Blanco *et al.*, 2017). Fluoroquinolones are one of the most commonly used antimicrobial veterinary drugs for livestock (Margalida & Bogliani, 2014), and the ingestion of fluoroquinolones and other pharmaceuticals can influence embryo development and reduce hatching success (Espín *et al.*, 2016; Hruba *et al.*, 2019). With livestock carcasses still being commonly used at supplementary feeding stations (Blanco *et al.*, 2017), understanding the impact of pharmaceuticals on hatching success in wild birds remains a priority.

## Mechanisms of hatching failure

Despite ample evidence that environmental change is driving increased rates of hatching failure in threatened birds, a clear understanding of the mechanistic drivers of egg failure remains elusive. The first step towards resolving this issue is to identify whether hatching failure is due to (1) fertilization failure, or (2) failure of a fertilized egg to develop into a hatched chick (i.e. embryo mortality). These two types of failure can have very different causes, so distinguishing between them is essential if we are to identify (and act upon) the ecological and/or behavioural drivers of hatching failure. Only a handful of studies distinguish between fertilization failure and embryo mortality as causes of reproductive failure in birds, and confusingly, ornithologists often universally refer to any undeveloped eggs as 'infertile' (e.g. Wetton & Parkin, 1991; Morrow, Arnqvist & Pitcher, 2002).

### Fertilization failure

Fertilization is the process of sperm and egg pronuclei fusing to form a viable zygote (syngamy). Therefore, an infertile egg is one where the female pronucleus has not fused with a male pronucleus. However, infertility is often used interchangeably to describe both embryo mortality and fertilization failure, possibly due to historic difficulties in distinguishing between the two. Birkhead *et al.* (2008) described a method by which fertilization failure and early embryo mortality can be unequivocally distinguished in unhatched bird eggs, by microscopically examining the egg contents for (1) sperm on the perivitelline layer surrounding the ovum, (2) penetration points in the perivitelline layer indicating the entrance of sperm into the egg, and (3) embryonic cells/tissue in the germinal disc of the ovum, indicating the onset of development (Figure 1). This method has been used on a range of bird species and demonstrated to be relatively robust to egg degradation (Hemmings, West, & Birkhead, 2012), making it well-suited for use on eggs of endangered wild birds, which must typically be left in the nest until after other eggs hatch to eliminate the risk of removing a viable egg. We have developed step-by-step protocols and video demonstrations of this method that are openly available via https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds. For clarity, we define infertility as fertilization failure (i.e. no syngamy) in this review, and when we talk about the causes of infertility, we refer to any processes contributing to fertilization failure.

In birds, fertilization failure is commonly assumed to be the result of a lack of sperm (Hemmings & Birkhead, 2015) or poor sperm function (Brillard, 1990; Lifjeld *et al.*, 2007), that is a problem with the male. However, there is little evidence explicitly linking sperm traits with hatching success in birds. Fertilization failure could also be female-mediated;

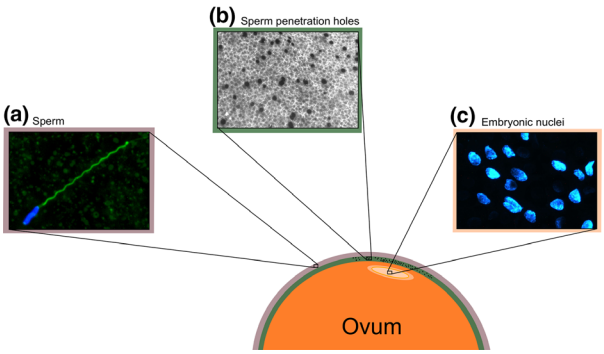


**Figure 1** Microscopic examination of undeveloped eggs allows us to distinguish between fertilization failure and embryo mortality as the cause of hatching failure. (a) Zebra Finch (*Taeniopygia guttata*) sperm stained with fluorescent dyes and imaged at 200x magnification. Sperm can be found on the PVL of unhatched eggs several weeks after failure. (b) Penetration points left by sperm that have entered the ovum, imaged with darkfield microscopy at 200× magnification. (c) Embryonic cells after 24 hours incubation, stained with a fluorescent dye and imaged at 400x magnification. Cell division begins approximately 2 hours after fertilization, and by the time the egg is laid, the germinal disc typically contains thousands of embryonic cells. Polyspermy (where multiple sperm enter the ovum) is part of the normal process of fertilization in birds and is required for normal early embryo development. Diagram not to scale

recent research has revealed that females exert far more control over post-copulatory processes than was previously assumed (Pizzari & Birkhead, 2000; Hemmings & Birkhead, 2017), and that variation in female reproductive traits may have a substantial impact on fertilization success. For example, the avian vagina is thought to be highly selective, with only 1% of sperm successfully passing the vagina and entering storage. Therefore, sperm selection in the female reproductive tract (cryptic female choice) can influence which sperm are available during fertilization (Sasanami *et al*., 2013). Ideally, this process would ensure only good quality sperm can fertilize the egg, theoretically improving fertilization success but also potentially enhancing offspring quality. It has been found in other taxa, for example, that cryptic female choice improves both egg fertilization rate and embryo survival (Rosengrave *et al*., 2016). The exact mechanisms of sperm selection are still unclear in birds, but some females are known to preferentially eject the sperm of undesirable males (Pizzari & Birkhead, 2000), and immunological activity within the vagina can influence sperm viability and transport (Bakst, Wishart, & Brillard, 1994). If these processes are too selective, insufficient sperm may reach the site of fertilization (Hemmings & Birkhead, 2015). In domestic birds, fertilization failure has also been shown to be associated with female age (Bramwell *et al*., 1996), female reproductive disorders (Srinivasan *et al*., 2014), aspects of the female's environment (such as diet and stress) (Lewis, 2004; Walzem & Chen, 2014) and genetic factors that may influence the receptivity of the oviduct and/or egg to sperm (Bernier, Spencer, & Swartwood, 1951). Fertilization failure may also result from behavioural incompatibilities between males and females that impede successful courtship and copulation.

## Embryo mortality

If an ovum is successfully fertilized, hatching failure may still occur as a result of embryo mortality. Embryo mortality can occur at any stage of development (including prior to oviposition) and for a variety of reasons (Figure 2), although deaths are more common during the early and late stages (Romanoff, 1949). Early embryo mortality (within 72 hours of fertilization) is commonly associated with lethal genetic factors, such as chromosomal abnormalities (Shook, Stephenson, & Biellier, 1971). Genetic perturbations are more likely in inbred individuals, and accordingly, inbreeding has been shown to significantly depress early embryo survival (Hemmings, Slate, & Birkhead, 2012). However, the mechanisms by which inbreeding depresses embryo development remain largely unknown. While most genetic problems manifest early in development, some result in death at a later stage of development, typically due to gross morphological abnormalities (Romanoff, 1949).

Although sperm quality is more typically expected to influence fertilization success, prolonged sperm storage in the male or female reproductive tract before fertilization has been shown to increase the incidence of early embryo mortality (Lodge, Fechheimer, & Jaap, 1971). This effect may be explained by age-related deterioration of sperm and/or a reduction in the number of sperm surviving to reach and penetrate the ovum (Eslick & McDaniel, 1992). Fewer viable sperm may limit the scope for physiological polyspermy, which is essential for normal early embryo development in birds (Hemmings & Birkhead, 2015).

In the early stages of development, embryos are vulnerable to fluctuations in ambient climatic conditions (particularly elevated temperatures) and trans-shell infections during the period between oviposition and incubation onset (Meijerhof, 1992). In many species that lay a clutch of eggs, incubation does not begin until the end of the egg-laying period to ensure synchronous hatching, so eggs laid earlier in the clutch have a longer pre-incubation exposure time. Early embryo mortality also appears to be more common when (1) females are younger (Fairchild *et al*., 2002); (2) females have greater body weight (Coleman & Siegel, 1966) and (3) eggs are small and/or poor quality (including the eggshell) (Lerner *et al*., 1993), which can be the result of poor female condition or stress/disturbance during egg production (Reynard & Savory, 1999).

Mid-development embryo mortality is relatively infrequent, although hyperthermia at this stage can result in developmental arrest or malformations (Christensen, 2001). The nature of these malformations depends on the stage at which the embryo is exposed to high temperatures. For example, around day 3 of incubation, during early brain formation, elevated temperatures can lead to abnormal brain and neural tube development (Alsop, 1919), whereas a week or so into development, high temperatures are more likely to lead to circulatory system failure, for example heart enlargement and cardiac arrest. Romanoff (1949) identified a critical period in the mid-stage development (at 12–14 days of incubation) of Domestic Fowl (*Gallus gallus domesticus*) when embryo mortality can peak if the maternal diet during egg production is deficient in animal protein, vitamins and minerals. Towards the end of development, high or low temperatures, as well as insufficient egg turning, can increase the incidence of embryonic malpositioning, limiting the ease with which the developed chick can successfully break out of the egg.

Although it is relatively easy to identify by eye whether a freshly laid egg is fertilized (Christensen, 2001), the structure of the blastoderm degenerates rapidly following early developmental arrest, particularly in the warm conditions of a nest. Therefore, if an embryo from a wild nest dies within the first 72 hours of development, and several days or weeks elapse before it is collected, the egg can be mistaken as unfertilized upon macroscopic examination (Birkhead *et al*., 2008). Using fluorescence microscopy methods, Hemmings & Evans (2020) found that early embryo deaths were mistaken for fertilization failure in 52% of Blue Tit (*Cyanistes caeruleus*) and 33% of Great Tit (*Parus major*) eggs left in the nest for 2 weeks after hatching. The fact that early embryo mortality can be so easily mistaken for fertilization failure in wild populations is of particular concern, given that the majority of embryo mortalities may happen during these early stages of development (Christensen, 2001).

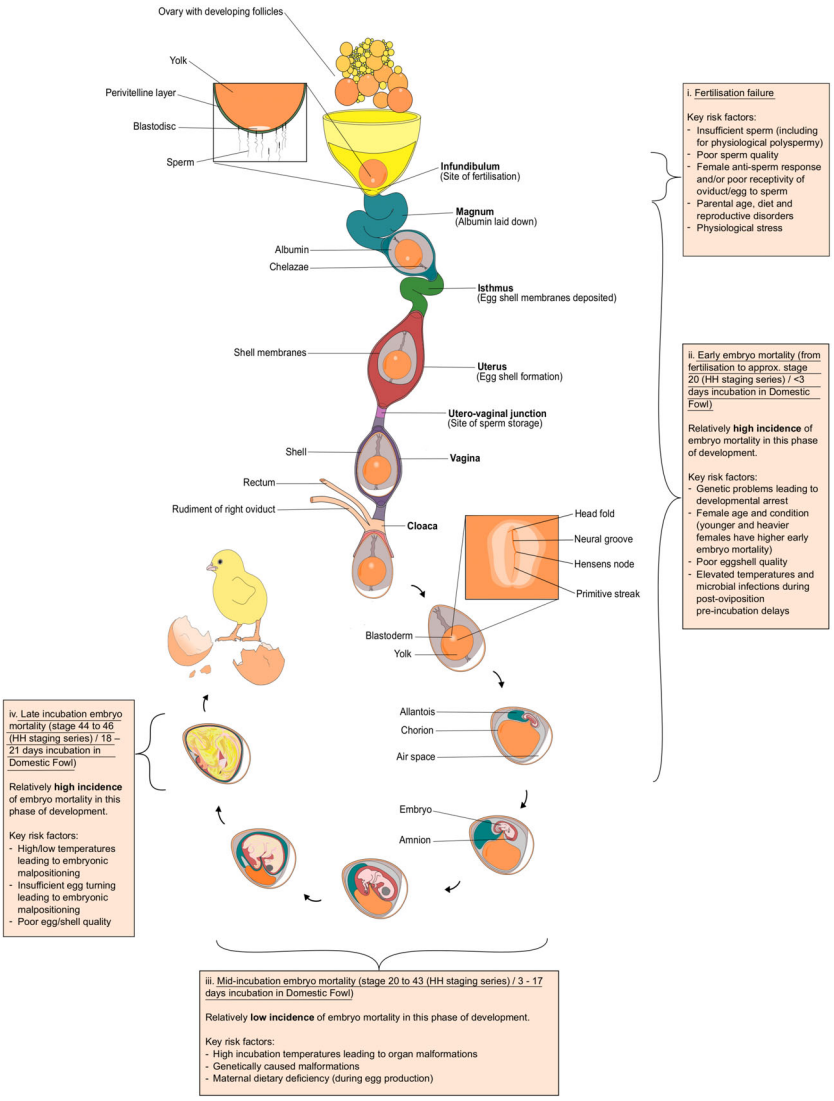


**Figure 2** Key risk factors associated with egg failure at different stages of egg formation and embryo development. I. Fertilization failure refers to factors that reduce the likelihood of sperm reaching and penetrating the ovum; II. Early embryo mortality refers to embryo death occurring between fertilization and approximately developmental stage 20 (~3 days incubation in Domestic Fowl). III. Mid-incubation mortality refers to embryo death during developmental stages 20-43 (~3-17 days incubation in Domestic Fowl); IV. Late incubation mortality refers to embryo death during developmental stages 44-46 (~18-21 days incubation in Domestic Fowl). Developmental stages and incubation phases are provided as a guide but vary depending on developmental rate and mode of different species. Embryo death is most common during the early (<3 days incubation) and late (pre-hatch) stages of development. Diagram not to scale

## Hatching failure in wild populations

Despite differences in the mechanisms that cause fertilization failure versus embryo mortality, the majority of studies of hatching failure in wild birds consider only whether or not eggs hatch, without investigating the underlying cause of failure and/or the stage at which the embryos died (e.g. Spottiswoode & Møller, 2004). Of those studies that have attempted to look at embryo mortality rates in wild birds, most have assumed undeveloped eggs to be unfertilized and therefore restricted their analyses to analysing mid- and late-term embryos (Jamieson & Ryan, 2000; Brekke *et al.*, 2010). Results from the limited number of studies that have distinguished between fertilization failure and early embryo death as causes of hatching failure in wild birds suggest that early embryo mortality is more common (Hemmings & Evans, 2020). Hemmings, West, & Birkhead (2012) microscopically examined eggs classed as 'infertile' from five endangered species and found that only 26% of these eggs were truly unfertilized. If extrapolated to another study such

as Jamieson & Ryan (2000), which compares infertility rates in New Zealand endangered species, this suggests that infertility may be strongly overestimated, while the incidence of early embryo mortality is underestimated (Figure 3).

Recognizing the role of early embryo mortality in the hatching failure of wild populations can improve conservation research but is also important for studies in other fields. For example, a study on a wild population of Eurasian Tree Sparrows (*Passer montanus*) – one of the few studies that has accurately discriminated between unfertilized eggs and early embryo mortality – found that the female-biased secondary sex ratio in this population was due to higher mortality of male embryos, most (62%) of which occurred at the early embryo stage (Kato *et al.*, 2017). Previous studies have attributed skewed sex ratios to temperature-dependent sex-biased embryo mortality (Eiby, Wilmer, & Booth, 2008) and biased parental investment (Spelt & Pichegru, 2017), but failure to consider individuals that die very early in the population creates a potential bias in these studies.

Accurate monitoring of early embryo mortality in wild populations can also provide important and formerly lacking data on extra-pair paternity. The role of extra pair paternity in connection with reproductive success is controversial; there is some evidence that engaging in extra-pair copulations is a female strategy for directly improving fitness via decreased hatching failure (Yuta *et al.*, 2018). However, there is opposing evidence for whether within-pair or extra-pair offspring have higher fitness themselves (Sardell *et al.*, 2012; Hsu *et al.*, 2014) and meta-analyses have come to contradictory conclusions about the correlation between extra-pair paternity and hatching success rates across species (Morrow, Arnqvist, & Pitcher, 2002; Reding, 2015). The paternity assignment of early embryos, previously assumed to be unfertilized eggs, provides more accurate data on paternity and reopens lines of enquiry on this issue – not only on the occurrence of extra-pair paternity, but also the consequences for hatching and survival of extra-pair offspring, their distribution in the laying order, differential parental investment and other related questions.

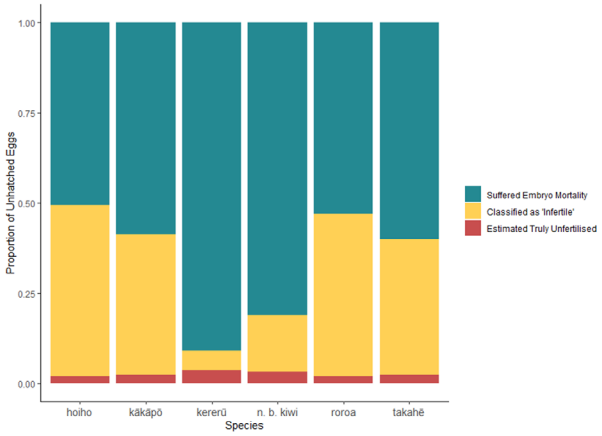


**Figure 3** The proportion of failed eggs classified as infertile in six endangered bird species (Northern Brown Kiwi (*Apteryx mantelli*), Roroa (Great Spotted Kiwi, *Apteryx haastii*), Kākāpō (*Strigops habroptilus*), Kererū (*Hemiphaga novaeseelandiae*), Takahē (*Porphyrio hochstetteri*), and Hoiho (Yellow-eyed Penguin, *Megadyptes antipodes*; data from Jamieson & Ryan (2000), Table 1), and estimated proportion of truly infertile eggs based on results of Hemmings, West, & Birkhead (2012), who found on average 74% of undeveloped eggs from endangered species that were classed as unfertilized by ornithologists actually showed evidence of fertilization/development

## Conservation management interventions for hatching failure: benefits and challenges

Birds that are bred in captivity for conservation management purposes often suffer notably high levels of hatching failure. However, unlike in the wild, where unhatched eggs tend to be fertilized but suffer early embryo mortality, fertilization failure may be a common cause of hatching failure in captive birds (Hemmings, West, & Birkhead, 2012). While captive birds benefit from medical care, a stable food supply and absence of predation (Mason, 2010), captivity can also be stressful due to frequent human disturbance and handling, unnatural or inadequate environment (e.g. artificial lighting), atypical group sizes, and forced mate pairing or separation (Morgan & Tromborg, 2007; Griffith *et al.*, 2017; Fischer & Romero, 2019). Such captive stress could lead to fewer breeding attempts, reduced parental investment/abnormal parental behaviour, and overall, reduced production of successful eggs. For example, in Houbara Bustards (*Chlamydotis [undulata] macqueenii*) hatching failure is higher in captivity than in the wild (Saint Jalme *et al.*, 1996), and captive (domesticated) Zebra Finches (*Taeniopygia guttata*) experience around twice the level of hatching failure reported for their wild counterparts (Hemmings, Slate, & Birkhead, 2012). Elevated rates of hatching failure impact the effectiveness of captive-breeding programmes, so it is important that management techniques are implemented to counteract these issues and improve egg hatchability (Supplementary material).

Egg manipulations are commonly used by conservation programmes of endangered birds to improve hatching success and population growth. A common conservation management practice for both captive and wild populations is 'egg pulling' – removal of eggs from nests for artificial incubation and/or fostering. These eggs are then either returned to the wild at a later stage of incubation or hatched in captivity, with the chicks being captive-reared and either retained for breeding programmes or released into the wild as juveniles or adults. Egg pulling may be employed if there are 'surplus' eggs, for example in the Whooping Crane (*Grus americana*) where two eggs are typically laid but only one chick usually survives (Supplementary Material; Kuyt, 1996). Eggs may also be pulled if they are at risk in the nest, for example to prevent incubating Peregrine Falcons (*Falco peregrinus*) from accidentally smashing eggs that were thin-shelled due to DDE contamination (e.g. Burnham

*et al.*, 1988). Alternatively, eggs may be removed to encourage the breeding pair to lay a replacement clutch, increasing the overall number of eggs laid in the population (e.g. Wood & Collopy, 1993). However, egg fertility, hatchability and quality have all been shown to decline in replacement clutches (e.g. Jones *et al.*, 1994) and forced re-clutching may negatively impact fledgling survival (e.g. Parmley *et al.*, 2015) and/or future reproductive success of adults (e.g. Wood & Collopy, 1993). This indicates that egg pulling can have important costs as well as benefits for breeding management, and accordingly, some conservation protocols enforce limitations on the number of replacement clutches that can be laid in a season.

Although removing eggs for artificial incubation is generally considered the safest option for conservation managers, artificially incubated eggs often experience lower hatching success than eggs left in the wild, and therefore represent an important source of mortality in captive-breeding programmes (e.g. Sancha *et al.*, 2004). While many aspects of the artificial incubation environment can be tightly controlled, what is lacking is the fine-scale control and adjustments that may be provided by parent birds throughout development as they respond to the developing embryo's requirements (Tong *et al.*, 2013). Artificial incubation will also inevitably lack factors that natural nesting environments and parental incubation provide such as growth of beneficial microbes, periodic cooling, natural turning patterns and stimulation provided by parental and sibling vocalizations (Deeming, 2002). One technique that has been shown to increase the hatchability of wild eggs in artificial incubation is delaying the removal of eggs from the nest (e.g. Burnham, 1983), suggesting that allowing a small amount of early incubation by the parent may be beneficial. However, delayed egg removal can also increase predation risk and exposure to adverse climatic conditions and may reduce the likelihood that parents will lay a replacement egg or clutch, which is often the primary objective of this intervention.

Artificial incubation is also used for eggs produced by birds in captive breeding programmes. Hatching success of captive-laid eggs under artificial incubation is often lower than that of wild-laid eggs (e.g. Burnham, 1983), but this may reflect lower rates of fertilization success in captive birds. Indeed, wild-laid Whooping Crane eggs (Supplementary Material) had greater hatching success than captive-laid eggs even when they were both naturally incubated (by foster parents; Kuyt, 1996). Differences between wild and captive-laid eggs may also be a consequence of the presence or absence of pre-collection incubation, respectively (see above), and/or health problems affecting egg/embryo quality in the captive population. For example, a sudden increase in late-incubation embryo deaths in captive Kakī (Black Stilt, *Himantopus novaezelandiae*) eggs, but not wild-laid eggs subjected to the same artificial incubation environment, indicated differences in egg quality between captive and wild birds. This was subsequently shown to be the result of iodine deficiency in the captive population (Sancha *et al.*, 2004).

A major risk to eggs during the incubation period are trans-shell microbial infections, which can lead to embryo mortality. Parental incubation has been shown to limit bacterial and fungal growth on eggshells relative to unincubated eggs, reducing the risk of infection and increasing hatching success (Cook *et al.*, 2005). However, the precise mechanisms underpinning this effect remain unclear. In the absence of parental incubation, cleaning eggs with alcohol has been shown to reduce trans-shell infection and increase hatching success (Cook *et al.*, 2005), and egg-cleaning before artificial incubation is common practice within some areas of the poultry industry (Rideout, 2012). However, support for egg-cleaning is mixed, since resulting damage to the shell cuticle could potentially reduce natural barriers against microorganisms (Baggott & Graeme-Cook, 2002).

Fostering of eggs is sometimes used in breeding management practices in combination with, or as an alternative to, artificial incubation. A study comparing parentally incubated, fostered and artificially incubated wild-laid Killdeer (*Charadrius vociferous*) eggs showed that hatching success was similar after parental incubation and fostering (in this case by another species, Spotted Sandpipers *Actitus macularia*) (Powell & Cuthbert, 1993). Artificial incubation resulted in significantly higher hatching success than both parental incubation and fostering, but this was primarily because a large proportion of wild nests were predated rather than due to failure in artificial incubation. While fostering by both conspecifics and heterospecific parents has been successful (e.g. Byrd *et al.*, 1984), fostering by heterospecifics carries the risk of incorrect imprinting (e.g. Butler & Merton, 1992) and inter-species disease transfer (e.g. Snyder et al., 1985). Hence, fostering by conspecifics is generally preferred where possible.

Conditions in captivity may influence reproductive behaviour, ultimately resulting in decreased fertilization success (Saint Jalme *et al.*, 1996; Hemmings, West, & Birkhead, 2012). Captive birds are often kept in pairs or small groups, limiting the potential for mate choice and extra-pair copulations, and potentially leading to a higher incidence of incestuous and/or same-sex pairings than found in the wild (Driscoll, 2008). Commonly in captive breeding programmes, unsuccessful individuals are separated and provided with alternative mates, a technique which may also be used to manage genetic diversity. However, multiple studies in both wild and captive populations have indicated that birds that retain their mates over multiple seasons have greater reproductive success than those that 'divorce' and change mate (e.g. Yamamoto *et al.*, 1989), and several studies of captive birds (albeit with small sample sizes) have shown that reproductive success – particularly fertilization success – improves with increasing time spent as a pair (e.g. Brosset, 1981). Hence, there is a trade-off in terms of management decisions between allowing sufficient time for captive pairs or groups to establish normal socio-sexual behaviour, gain experience and improve their reproductive success, and avoiding the risk of wasted mating opportunities. This is particularly important in seasonal and/or unpredictable breeders.

To address issues with reproductive behaviour and timing, artificial insemination has been introduced in many captive populations, and in the special case of the free-living Kākāpō

(*Strigops habroptilus*) (Supplementary Material). Artificial insemination can compensate for a lack of copulation, an absence of extra-pair copulations, and/or unsuitable or unsuccessful pairings. For example, in a captive-bred population of Houbara Bustard, 'natural breeding' scenarios yielded 20-50% fertility (in this study, fertility refers to eggs that showed an obvious sign of embryonic development), while artificial insemination achieved up to 85% fertility (Saint Jalme *et al*., 1996). It has been shown that even when fertility levels are high (80-85%) they can be improved by an additional 5-10% through artificial insemination, with the best results being obtained from repeated deep inseminations of a large volume of semen as soon as possible after collection (Gee *et al*., 2004). The application of artificial insemination can be expanded through the use of frozen semen, which removes the temporal and spatial constraints imposed by the decline in sperm function over time post-ejaculation (Lodge, Fechheimer, & Jaap, 1971). However, the use of frozen semen results in lower egg fertilization rates (e.g. Parks & Hardaswick, 1987; Gee *et al*., 2004) and improvements in cryopreservation methods are essential to make this a viable management approach. Despite its benefits, artificial insemination is labour intensive and invasive, hence many programmes continue to focus on improving fertilization success in natural breeding.

## Conclusions and guidelines for best practice

Hatching failure is one of the most crucial factors limiting the recovery of threatened bird populations. Here we have highlighted the key drivers of hatching failure and explored how these might differ between wild and managed/captive populations. Our overarching conclusion is that a better understanding of the mechanistic causes of hatching failure is required in order to ensure conservation management interventions are appropriately targeted. Distinguishing accurately between infertility and early embryo death and the rates at which each of these occur will enable bird conservation managers to adapt their approaches and provide more tailored solutions to egg failure. We have developed a set of openly available protocols and video demonstrations to facilitate the integration of egg examination techniques into conservation management (https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds), and we advocate the use of these methods for the following reasons. First, these techniques allow us to establish if sufficient sperm are reaching eggs. The absence of sperm on the perivitelline layer of unhatched eggs strongly indicates a male sperm production or copulation problem, facilitating quick intervention. In captivity, for example, unsuccessful males (no sperm reaching eggs) can be removed to allow the female to form a new pair bond with a male of proven fertility within the same breeding season. Alternatively, females could be artificially inseminated with sperm from a proven male. Either approach would maximize the production of fertilized eggs within a season. Second, the identification of male fertility status from the presence/absence of sperm on eggs provides crucial information for translocation decisions – inclusion of an infertile male could potentially threaten the successful establishment of a small founder population. Third, if undeveloped eggs are fertilized but suffer early embryo death, management interventions can shift focus to incubation conditions and maternal health/nutrition to ensure optimal conditions for early embryo survival, as well as considering the genetic compatibility of the parents. Methods for examining unhatched eggs have so far been used to inform the management of a small number of captive and managed bird populations (e.g. Hemmings, West, & Birkhead, 2012; Croyle, Durrant, & Jensen, 2015). We hope that conservation practitioners will make use of the open resources now available (https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds), and that examinations of unhatched eggs will be widely adopted in the future to maximize our understanding of avian reproductive failure.

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## References

Aldredge, R.A., Leclair, S.C. & Bowman, R. (2012). Declining egg viability explains higher hatching failure in a suburban population of the threatened Florida Scrub-jay *Aphelocoma coerulescens*. *J. Avian Biol.* **43**, 369–375.

Alsop, F.M. (1919). The effect of abnormal temperatures upon the developing nervous systems in the chick embryos. *Anat. R.* **15**, 307–331.

Baggott, G.K. & Graeme-Cook, K. (2002). Microbiology of natural incubation. In *Avian Incubation: Behaviour, Environment and Evolution*: 179–191, Deeming, D.C. (Ed.). New York: Oxford University Press.

Bakst, M.R., Wishart, G. & Brillard, J.P. (1994). Oviducal sperm selection, transport, and storage in poultry. *Poult. Sci. Rev.* **5**, 117–143.

# Appendix 1



Bernanke, J. & Köhler, H.-R. (2009). The impact of environmental chemicals on wildlife vertebrates. *Rev. Environ. Contam. Toxicol.* **198**, 1–47.

Bernier, P.E., Spencer, J.V. & Swartwood, J.L. (1951). The relative effects of inbreeding and outbreeding on reproduction in the domestic fowl. *Hilgardia* **20**, 529–628.

BirdLife International. (2020). *State of the world's birds: taking the pulse of the planet*. Cambridge, UK: Birdlife International.

Birkhead, T.R., Hall, J., Schut, E. & Hemmings, N. (2008). Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis* **150**, 508–517.

Blanco, G., Junza, A. & Barrón, D. (2017). Occurrence of veterinary pharmaceuticals in golden eagle nestlings: unnoticed scavenging on livestock carcasses and other potential exposure routes. *Sci. Total Environ.* **586**, 355–361.

Borgeest, C., Greenfeld, C., Tomic, D. & Flaws, J.A. (2002). The effects of endocrine disrupting chemicals on the ovary. *Front. Biosci.* **7**, 1941–1948.

Bramwell, R.K., Mcdaniel, C.D., Wilson, J.L. & Howarth, B. (1996). Age effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. *Poult. Sci.* **75**, 755–762.

Brekke, P., Bennett, P.M., Wang, J., Pettorelli, N. & Ewen, J.G. (2010). Sensitive males: inbreeding depression in an endangered bird. *Proc. R. Soc. Lond. B: Biol. Sci.* **277**, 3677–3684.

Brillard, J.P. (1990). *Control of fertility in birds*. Paris: INRA.

Briskie, J.V. & Mackintosh, M. (2004). Hatching failure increases with severity of population bottlenecks in birds. *Proc. Nat. Acad. Sci.* **101**, 558–561.

Brosset, A. (1981). Breeding the Black Sparrow Hawk *Accipiter melanoleucus* in captivity. *Raptor Res.* **15**, 58–64.

Burnett, L.J., Sorenson, K.J., Brandt, J., Sandhaus, E.A., Ciani, D., Clark, M., David, C., Theule, J., Kasielke, S. & Risebrough, R.W. (2013). Eggshell thinning and depressed hatching success of California Condors reintroduced to Central California. *The Condor* **115**, 477–491.

Burnham, W. (1983). Artificial incubation of falcon eggs. *J. Wildl. Manage.* **47**, 158–168.

Burnham, W.A., Heinrich, W., Sanfort, C., Levine, E., O'Brien, D. & Konkel, D. (1988). Recovery effort for the Peregrine Falcon in the Rocky Mountains. In *Peregrine Falcon Populations, Their Management and Recovery*: 565–575, Cade, T.J., Enderson, J.H., Thelander, C.G. & White, C.M. (Eds.). Boise, Idaho: The Peregrine Fund Inc.

Butler, D. & Merton, D. (1992). *The Black Robin: Saving the world's most endangered bird*. Oxford: Oxford University Press.

Byrd, G.V., Sincock, J.L., Telfer, T.C., Moriarty, D.I. & Brady, B.G. (1984). A cross-fostering experiment with Newell's race of Manx Shearwater. *J. Wildl. Manage.* **48**, 163–168.

Christensen, V.L. (2001). Factors associated with early embryonic mortality. *Worlds Poult. Sci. J.* **57**, 357–372.

Coleman, J.W. & Siegel, P.B. (1966). Selection for body weight at eight weeks of age: 5. Embryonic stage at oviposition and its relationship to hatchability. *Poult. Sci.* **45**, 1003–1011.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R.A. & Arendt, W.J. (2005). Microbial infection affects egg viability and incubation behavior in a tropical passerine. *Behav. Ecol.* **16**, 30–36.

Croyle, K.E., Durrant, B.S. & Jensen, T. (2015). Detection of oocyte perivitelline membrane-bound sperm: a tool for avian collection management. *Conserv. Physiol.* **3**, cou060. https://doi.org/10.1093/conphys/cou060.

Cuthbert, R.J., Taggart, M.A., Prakesh, V., Chakraborty, S.S., Deori, P., Galligan, T., Kulkarni, M., Ranade, S., Saini, M., Sharma, A.K., Shringarpure, R. & Green, R.E. (2014). Avian scavengers and the threat from veterinary pharmaceuticals. *Philos. Trans. R. Soc. B: Biol. Sci* **369**, 20130574. https://doi.org/10.1098/rstb.2013.0574.

Deeming, D.C. (Ed.). (2002). *Avian incubation: behaviour, environment and evolution*. Oxford: Oxford University Press.

Driscoll, E.V. (2008). Bisexual species. *Sci. Am. Mind* **19**, 68–73.

Dunn, P. (2019). Changes in timing of breeding and reproductive success in birds. In *Effects of climate change on birds*: 108–119.Dunn, P. & Møller, A.P. (Eds.), Oxford: Oxford University Press.

Eiby, Y.A., Wilmer, J.W. & Booth, D.T. (2008). Temperature-dependent sex-biased embryo mortality in a bird. *Proc. R. Soc. B: Biol. Sci.* **275**, 2703–2706.

Eslick, M.L. & McDaniel, G.R. (1992). Interrelationships between fertility and hatchability of eggs from broiler breeder hens. *J. Appl. Poult. Sci.* **1**, 156–159.

Espín, S., García-Fernández, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martínez-López, E., Coeurdassier, M., Eulaers, I., Fritsch, C., Gómez-Ramírez, P. & Jaspers, V.L.B. (2016). Tracking pan-continental trends in environmental contamination using sentinel raptors—what types of samples should we use? *Ecotoxicology* **25**, 777–801.

Fairchild, B.D., Christensen, V.L., Grimes, J.L., Wineland, M.J. & Bagley, L.G. (2002). Hen age relationship with embryonic mortality and fertility in commercial turkeys. *J. Appl. Poult. Res.* **11**, 260–265.

Ferreira, S.M., Hansen, K.M., Parrish, G.R., Pierce, R.J., Pulham, G.A. & Taylor, S. (2005). Conservation of the endangered New Zealand fairy tern. *Biol. Conserv.* **125**, 345–354.

Fischer, C.P. & Romero, L.M. (2019). Chronic captivity stress in wild animals is highly species-specific. *Conserv. Physiol.* **7**, 1–38.

Fry, D.M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **103**, 165–171.

Gee, G.F., Bertschinger, H., Donoghue, A.M., Blanco, J. & Soley, J. (2004). Reproduction in nondomestic birds:

physiology, semen collection, artificial insemination and cryopreservation. *Avian Poult. Biol. Rev.* **15**, 47–101.

Giesy, J.P., Feyk, L.A., Jones, P.D., Kannan, K. & Sanderson, T. (2003). Review of the effects of endocrine-disrupting chemicals in birds. *Pure Appl. Chem.* **75**, 2287–2303.

Griffith, S.C., Crino, O.L., Andrew, S.C., Nomano, F.Y., Adkins-Regan, E., Alonso-Alvarez, C., Bailey, I.E., Bittner, S.S., Bolton, P.E., Boner, W., Boogert, N., Boucaud, I.C.A., Briga, M., Buchanan, K.L., Caspers, B.A., Cichoń, M., Clayton, D.F., Derégnaucourt, S., Forstmeier, W., Guillette, L.M., Hartley, I.R., Healy, S.D., Hill, D.L., Holveck, M.J., Hurley, L.L., Ihle, M., Tobias Krause, E., Mainwaring, M.C., Marasco, V., Mariette, M.M., Martin-Wintle, M.S., McCowan, L.S.C., McMahon, M., Monaghan, P., Nager, R.G., Naguib, M., Nord, A., Potvin, D.A., Prior, N.H., Riebel, K., Romero-Haro, A.A., Royle, N.J., Rutkowska, J., Schuett, W., Swaddle, J.P., Tobler, M., Trompf, L., Varian-Ramos, C.W., Vignal, C., Villain, A.S. & Williams, T.D. (2017). Variation in reproductive success across captive populations: methodological differences, potential biases and opportunities. *Ethology* **123**, 1–29.

Grisham, B.A., Godar, A.J., Boal, C.W. & Haukos, D.A. (2016). Interactive effects between nest microclimate and nest vegetation structure confirm microclimate thresholds for Lesser Prairie-Chicken nest survival. *Condor* **118**, 728–746.

Grueber, C.E., Hogg, C.J., Ivy, J.A. & Belov, K. (2015). Impacts of early viability selection on management of inbreeding and genetic diversity in conservation. *Mol. Ecol.* **24**, 1645–1653.

Heber, S. & Briskie, J.V. (2010). Population bottlenecks and increased hatching failure in endangered birds. *Conserv. Biol.* **24**, 1674–1678.

Helander, B., Olsson, A., Bignert, A., Asplund, L. & Litzén, K. (2002). The role of DDE, PCB, coplanar PCB and eggshell parameters for reproduction in the white-tailed sea eagle (*Haliaeetus albicilla*) in Sweden. *Ambio* **31**, 386–403.

Hemmings, N. & Birkhead, T.R. (2015). Polyspermy in birds: sperm numbers and embryo survival. *Proc. R. S. B: Biol. Sci.* **282**, 20151682. https://doi.org/10.1098/rspb.2015.1682.

Hemmings, N. & Birkhead, T.R. (2017). Differential sperm storage by female zebra finches Taeniopygia guttata. *Proc. R. S. B: Biol. Sci.* **284**, 20171032. https://doi.org/10.1098/rspb.2017.1032.

Hemmings, N. & Evans, S. (2020). Unhatched eggs represent the invisible fraction in two wild bird populations. *Biol. Lett.* **16**, 20190763. https://doi.org/10.1098/rsbl.2019.0763.

Hemmings, N.L., Slate, J. & Birkhead, T.R. (2012). Inbreeding causes early death in a passerine bird. *Nat. Commun.* **3**, 863. https://doi.org/10.1038/ncomms1870.

Hemmings, N.L., West, M. & Birkhead, T.R. (2012). Causes of hatching failure in endangered birds. *Biol. Lett.* **8**, 964–967.

Hernández, M., Colomer, M., Pizarro, M. & Margalida, A. (2018). Changes in eggshell thickness and ultrastructure in the Bearded Vulture (*Gypaetus barbatus*) Pyrenean population: A long-term analysis. *Sci. Total Environ.* **624**, 713–721.

Hruba, Hana, Abdelsalam, Ehdaa Eltayeb Eltigani, Anisimov, Nikolay, Bandouchova, Hana, Havelkova, Barbora, Heger, Tomas, Kanova, Miroslava, Kovacova, Veronika, Nemcova, Monika, Piacek, Vladimir, Sedlackova, Jana, Vitula, Frantisek & Pikula, Jiri (2019). Reproductive toxicity of fluoroquinolones in birds. *BMC Vet Res* **15**, 209.

Hsu, Y.-H., Schroeder, J., Winney, I., Burke, T. & Nakagawa, S. (2014). Costly infidelity: low lifetime fitness of extra-pair offspring in a passerine bird. *Evolution* **68**, 2873–2884.

IUCN. (2020). The IUCN. Red List of Threatened Species. Version 2020–1. https://www.IUCN.redlist.org.

Jamieson, I.G. & Ryan, C.J. (2000). Increased egg infertility associated with translocating inbred Takahe (*Porphyrio hochstetteri*) to island refuges in New Zealand. *Biol. Conserv.* **94**, 107–114.

Jones, C.G., Heck, W.R., Lewis, R.E., Mungroo, Y., Slade, G. & Cade, T.J. (1994). The restoration of the Mauritius Kestrel *Falco punctatus* population. *The Ibis* **137**, 173–180.

Kato, T., Matsui, S., Terai, Y., Tanabe, H., Hashima, S., Kasahara, S., Morimoto, G., Mikami, O.K., Ueda, K. & Kutsukake, N. (2017). Male-specific mortality biases secondary sex ratio in Eurasian Tree Sparrows *Passer montanus*. *Ecol. Evol.* **7**, 10675–10682.

Keller, L.F. (1998). Inbreeding and its fitness effects in an insular population of Song Sparrows (*Melospiza melodia*). *Evolution* **52**, 240–250.

Keller, L.F. & Waller, D.M. (2002). Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**, 230–241.

Kuyt, E. (1996). Reproductive manipulation in the Whooping Crane *Grus americana*. *Bird Conserv. Int.* **6**, 3–10.

Lara, L. & Rostagno, M. (2013). Impact of heat stress on poultry production. *Animals* **3**, 356–369.

Leighton, F.A. (1993). The toxicity of petroleum oils to birds. *Environ. Rev.* **1**, 92–103.

Lerner, S.P., French, N., McIntyre, D. & Baxter, C. (1993). Age-related changes in egg production, fertility, embryo mortality, and hatchability in commercial turkey flocks. *Poult. Sci.* **72**, 1025–1039.

Lewis, P.D. (2004). Responses of domestic fowl to excess iodine: a review. *Br. J. Nut.* **91**, 29–39.

Lifjeld, J., Laskemoen, T., Fossøy, F., Johnsen, A. & Kleven, O. (2007). Functional infertility among territorial males in two passerine species, the Willow Warbler *Phylloscopus trochilus* and the Bluethroat *Luscinia svecica*. *J. Avian Biol.* **38**, 267–272.

Lindsay, W.R., Madsen, T., Wapstra, E., Lillie, M., Loeb, L., Ujvari, B. & Olsson, M. (2020). Long term effects of outbreeding: experimental founding of island population eliminates malformations and improves hatching success in sand lizards. *Biol. Conserv.* **249**, 108710. https://doi.org/10.1016/j.biocon.2020.108710.

Lodge, J.R., Fechheimer, N.S. & Jaap, R.G. (1971). The relationship of *in vivo* sperm storage interval to fertility and

embryonic survival in the chicken. *Biol. Reprod.* **5**, 252–257.

Margalida, A., Bogliani, G., Bowden, C.g r, Donazar, J.a, Genero, F., Gilbert, M., Karesh, W.b, Kock, R., Lubroth, J., Manteca, X., Naidoo, V., Neimanis, A., Sanchez-Zapata, J.a, Taggart, M.a, Vaarten, J., Yon, L., Kuiken, T. & Green, R.e (2014). One Health approach to use of veterinary pharmaceuticals. *Science* **346**, 1296–1298.

Mason, G.J. (2010). Species differences in responses to captivity: stress, welfare and the comparative method. *Trends Ecol. Evol.* **25**, 713–721.

Meijerhof, R. (1992). Pre-incubation holding of hatching eggs. *Worlds Poult. Sci. J.* **48**, 57–68.

Morgan, K.N. & Tromborg, C.T. (2007). Sources of stress in captivity. *Appl. Anim. Behav. Sci.* **102**, 262–302.

Morrow, E.H., Arnqvist, G. & Pitcher, T.E. (2002). The evolution of infertility: does hatching rate in birds coevolve with female polyandry? *J. Evol. Biol.* **15**, 702–709.

van Oosten, H.H., van den Burg, A.B., Arlt, D., Both, C., van den Brink, N.W., Chiu, S., Crump, D., Jeppsson, T., de Kroon, H., Traag, W. & Siepel, H. (2019). Hatching failure and accumulation of organic pollutants through the terrestrial food web of a declining songbird in Western Europe. *Sci. Total Environ.* **650**, 1547–1553.

Ortego, J., Cordero, P.J., Aparicio, J.M. & Calabuig, G. (2010). Parental genetic characteristics and hatching success in a recovering population of Lesser Kestrels. *J. Ornithol.* **151**, 155–162.

Ottinger, M.A., Quinn, M.J., Lavoie, E., Abdelnabi, M.A., Thompson, N., Hazelton, J.L., Wu, J.M., Beavers, J. & Jaber, M. (2005). Consequences of endocrine disrupting chemicals on reproductive endocrine function in birds: establishing reliable end points of exposure. *Domest. Anim. Endocrinol.* **29**, 411–419.

Parks, J.E. & Hardaswick, V. (1987). Fertility and hatchability of falcon eggs after insemination with frozen Peregrine Falcon semen. *J. Raptor Res.* **21**, 70–72.

Parmley, E.J., Pearl, D.L., Vogt, N.A., Yates, S., Campbell, G.D., Steiner, J., Imlay, T.L., Hollamby, S., Tuininga, K. & Barker, I.K. (2015). Factors influencing mortality in a captive breeding population of Loggerhead Shrike, Eastern subspecies (*Lanius ludovicianus* ssp.) in Canada. *BMC Vet. Res.* **11**, 129.

Patisaul, H.B., Fenton, S.E. & Aylor, D. (2018). Animal models of endocrine disruption. *Best Pract. Res. Clin. Endocrinol. Metab.* **32**, 283–297.

Pizzari, T. & Birkhead, T.R. (2000). Female feral fowl eject sperm of subdominant males. *Nature* **405**, 787–789.

Powell, A.N. & Cuthbert, F.J. (1993). Augmenting small populations of plovers: an assessment of cross-fostering and captive-rearing. *Conserv. Biol.* **7**, 160–168.

Reding, L. (2015). Increased hatching success as a direct benefit of polyandry in birds. *Evolution* **69**, 264–270.

Reynard, M. & Savory, J. (1999). Stress-induced oviposition delays in laying hens: duration and consequences for eggshell quality. *Br. Poult. Sci.* **40**, 585–591.

Rideout, B.A. (2012). Investigating embryo deaths and hatching failure. *Vet. Clin. North Am. Exot. Anim. Pract.* **15**, 155–162.

Romanoff, A.L. (1949). Critical periods and causes of death in avian embryonic development. *Auk* **66**, 264–270.

Rosengrave, P., Montgomerie, R. & Gemmell, N. (2016). Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. *Proc. R. Soc. B.: Biol. Sci.* **283**, 20160001. https://doi.org/10.1098/rspb.2016.0001.

Saint Jalme, M., Combreau, O., Seddon, P.J., Paillat, P., Gaucher, P. & van Heezik, Y. (1996). Restoration of *Chlamydotis undulata macqueenii* (Houbara Bustard) populations in Saudi Arabia: a progress report. *Restor. Ecol.* **4**, 81–87.

Sancha, E., van Heezik, Y., Maloney, R., Alley, M. & Seddon, P. (2004). Iodine deficiency affects hatchability of endangered captive Kakī (Black Stilt, *Himantopus novaezelandiae*). *Zoo Biol.* **23**, 1–13.

Sardell, R.J., Arcese, P., Keller, L.F. & Reid, J.M. (2012). Are there indirect fitness benefits of female extra-pair reproduction? Lifetime reproductive success of within-pair and extra-pair offspring. *Am. Nat.* **179**, 779–793.

Sasanami, T., Matsuzaki, M., Mizushima, S. & Hiyama, G. (2013). Sperm storage in the female reproductive tract in birds. *J. Reprod. Dev.* **59**, 334–338.

Shook, J.G., Stephenson, A.B. & Biellier, H.V. (1971). Heritability estimates of differences in arbitrary and embryonic mortality traits in turkeys. *Poult. Sci.* **50**, 1255–1260.

Slatyer, R.A., Mautz, B.S., Backwell, P.R. & Jennions, M.D. (2012). Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. *Biol. Rev.* **87**, 1–33.

Snyder, B., Thilstead, J., Burgess, B. & Richard, M. (1985). Pigeon herpesvirus mortalities in foster reared Mauritius Pink Pigeons. *Annu.l Proc. Am. Assoc. Zoo Vet.* 69–70.

Spelt, A. & Pichegru, L. (2017). Sex allocation and sex-specific parental investment in an endangered seabird. *Ibis* **159**, 272–284.

Spottiswoode, C. & Møller, A.P. (2004). Genetic similarity and hatching success in birds. *Proc. R. Soc. Lond. B: Biol. Sci.* **271**, 267–272.

Srinivasan, P., Balasubramaniam, G.A., Gopala Krishna Murthy, T.R. & Balachandran, P. (2014). Prevalence and pathology of oviduct impaction in commercial white leghorn layer chicken in Namakkal region of India. *Vet. World* **7**, 553–558.

Tong, Q., Romanini, C.E., Exadaktylos, V., Bahr, C., Berckmans, D., Bergoug, H., Eterradossi, N., Roulston, N., Verhelst, R., McGonnell, I.M. & Demmers, T. (2013). Embryonic development and the physiological factors that coordinate hatching in domestic chickens. *Poult. Sci.* **92**, 620–628.

de Villemereuil, P., Rutschmann, A., Lee, K.D., Ewen, J.G., Brekke, P. & Santure, A.W. (2019). Little adaptive potential in a threatened passerine bird. *Curr. Biol.* **29**, 889–894.e3. https://doi.org/10.1016/j.cub.2019.01.072.

Walzem, R.L. & Chen, S. (2014). Obesity-induced dysfunctions in female reproduction: lessons from birds and mammals. *Adv. Nutr.* **5**, 199–206.

Weiser, E.L., Grueber, C.E., Kennedy, E.S. & Jamieson, I.G. (2016). Unexpected positive and negative effects of continuing inbreeding in one of the world's most inbred wild animals. *Evolution* **70**, 154–166.

Wetton, J. & Parkin, D. (1991). An association between fertility and cuckoldry in the House Sparrow, *Passer domesticus*. *Proc. R. Soc. B. Biol. Sci.* **245**, 227–233.

White, K.L., Eason, D.K., Jamieson, I.G. & Robertson, B.C. (2015). Evidence of inbreeding depression in the critically endangered parrot, the Kakapo. *Animal Conserv.* **18**, 341–347.

Wood, P.B. & Collopy, M.W. (1993). Effects of egg removal on Bald Eagle productivity in northern Florida. *J. Wildl. Manage.* **57**, 1–9.

Yamamoto, J.T., Shields, K.M., Millam, J.R., Roudybush, T.E. & Grau, C.R. (1989). Reproductive activity of force-paired Cockatiels (*Nymphicus hollandicus*). *Auk* **106**, 86–93.

Yuta, T., Nomi, D., Ihle, M. & Koizumi, I. (2018). Simulated hatching failure predicts female plasticity in extra-pair behavior over successive broods. *Behav. Ecol.* **29**, 1264–1270.

## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1

# Appendix 2:

## Supplementary material for Chapter 2: 'Physiological factors influencing female fertility in birds'

*Assersohn, K., Brekke, P., Hemmings, N. 2021 Physiological factors influencing female fertility in birds. R. Soc. Open Sci. 8: 202274. https://doi.org/10.1098/rsos.202274*

*This supplementary material can also be found published alongside the manuscript, and is included here in it's published form.*

# Supporting material for "Physiological factors influencing female fertility in birds"

**Appendix S1:** Specific methods and results for a systematic search of the avian fertility literature, undertaken to assess whether there has been more research effort towards either males or females, and whether the research effort in avian fertility has been dominated by studies on certain taxa, and on wild/captive populations.

**Figure S1:** Prisma diagram showing the process used during the literature search and categorisation in appendix S1.

**Table S1:** Specific details of the criteria that were used to categorise papers within appendix S1.

## Appendix S1: Methods and results for the Web of Science literature analysis in "Physiological factors influencing female fertility in birds"

### Methods

Articles investigating avian fertility were collected by performing a literature search in the Web of Science database (April 20th 2020). The search included the Web of Science Core Collection for all years (1900 – 2020), and with document type limited to articles. Search terms included "bird" OR "avian" in topic, AND "fertility" OR "infertility" in topic. Initial results yielded 1097 articles, and these were imported into the reference manager software Mendeley. Duplicates and meta-analyses were removed (leaving a total of 1093 studies) and articles were then categorised as: (i) focused on male fertility only ("male"); (ii) focused on female fertility only ("female"); (iii) focused on both male and female fertility ("both"); (iv) unrelated to avian fertility ("unrelated") (Figure 1). Criteria used to determine article category is given in Table S1. It is relevant to note that often an article's title did not reflect the methodology used, and further examination of the methods was often required to deduct whether the treatment was experienced by both sexes, and whether the physiological processes being investigated can be influenced by the other sex (for example if only egg production was investigated this would be categorised as a "female" paper).

To ensure that categorisations were reproducible, 10% of articles were chosen at random and, for this subset, category assigning was repeated by one of the co-authors, who was given the

categorisation criteria but did not know how their sample of papers had been originally categorised. The two authors consistently categorised 80% of the subsampled papers. The majority of disagreements occurred for papers that had been categorised as either "both" or "unrelated", suggesting that it was straightforward to distinguish between "male only" and "female only" categories, and to distinguish these from the "both" category. After a thorough examination of the categorisation disagreements, the criteria were amended to improve reproducibility for "both" and "unrelated" categories. The total list of articles was subsequently reviewed again for any discrepancies in light of the finalised criteria.

Statistical analysis was conducted in R (R Core Team, 2018). To assess the evidence that research effort in avian fertility is dominated by studies in males relative to females, we used a generalised linear model (with a Poisson distribution and logit link function, and p-values obtained from a likelihood ratio test), to investigate whether the number of avian fertility papers over time (1985-2020) is dependent on the study focal sex (i.e., category 'male', category 'female' or category 'both'). Only years between 1985 and 2020 were included in this analysis because prior to 1985 there were very few papers produced which introduced a large number of zeros into the data. To assess the evidence that the avian fertility research effort is dominated by studies in certain taxa, we then categorised all the papers (across all years) by the focal taxon and whether the study focused on captive or wild populations.

**Results**

After removing unrelated papers, the total number of papers published on avian fertility between September 1921 (the first published article appearing in this literature search) and April 2020 (the date of the literature search) was 718. Of these, 42% investigated both males and females (n = 304), 37% investigated males only (n = 268), and 20% investigated females only (n = 146). Overall, the number of papers on avian fertility by year is significantly affected by focal sex ($x^2$ = 15, df = 2, p<0.001), and the total number of papers published on avian fertility in males was 1.84 times that for females by April 2020 (Figure 1A in main text). Of all the articles (between 1921 and 2020), 79% investigated only captive populations only, of which 80% focused on Galliformes, and 54% looked exclusively at the domestic chicken (*Gallus gallus domesticus*) (Figure 1B in main text).

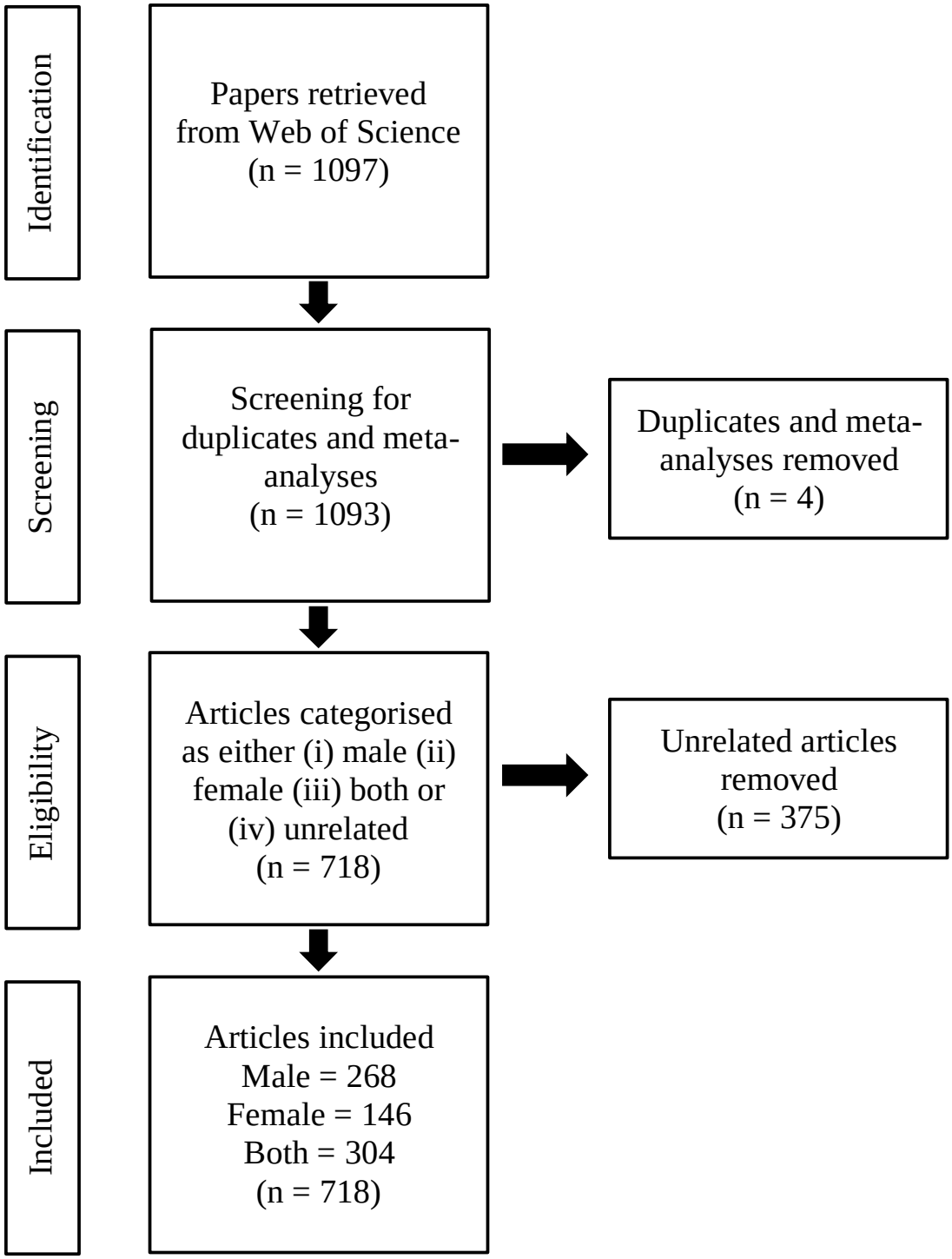


**Figure S1:** PRISMA diagram showing the literature search and categorisation process.

# Appendix 2

**Table S1:** Criteria used to categorise papers as studying: (i) "male" fertility only; (ii) "female" fertility only; (iii) "both" male and female fertility; or (iv) "unrelated" to male or female fertility in birds.

| Article category | Criteria for categorisation |
|---|---|
| (i) Male only | • Measured effects of a given treatment or experimental manipulation in males only i.e. study design prevented female effects from influencing the outcome of the treatment, or if female effects were controlled for.<br>• Investigated male fertility traits only e.g. male reproductive physiology, sperm traits, male mating behaviour.<br>• Assessed an intervention that influences an aspect of male fertility only. For example, sperm cryopreservation. |
| (ii) Female only | • Measured effects of a given treatment or experimental manipulation in females only i.e. study design prevented male effects from influencing the outcome of the treatment, or if male effects were controlled for.<br>• Investigated female fertility traits only e.g. female reproductive physiology, egg production, female mating behaviour.<br>• Assessed an intervention that influences an aspect of female fertility only. For example, egg cryopreservation. |
| (iii) Both | • Investigated avian fertility traits in both males and females e.g. female/male reproductive physiology, egg/sperm traits, female/male mating behaviour.<br>• Investigated avian fertility but did not differentiate between male or female effects e.g. If while investigating the effect of a particular treatment on egg fertility (such as diet) the treatment was experienced by both sexes.<br>• Investigated interventions that influence aspects of both male and female fertility in the same paper. For example, sperm cryopreservation and egg cryopreservation. |
| (iv) Unrelated | • A number of articles extracted in the original search did not actually investigate fertility and/or birds. In some cases, the article looked at birds but was not related to fertility (including articles related to egg properties that influence embryo development but not fertility). |

# Appendix 3:

## Supplementary material for ‘A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches’

# Supplementary material

Supplementary material for '**A sex-linked supergene with large effects on sperm traits has little impact on reproductive traits in female zebra finches**' (https://doi.org/10.1098/rspb.2023.2796), including a summary of the process of model interpretation: posterior predictive model checks, and probability of direction (PD) plots. Included is also: a summary of the initial attempted glmm models using a frequentist framework, a summary of the variance decomposition analysis, and samples sizes for parental genotype combinations in each analysis.

## Contents

## S1) Egg production

S1A - F) Model investigating the effects of **female** haplotype and haplotype sharing on egg production.

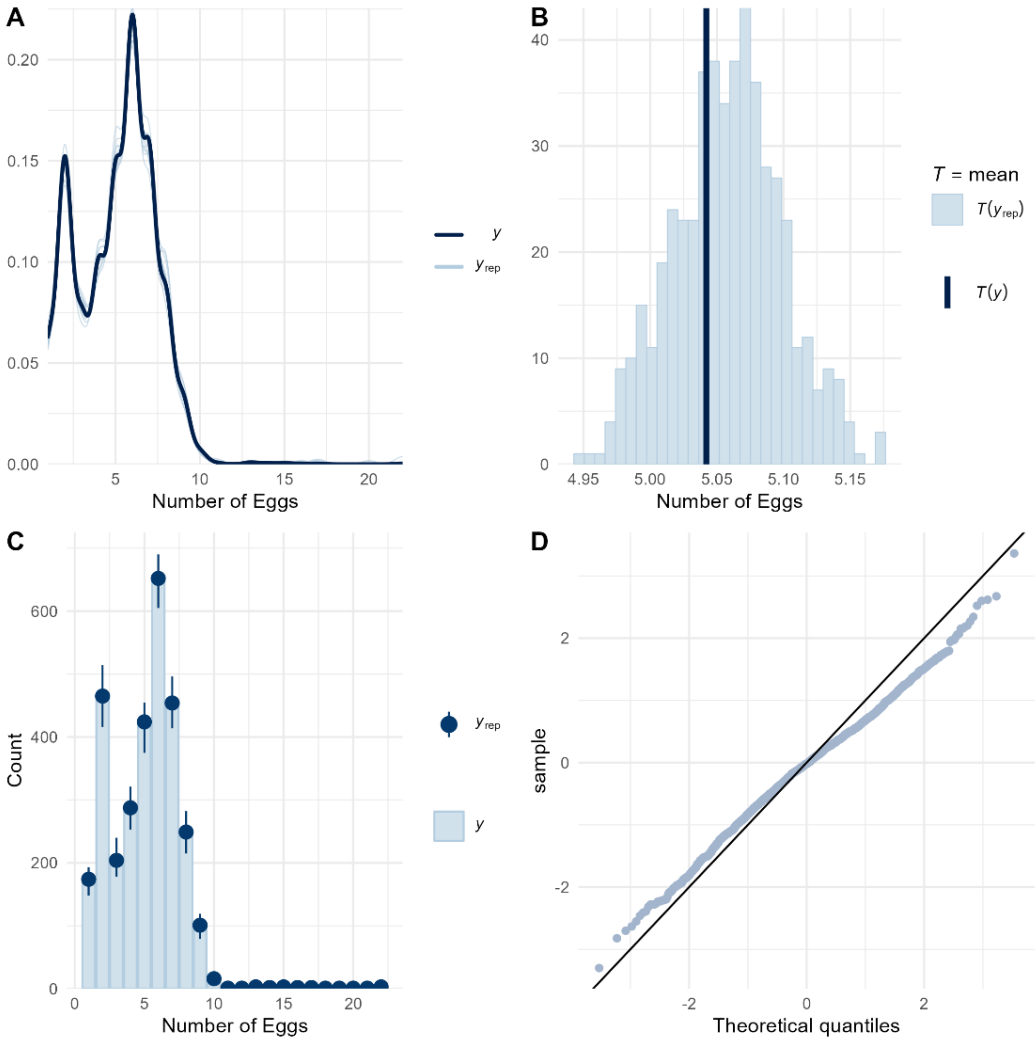


**Figure S1A:** Posterior predictive model checks for model investigating variation in the probability of laying different clutch sizes with respect to female inversion haplotypes and the number of parental haplotypes shared. **A (top left):** A density overlay plot, comparing the distributions of $y$ (observed data) and $y_{rep}$ (posterior predictions). **B (top right):** A check of central tendency, comparing the mean of the observed data to the distribution of posterior means. **C (bottom left):** Compares the counts (egg number) of observed data with the distribution of posterior estimated counts. **D (bottom right):** A test of residual normality, comparing the randomized quantile residuals of the posterior data with a theoretical normal distribution of residuals.

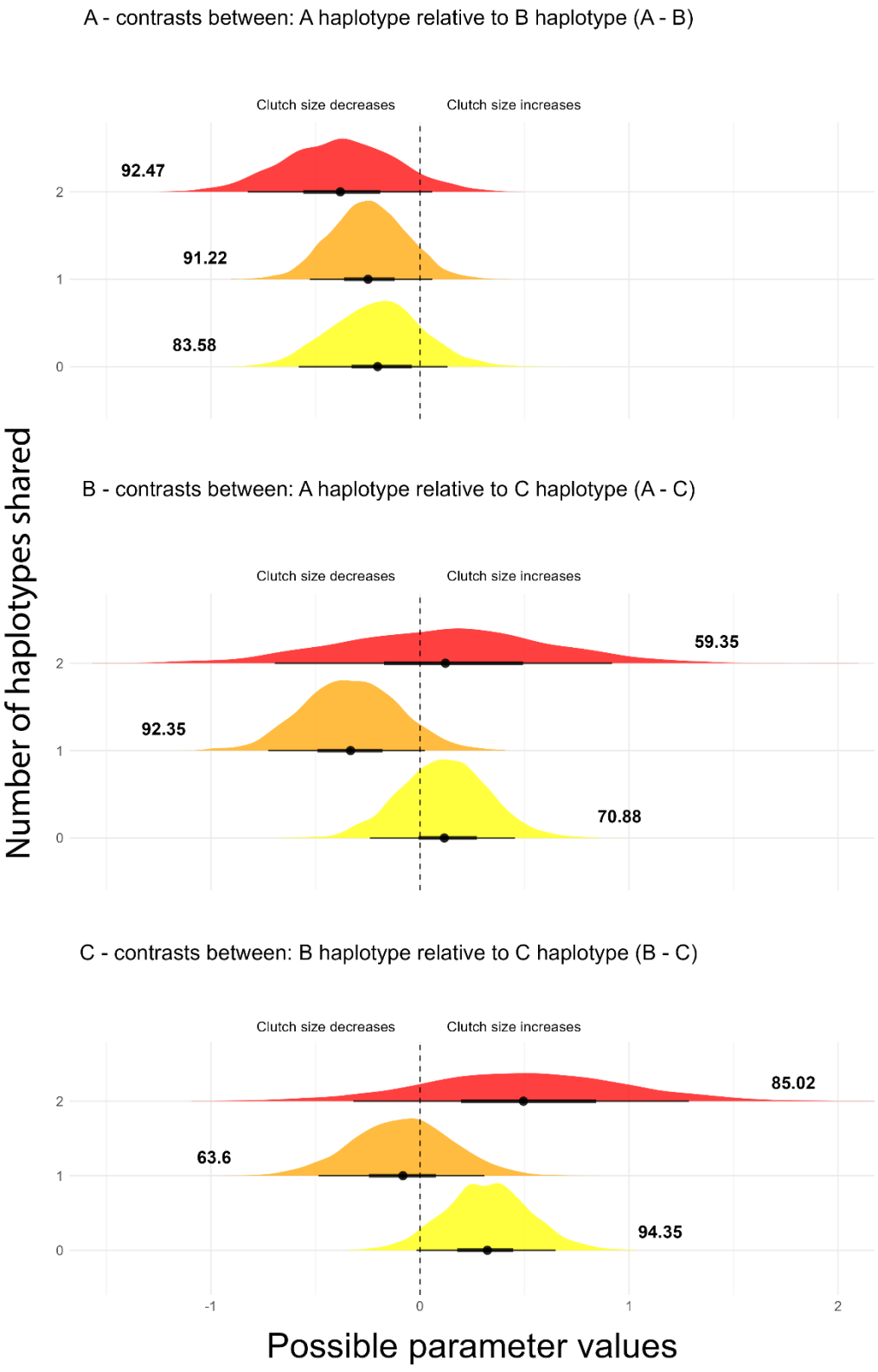


**Figure S1B:** Probability of direction (PD) plot for model investigating variation in the probability of laying different clutch sizes with respect to female inversion haplotype, where PD values are given to the left (if the median is negative) or right (if the median is positive)

of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between the A haplotype relative to the B haplotype (E.g., A0 – B0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with a decrease in clutch size relative to the B haplotype (and vice versa for positive effects). **B:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between the A haplotype relative to the C haplotype (E.g., A0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with a decrease in clutch size relative to the C haplotype (and vice versa for positive effects). **C:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between the B haplotype relative to the C haplotype (E.g., B0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the B haplotype is associated with a decrease in clutch size relative to the C haplotype (and vice versa for positive effects).

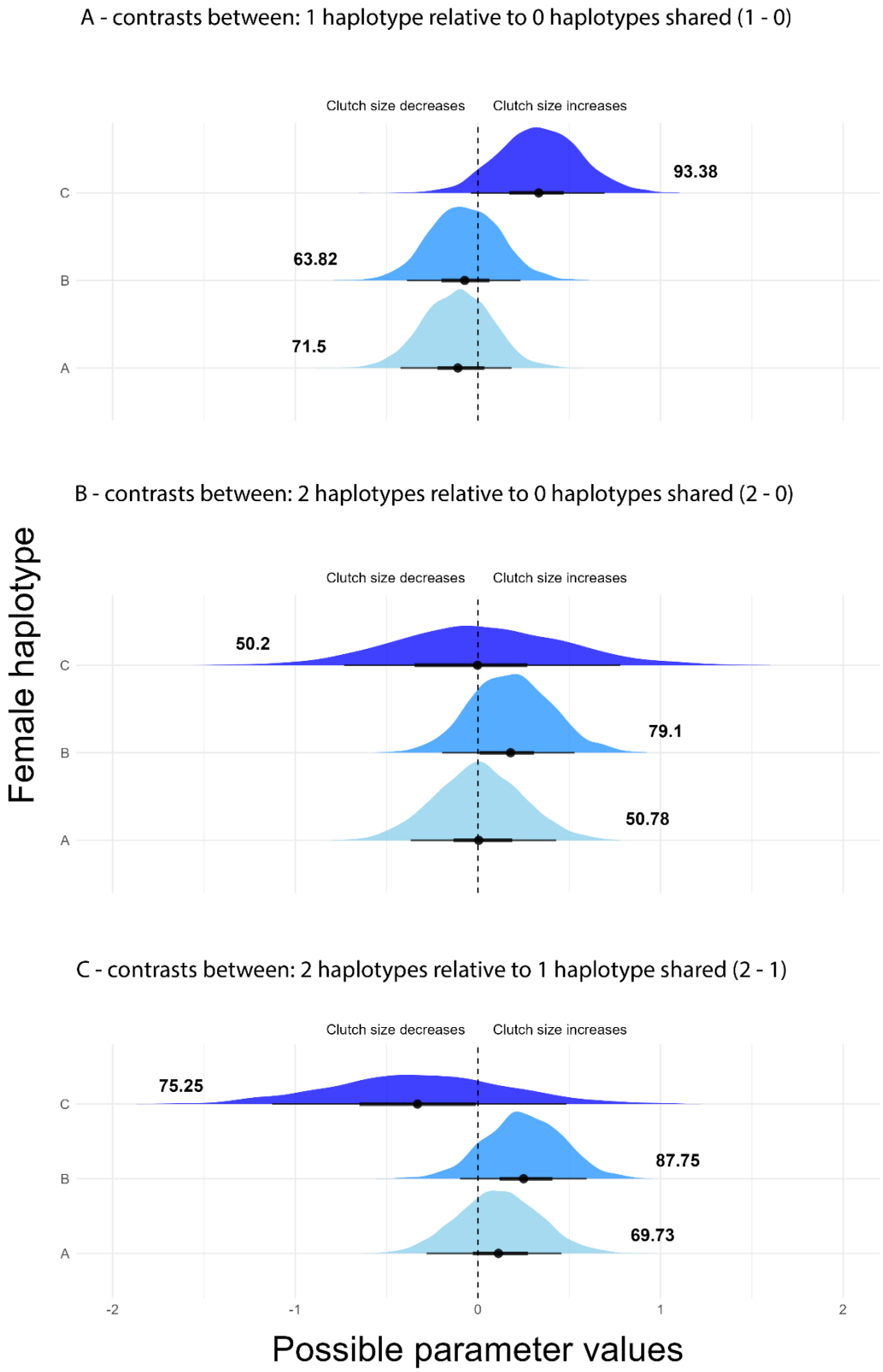


**Figure S1C:** Probability of direction (PD) plot for model investigating variation in the probability of laying different clutch sizes with respect to the number of parental haplotype sharing, where PD values are given to the left (if the median is negative) or right (if the

median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 1 haplotype shared relative to no haplotypes shared (E.g., A1 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 1 haplotype with the female is associated with a decrease in clutch size relative to sharing no haplotypes (and vice versa for positive effects). **B:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to no haplotypes shared (E.g., A2 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing both haplotypes with the female is associated with a decrease in clutch size relative to sharing no haplotypes (and vice versa for positive effects). **C:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to 1 haplotype shared (E.g., A2 – A1). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing both haplotypes with the female is associated with a decrease in clutch size relative to sharing 1 haplotype (and vice versa for positive effects).

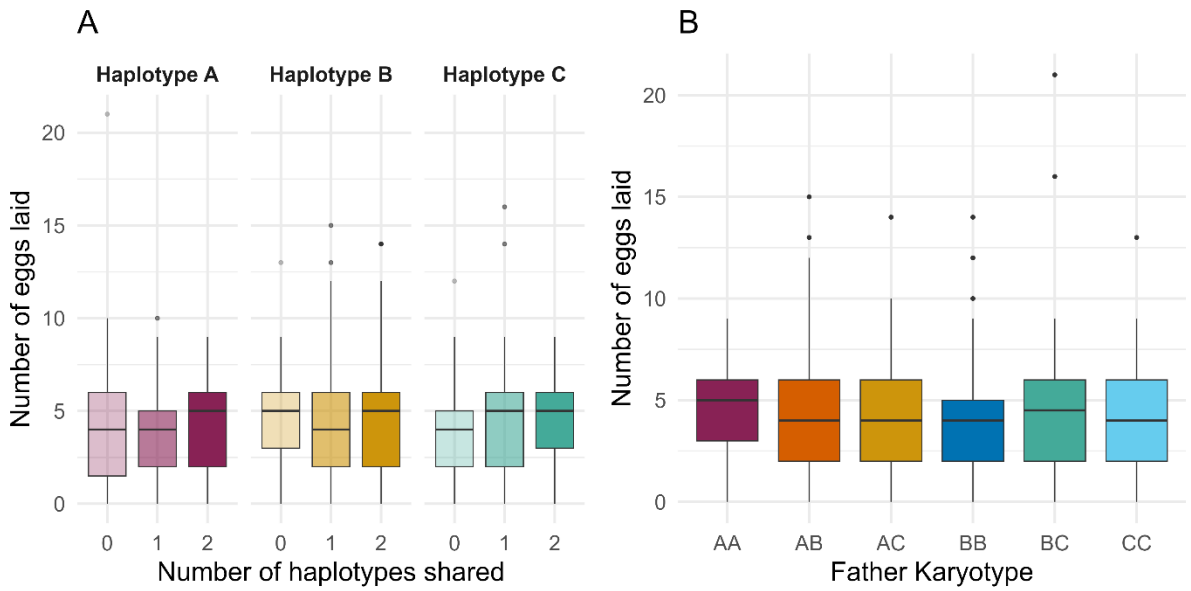


**Figure S1D: A**) The effect of mother haplotype and the number of haplotypes shared on egg production. **B)** The effect of father karyotype on egg production. Boxplots represent the interquartile range and the median of the raw data. Whiskers extend to the highest (Q3 + 1.5*IQR) and lowest values (Q1 – 1.5*IQR) and dots represent outliers.

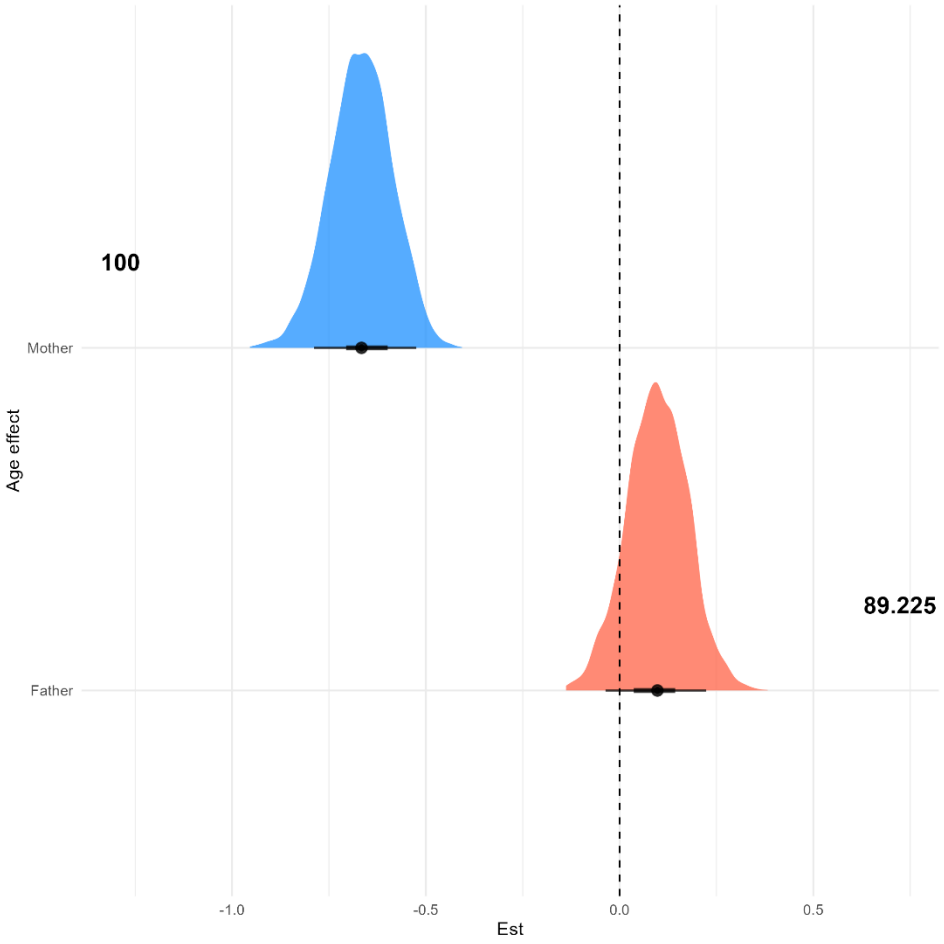


**Figure S1E:** Probability of direction (PD) plot showing the effect of male and female age on egg production, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). A negative effect would indicate that an increase in age is associated with a decrease in clutch size (and vice versa for positive effects).

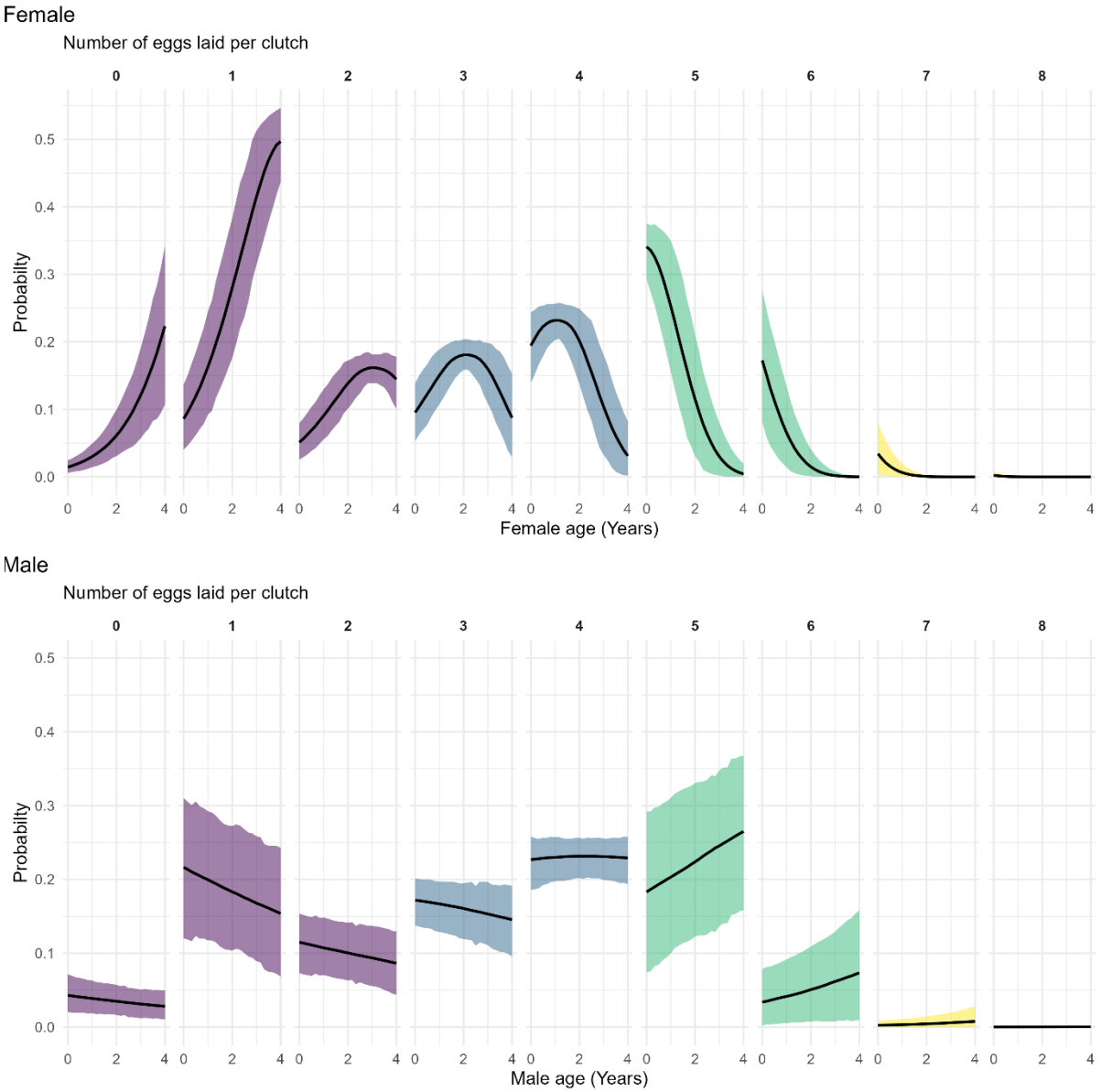


**Figure S1F:** The effect of female age (top, PD = 100) and male age (bottom, PD = 89.23) on the probability of laying a particular number of eggs per clutch. Each panel represents separate probabilities for each clutch size. Lines are the posterior medians and coloured ribbons represent the 90% highest density intervals (HDIs) for the average individual.

1G- H) Model investigating the effect of **male** karyotype on egg production.

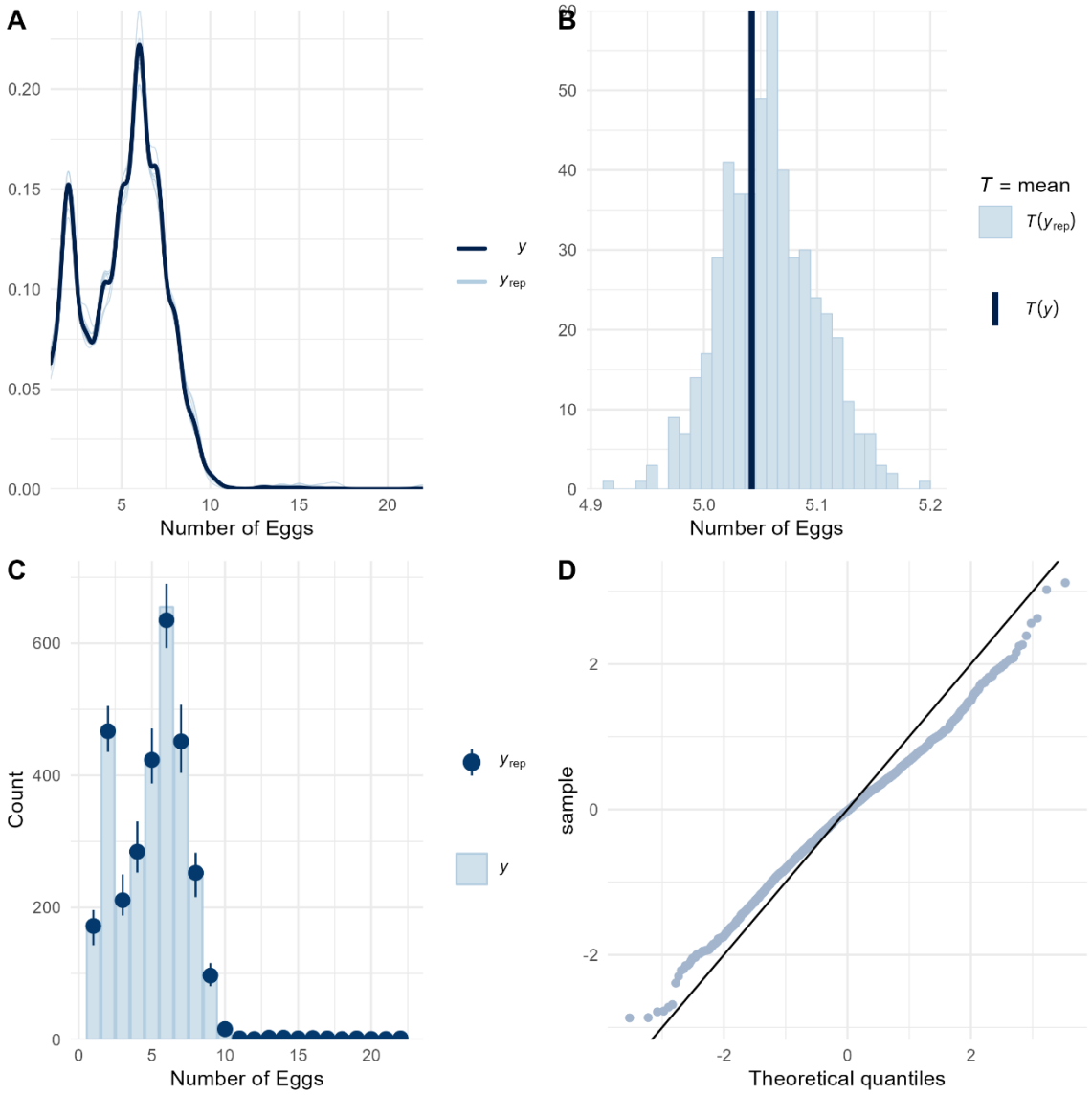


**Figure S1G:** Posterior predictive model checks for model investigating the effects of male karyotype on egg production. **A (top left):** A density overlay plot, comparing the distributions of $y$ (observed data) and $y_{rep}$ (posterior predictions). **B (top right):** A check of central tendency, comparing the mean of the observed data to the distribution of posterior means. **C (bottom left):** Compares the counts (egg number) of observed data with the distribution of posterior estimated counts. **D (bottom right):** A test of residual normality, comparing the randomized quantile residuals of the posterior data with a theoretical normal distribution of residuals.

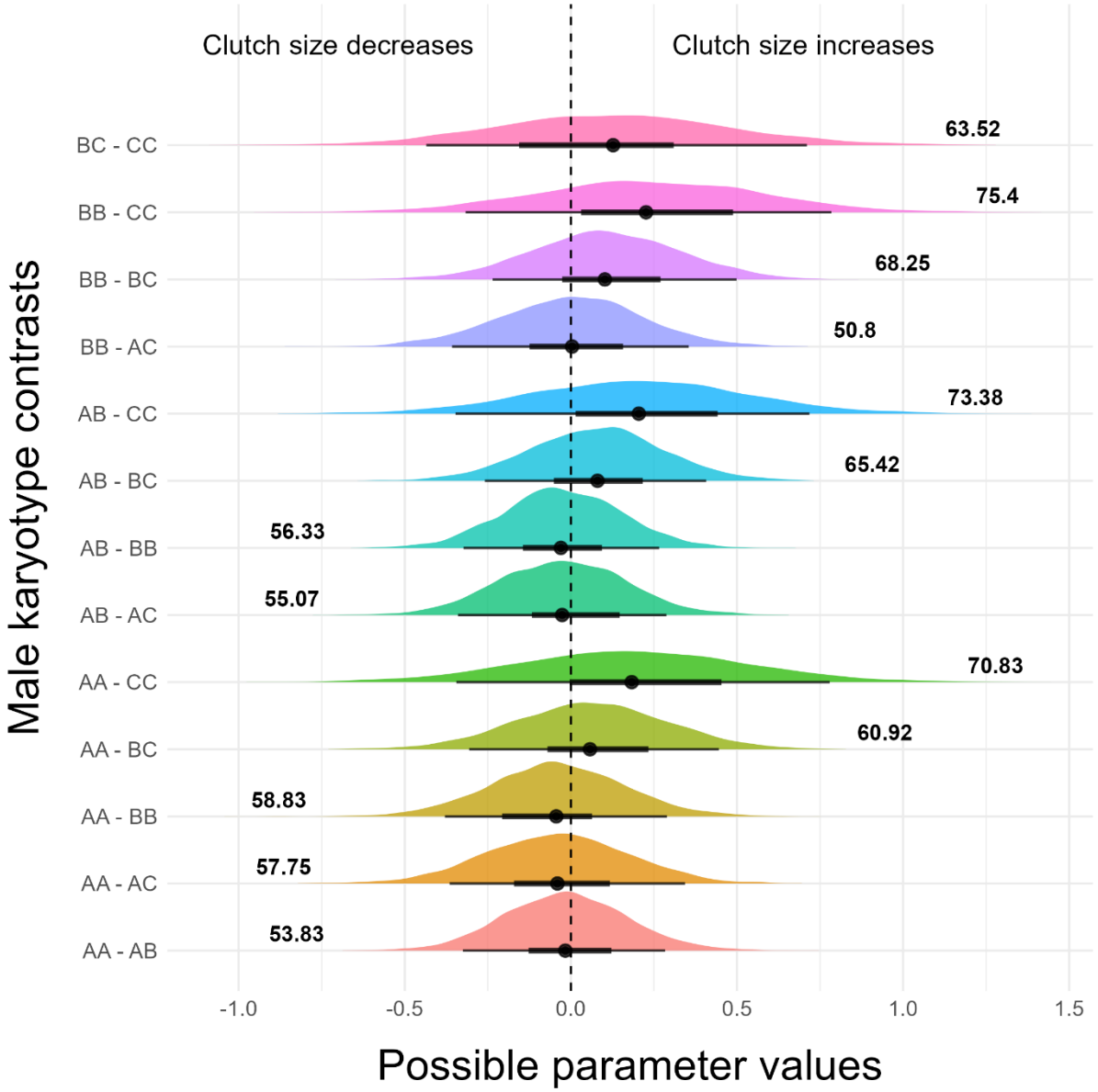


**Figure S1H:** Probability of direction (PD) plot showing the effect of male karyotype on egg production. The Y axis shows contrasts between male karyotypes, where, for example, AA – AB represents the effect of the AA karyotype relative to the AB karyotype on clutch size. A negative effect would indicate that AA karyotpes are associated with a decrease in clutch size relative to AB karyotypes (and vice versa for positive effects). PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

## S2) Fertility and early embryo development

S2A - E) Model investigating the effects of **female** haplotype and haplotype sharing on the probability of eggs developing past 3 days of incubation.

I.e., the probability that eggs were fertilised and survived early development relative to eggs that had failed before 3 days of incubation (and so failed due to either infertility or early embryo mortality).

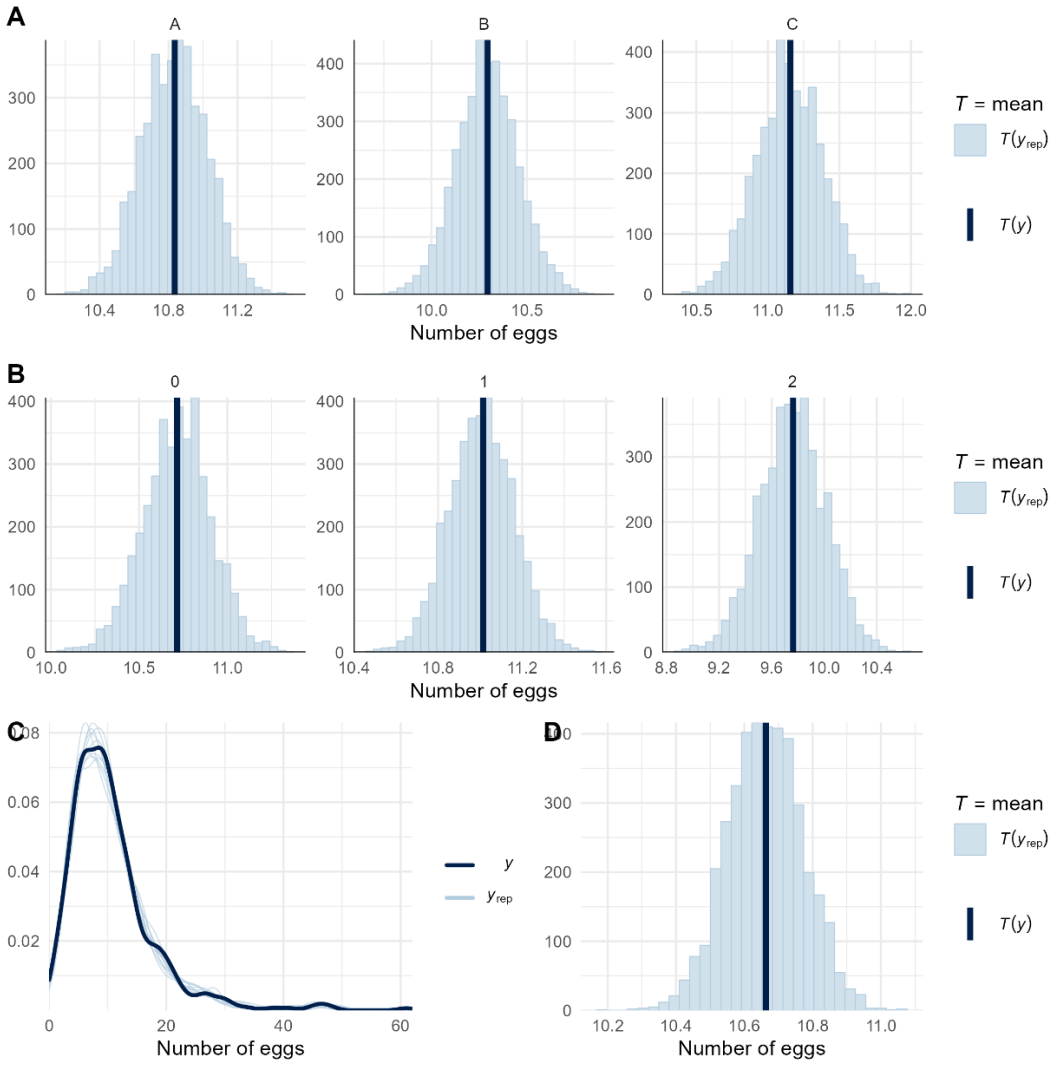


**Figure S2A:** Posterior predictive model checks for model investigating the effects of female haplotype on the probability of eggs developing past 3 days of incubation. **A (top):** A check of central tendency within groups of female haplotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (middle):** A check of central tendency within groups of haplotypes shared, comparing the mean of the observed data to

the distribution of posterior means. **C (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **D (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

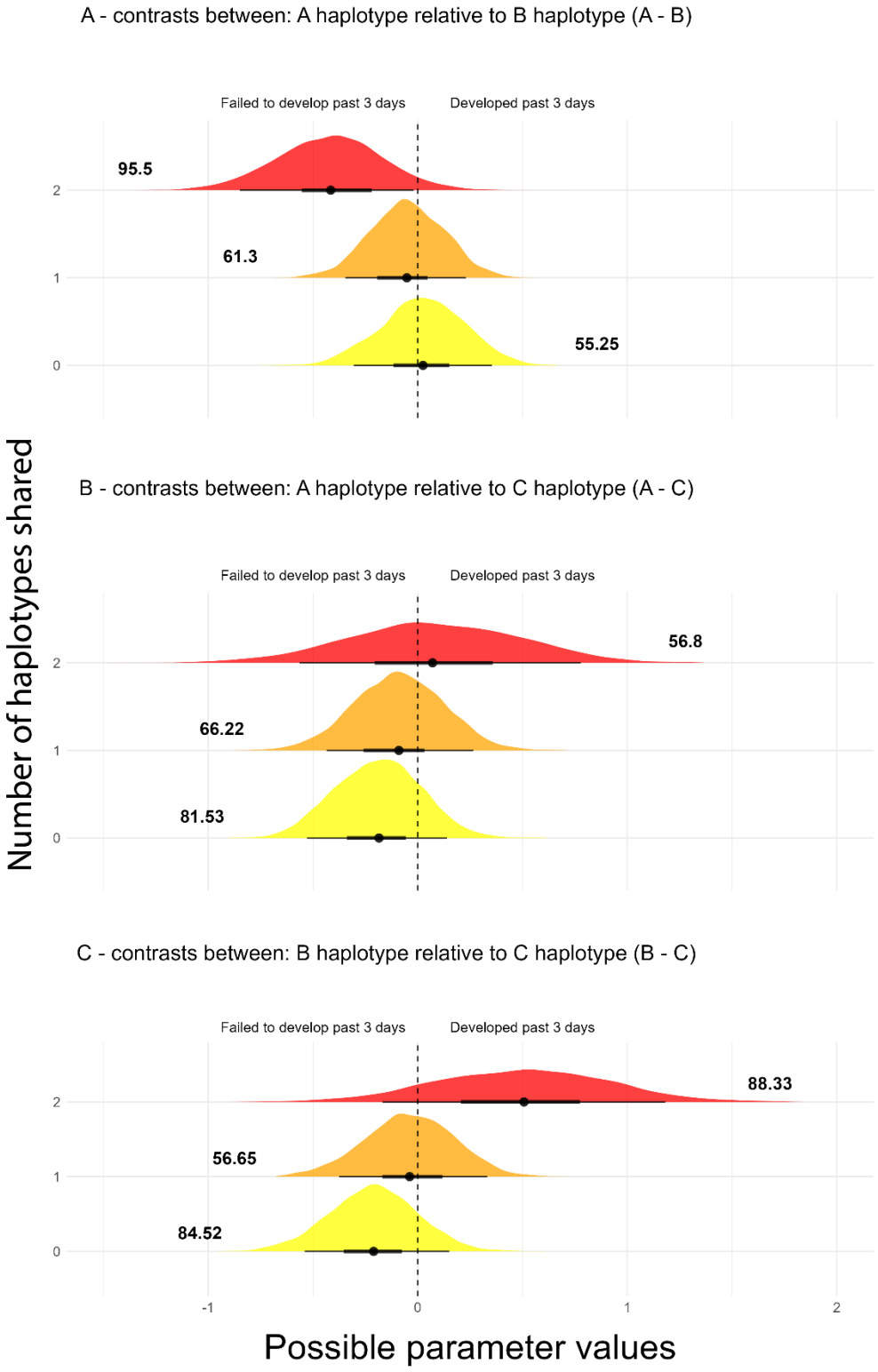


**Figure S2B:** Probability of direction (PD) plot showing the effect of female haplotype on the probability of eggs developing past 3 days of incubation, where PD values are given to the

left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts between the A haplotype relative to the B haplotype (E.g., A0 – B0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to the B haplotype (and vice versa for positive effects). **B:** Contrasts between the A haplotype relative to the C haplotype (E.g., A0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to the C haplotype (and vice versa for positive effects). **C:** Contrasts between the B haplotype relative to the C haplotype (E.g., B0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the B haplotype is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to the C haplotype (and vice versa for positive effects).

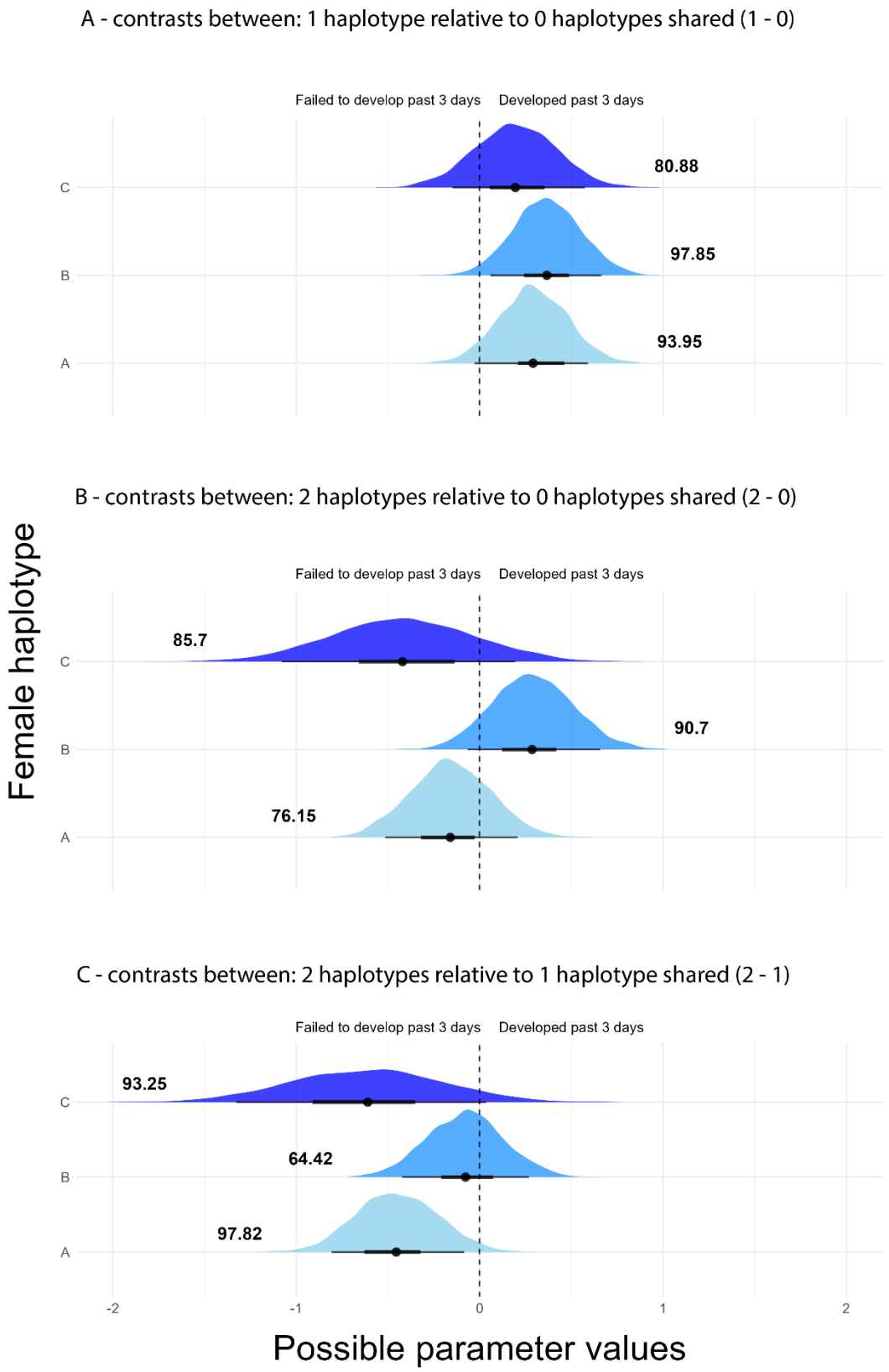


**Figure S2C:** Probability of direction (PD) plot showing the effect of haplotype sharing between males and females on the probability of eggs developing past 3 days of incubation, where PD values are given to the left (if the median is negative) or right (if the median is

positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 1 haplotype shared relative to no haplotypes shared (E.g., A1 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that that a male sharing 1 haplotype with the female is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to sharing no haplotypes (and vice versa for positive effects). **B:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to no haplotypes shared (E.g., A2 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that that a male sharing 2 haplotypes with the female is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to sharing no haplotypes (and vice versa for positive effects). **C:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to 1 haplotype shared (E.g., A2 – A1). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that that a male sharing 2 haplotypes with the female is associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to sharing 1 haplotype (and vice versa for positive effects).

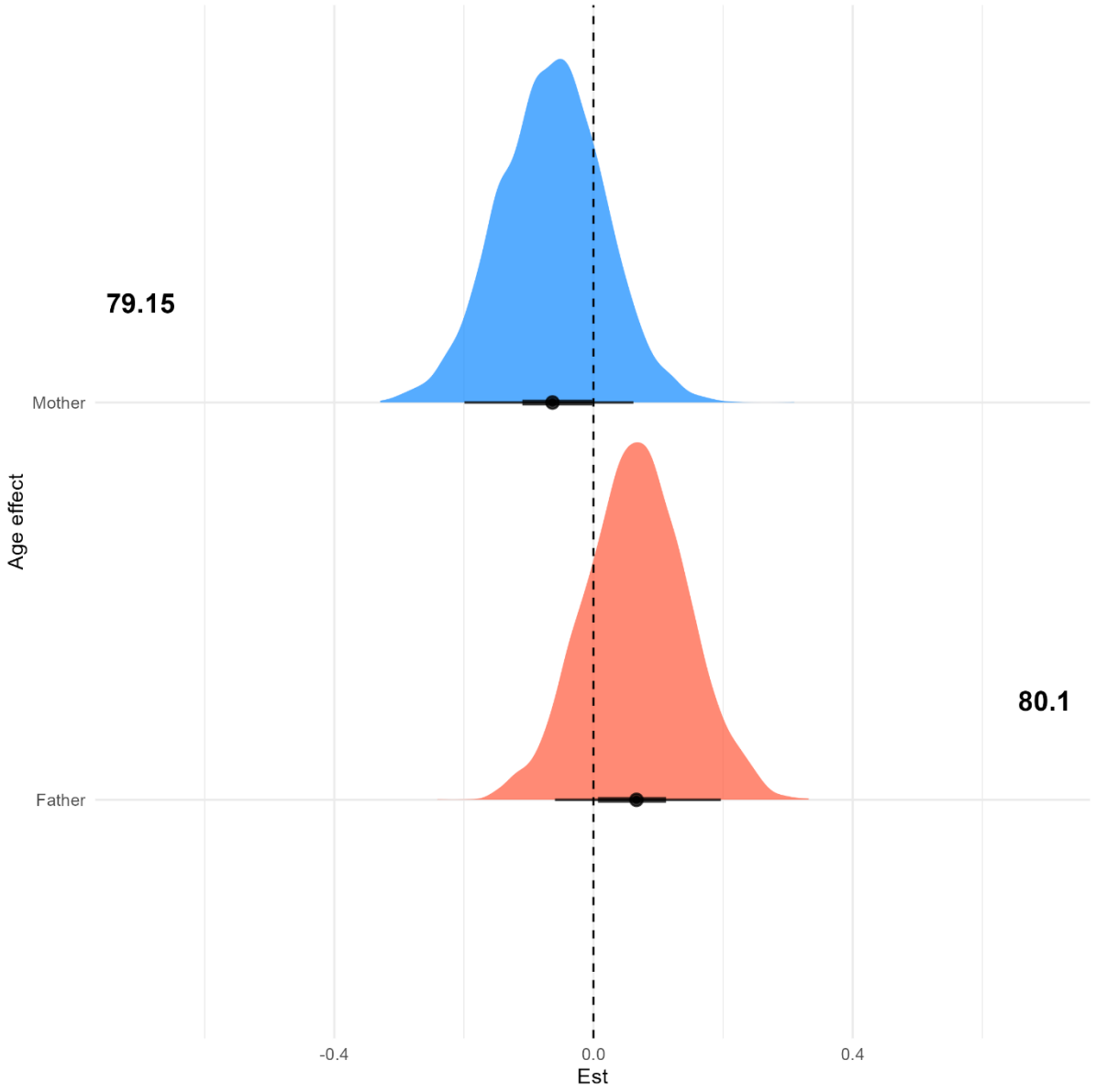


**Figure S2D:** Probability of direction (PD) plot showing the effect of male and female age on on the probability of eggs developing past 3 days of incubation, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

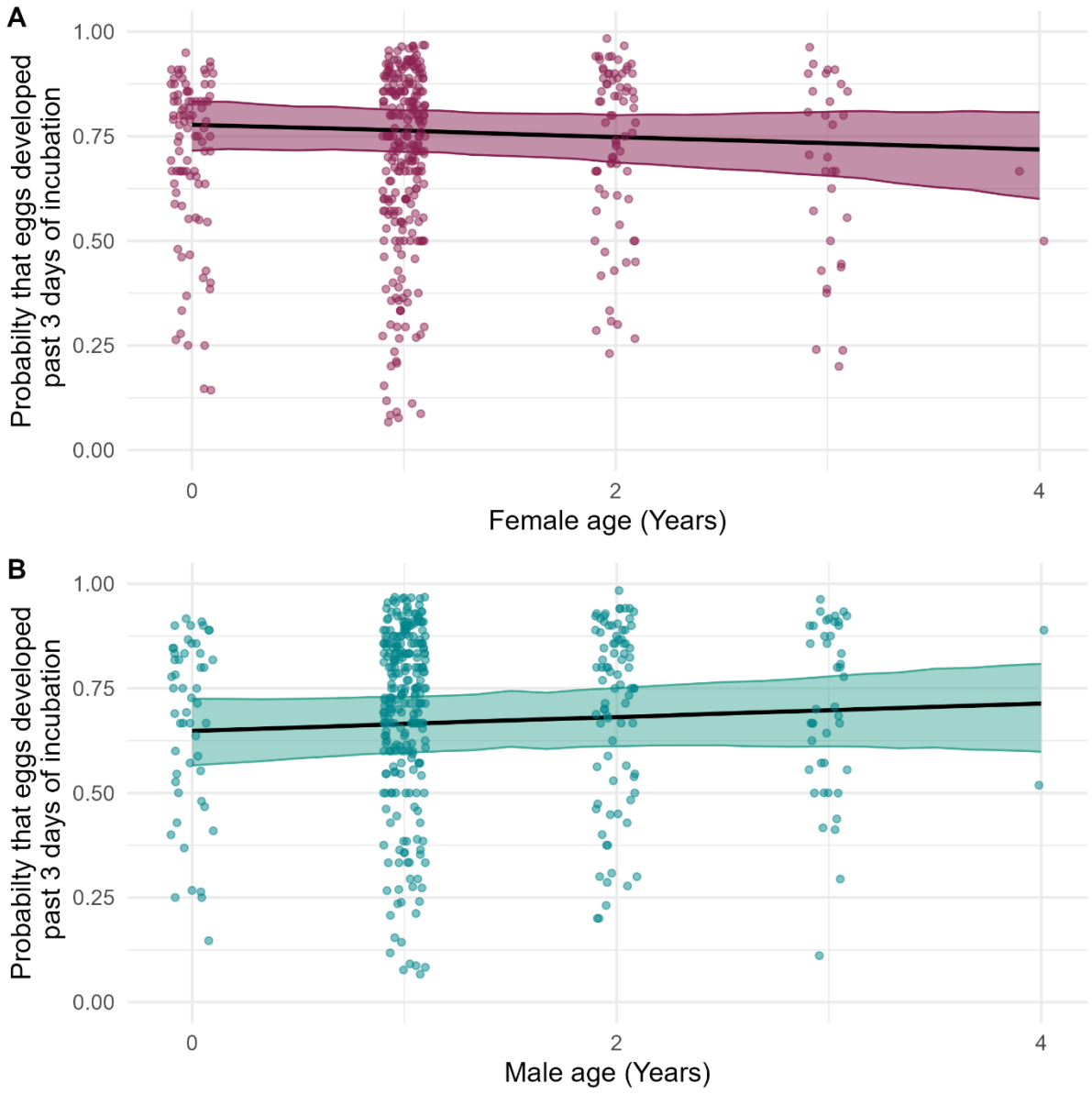


**Figure S2E:** The effect of female age (**A,** PD = 79.15) and male age (**B**, PD = 80.1) on the probability of eggs developing past 3 days of incubation. Lines are the posterior medians and coloured ribbons represent the 90% highest density intervals (HDIs) for the average individual. Points show the raw observed data and have been jittered to aid visualisation and prevent overlaying points.

S2F - G) Model investigating the effect of **male** karyotype on the probability of eggs developing past 3 days of incubation.

I.e., the probability that eggs were fertilised and survived early development relative to eggs that had failed before 3 days of incubation (and so failed due to either infertility or early embryo mortality).

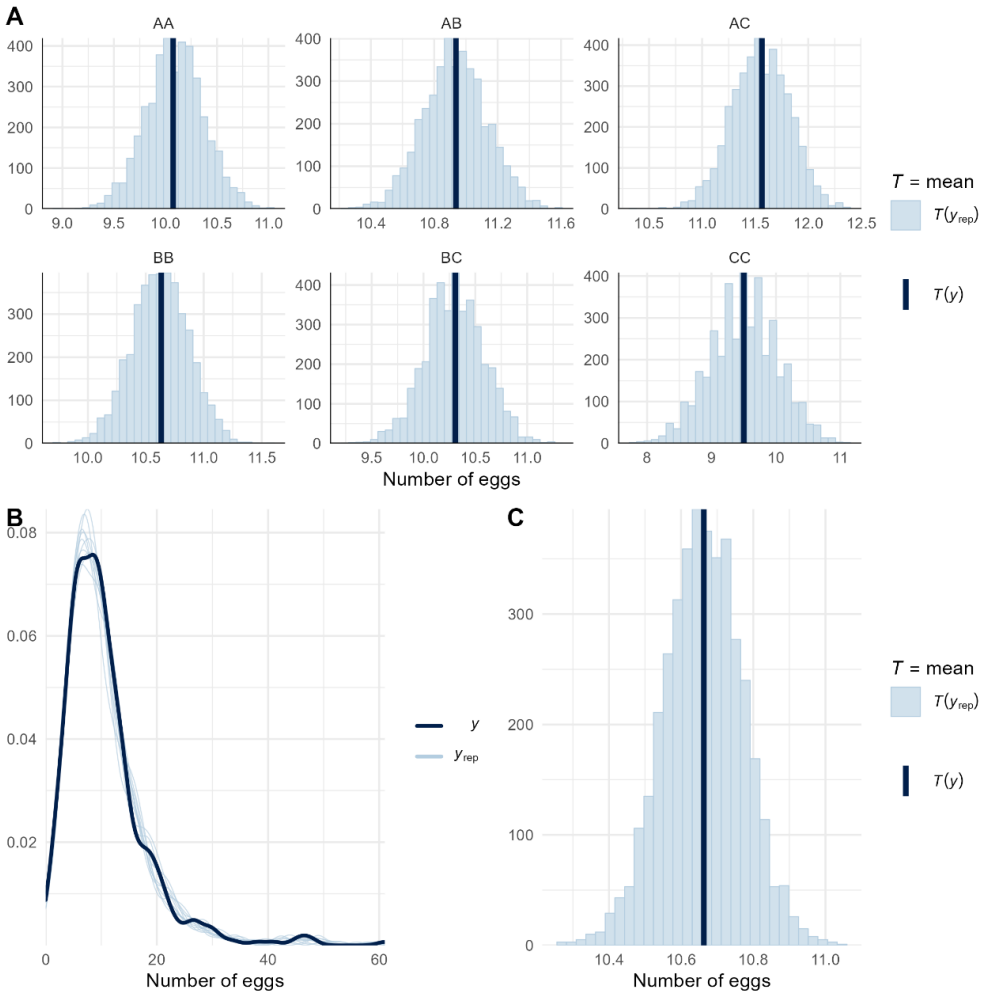


**Figure S2F:** Posterior predictive model checks for model investigating the effects of male karyotype on the probability of eggs developing past 3 days of incubation. **A (top):** A check of central tendency within groups of male karyotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **C (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

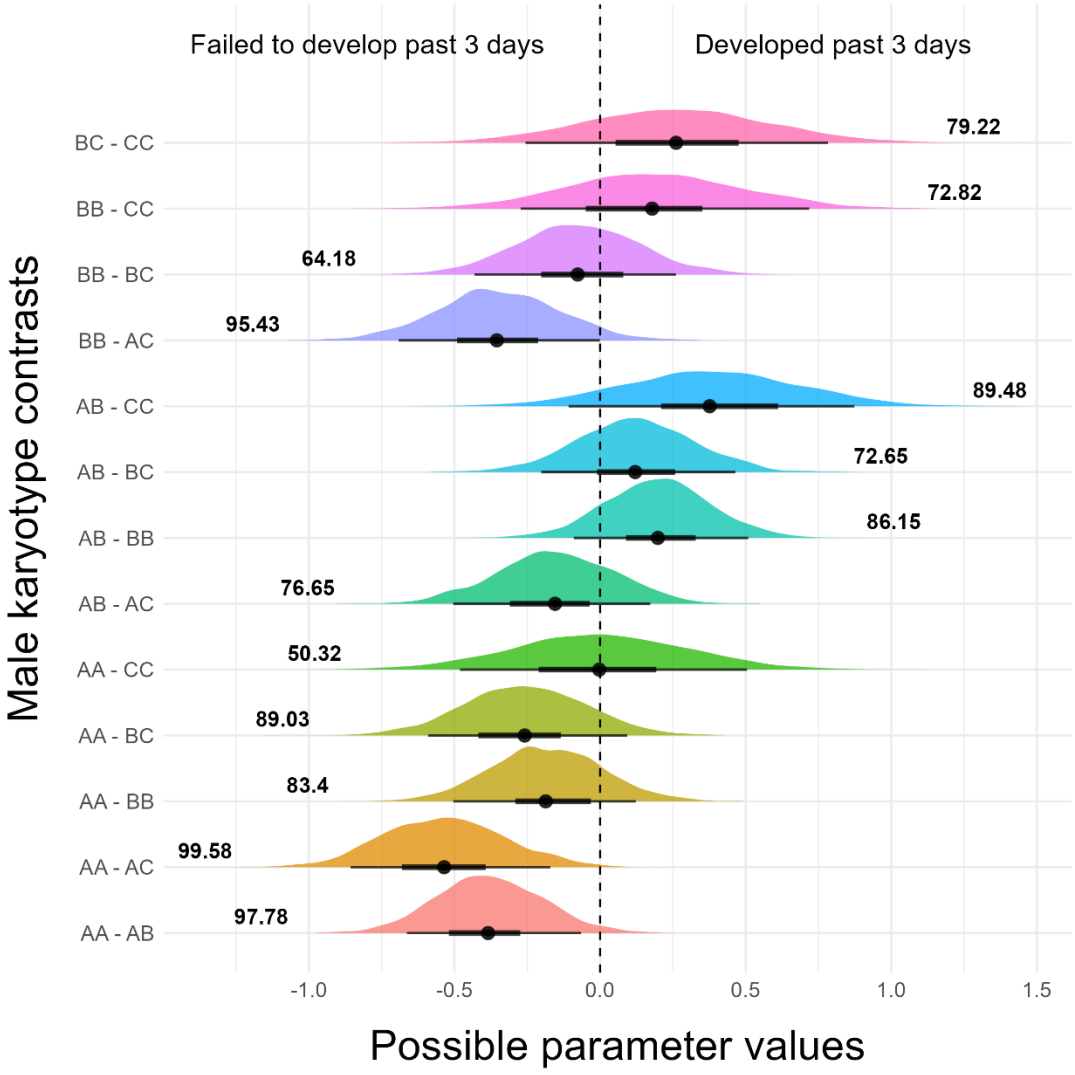


**Figure S2G:** Probability of direction (PD) plot showing the effect of male karyotype on the probability of eggs developing past 3 days of incubation. The Y axis shows contrasts between male karyotypes, where, for example, AA – AB represents the effect of the AA karyotype relative to the AB karyotype on the probability of eggs developing past 3 days of incubation. A negative effect would indicate that AA karyotpes are associated with an increase in the probability that eggs will fail to develop past 3 days of incubation (and so be infertile/suffer early embryo mortality) relative to AB karyotypes (and vice versa for positive effects). PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

## S3) Late development

S3A - E) Model investigating the effects of **female** haplotype and haplotype sharing on the probability of eggs surviving both early and late development (i.e. hatched), relative to eggs that survive early but not late development (i.e. embryo visible at 3 days of development, but failed to hatch).

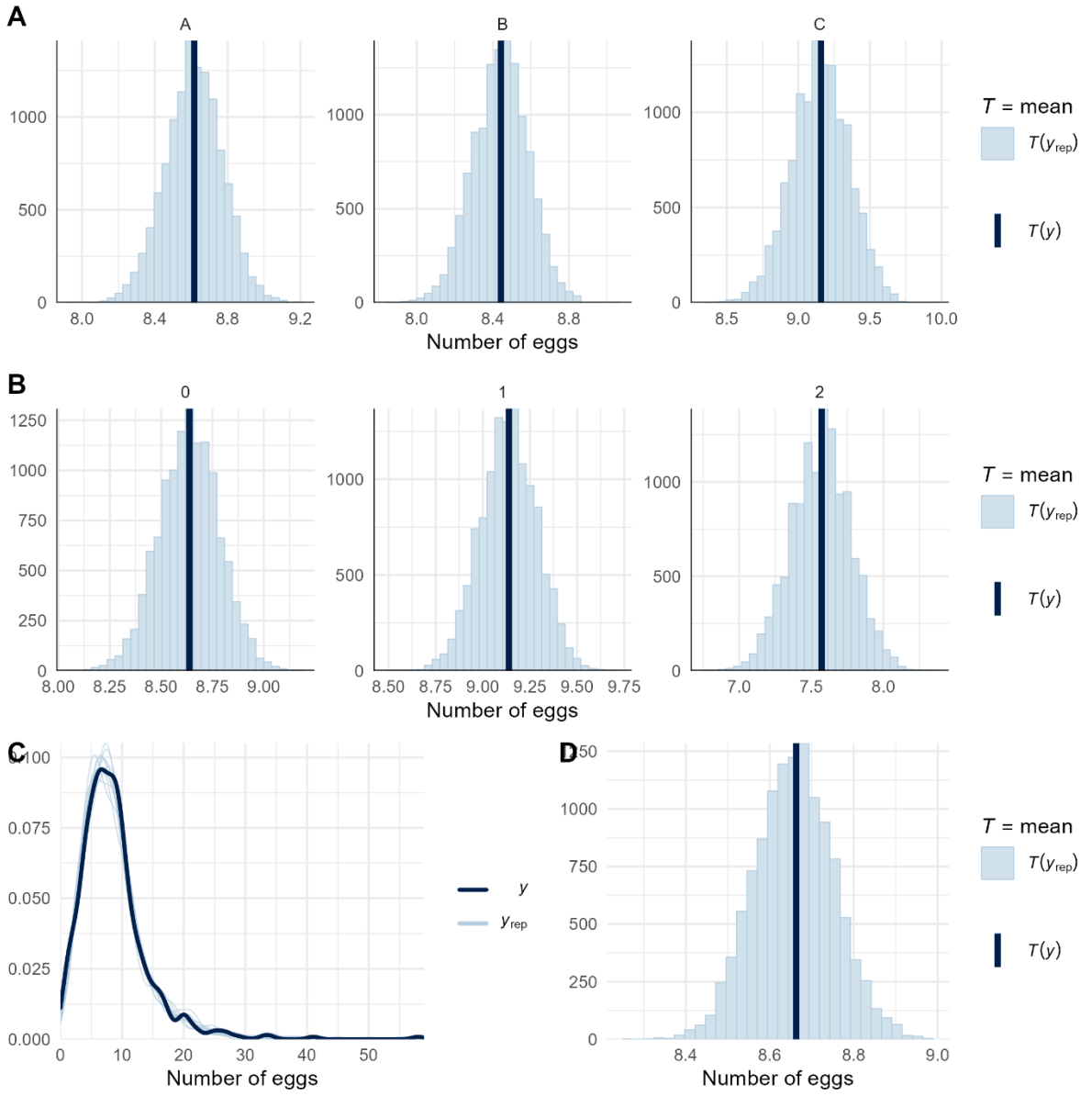


**Figure S3A:** Posterior predictive model checks for model investigating the effects of female haplotype on the probability of eggs surviving both early and late development. **A (top):** A check of central tendency within groups of female haplotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (middle):** A check of

central tendency within groups of haplotypes shared, comparing the mean of the observed data to the distribution of posterior means. **C (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **D (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

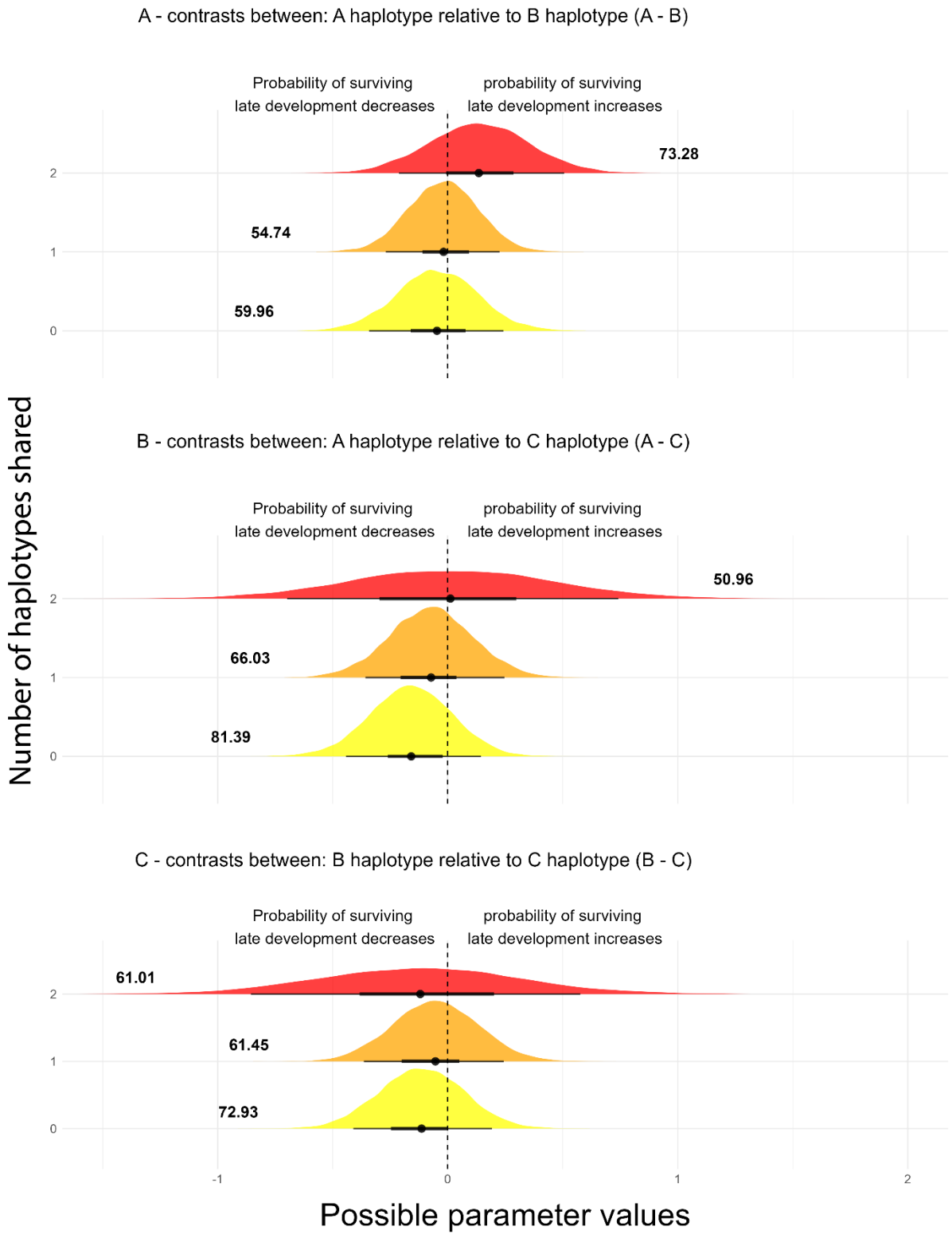


**Figure S3B:** Probability of direction (PD) plot showing the effect of female haplotype on the probability of eggs surviving both early and late development, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals

(90% HDI) (black lines). **A:** Contrasts between the A haplotype relative to the B haplotype (E.g., A0 – B0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2) Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will not survive late development relative to the B haplotype (and vice versa for positive effects). **B:** Contrasts between the A haplotype relative to the C haplotype (E.g., A0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2) Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will not survive late development relative to the C haplotype (and vice versa for positive effects). **C:** Contrasts between the B haplotype relative to the C haplotype (E.g., B0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the B haplotype is associated with an increase in the probability that eggs will not survive late development relative to the C haplotype (and vice versa for positive effects).

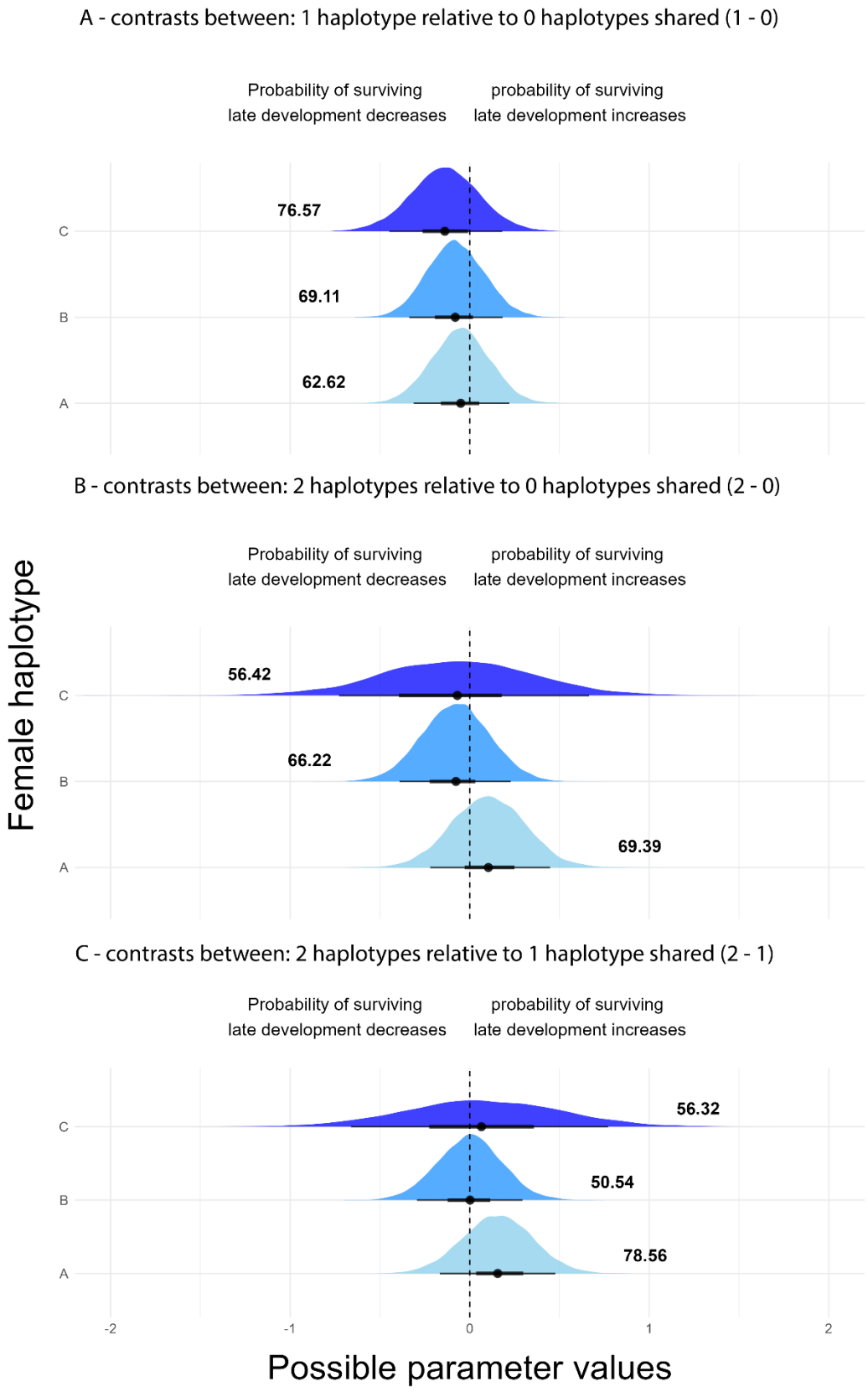


**Figure S3C:** Probability of direction (PD) plot showing the effect of haplotype sharing between males and females on the probability of eggs surviving both early and late development, where PD values are given to the left (if the median is negative) or right (if the

median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 1 haplotype shared relative to no haplotypes shared (E.g., A1 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 1 haplotype with the female is associated with an increase in the probability that eggs will not survive late development, relative to sharing no haplotypes (and vice versa for positive effects). **B:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to no haplotypes shared (E.g., A2 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 2 haplotypes with the female is associated with an increase in the probability that eggs will not survive late development, relative to sharing no haplotypes (and vice versa for positive effects). **C:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to 1 haplotype shared (E.g., A2 – A1). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 2 haplotypes with the female is associated with an increase in the probability that eggs will not survive late development, relative to sharing 1 haplotype (and vice versa for positive effects).

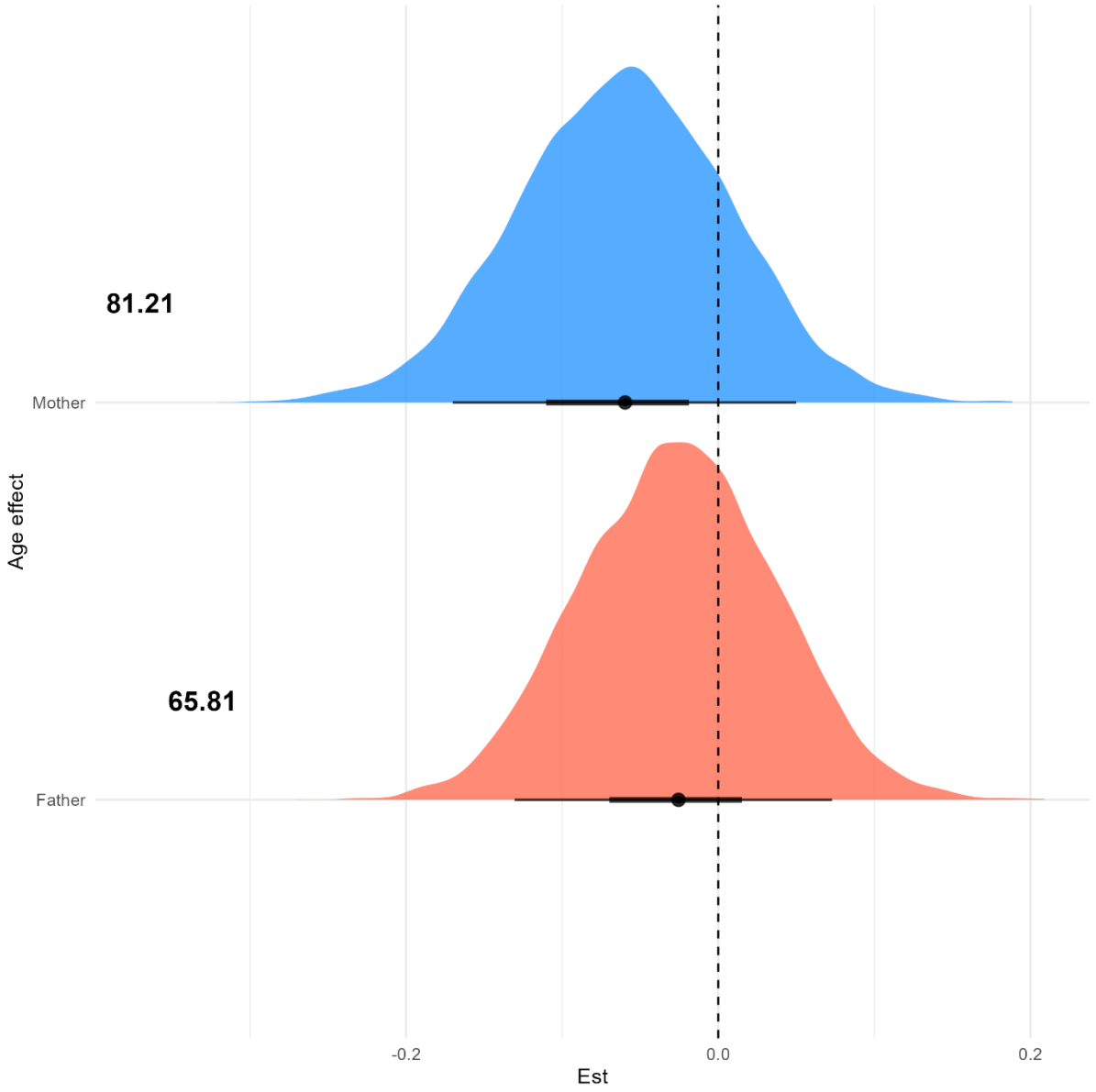


**Figure S3D:** Probability of direction (PD) plot showing the effect of male and female age on on the probability of eggs of eggs surviving both early and late development, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

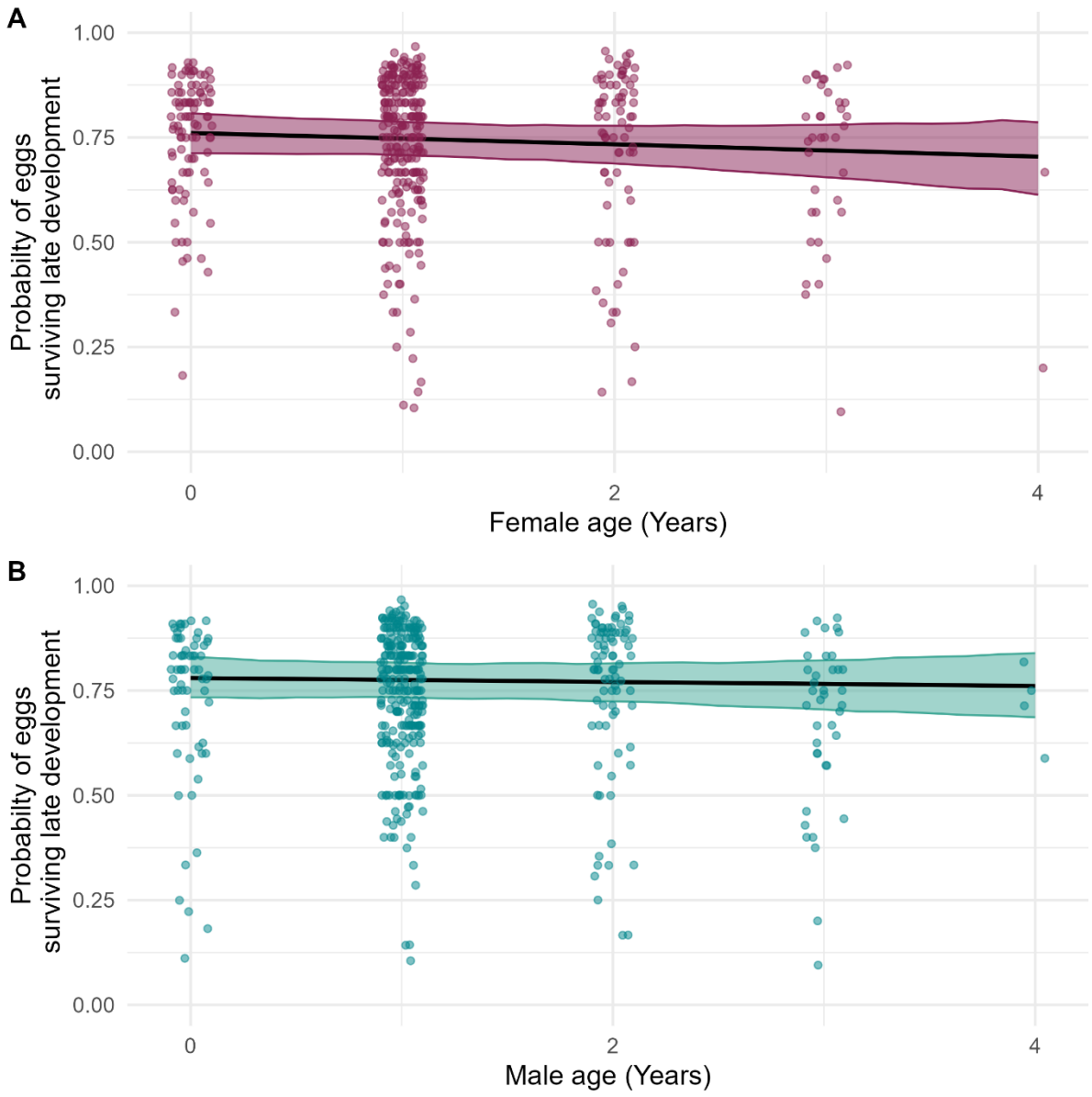


**Figure S3E:** The effect of female age (**A**, PD = 81.21) and male age (**B**, PD = 65.81) on the probability of eggs surviving late development. Lines are the posterior medians and coloured ribbons represent the 90% highest density intervals (HDIs) for the average individual. Points show the raw observed data and have been jittered to aid visualisation and prevent overlaying points.

S3F - G) Model investigating the effects of **male** haplotype and haplotype sharing on the probability of eggs surviving both early and late development (i.e. hatched), relative to eggs that survive early but not late development (i.e. embryo visible at 3 days of development, but failed to hatch).

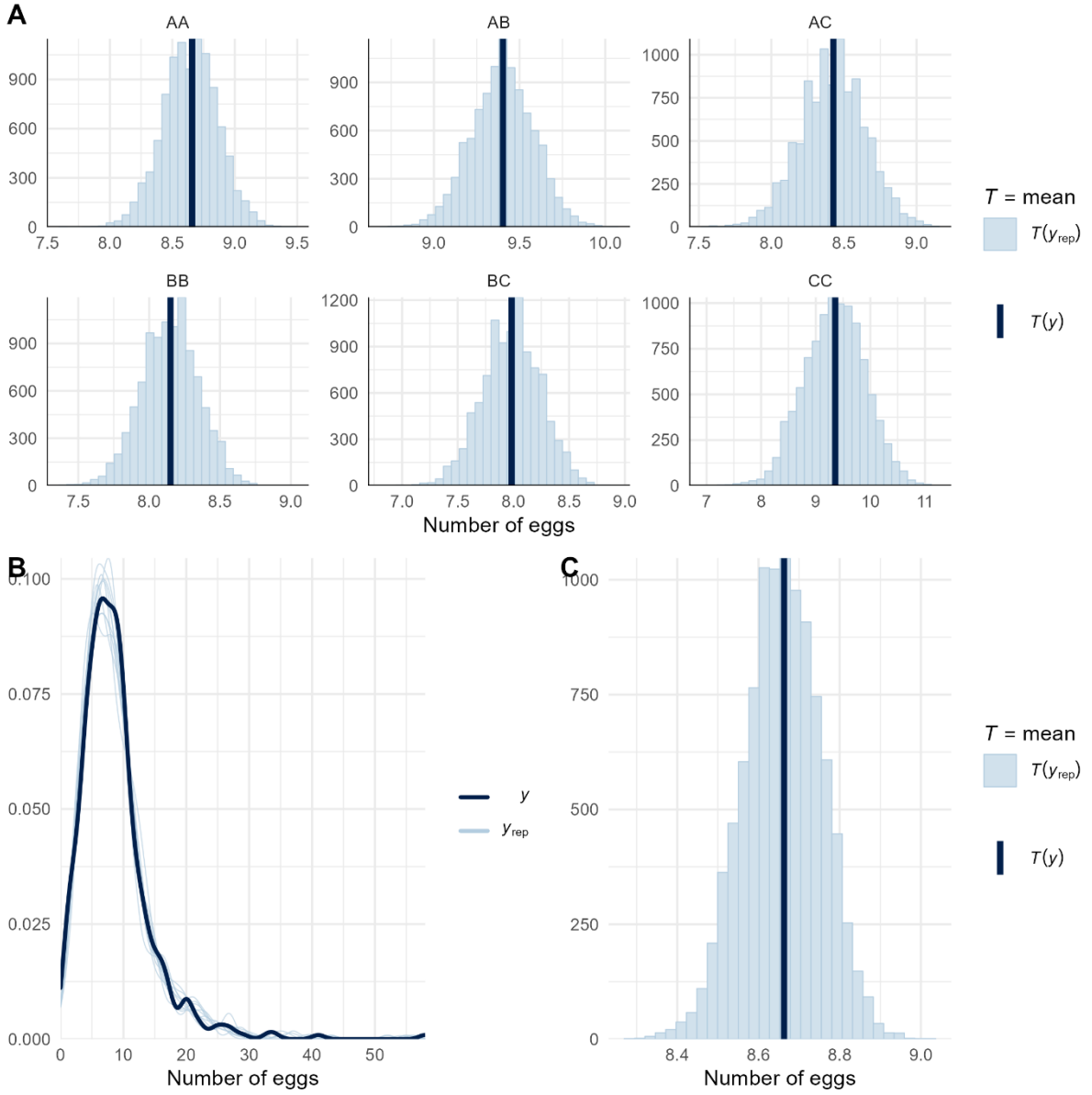


**Figure S3F:** Posterior predictive model checks for model investigating the effects of male karyotype on the probability of eggs of eggs surviving both early and late development. **A (top l):** A check of central tendency within groups of male karyotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **C (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

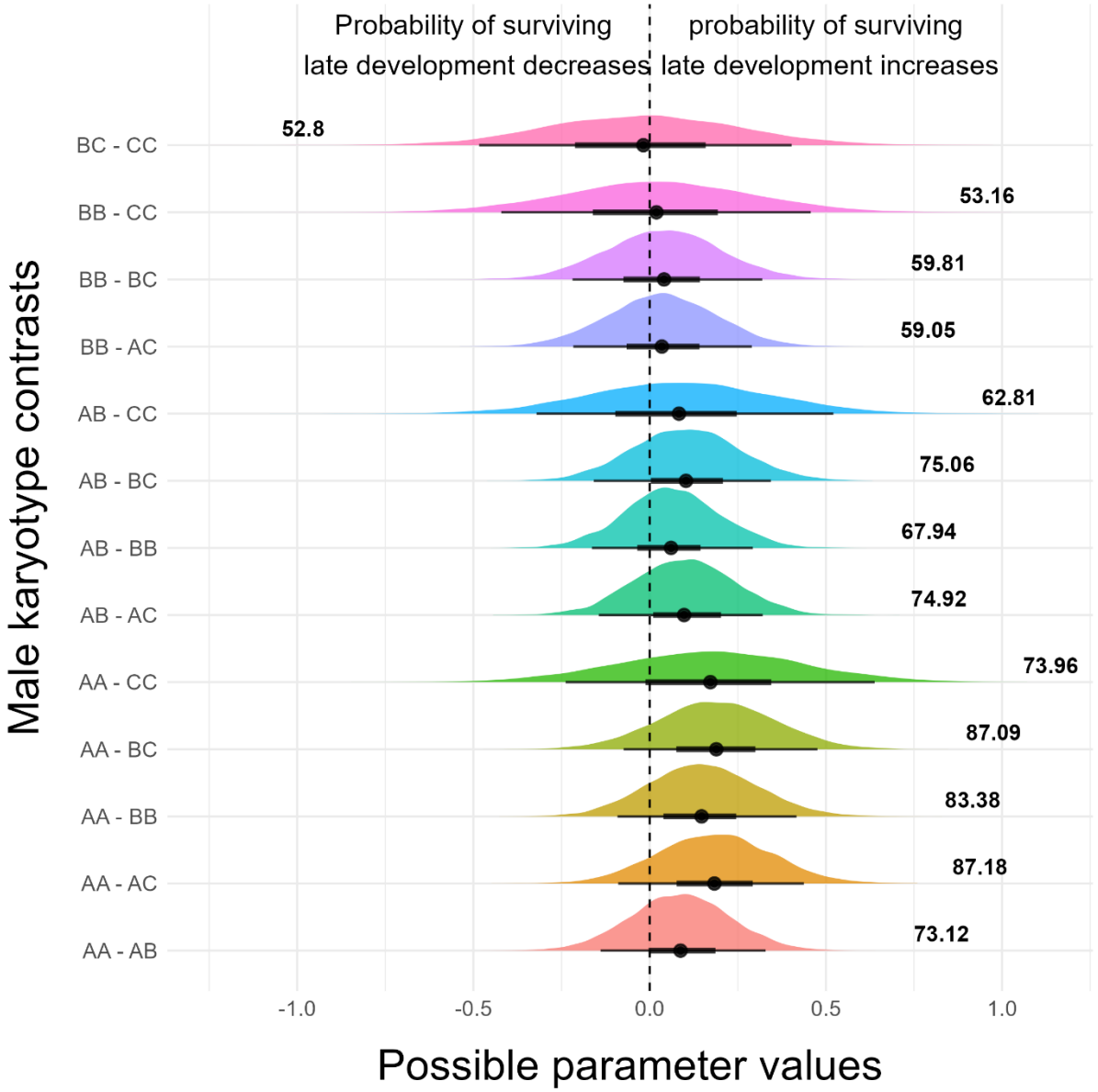


**Figure S3G:** Pobability of direction (PD) plot showing the effect of male karyotype on the probability of eggs surviving both early and late development. The Y axis shows contrasts between male karyotypes, where, for example, AA – AB represents the effect of the AA karyotype relative to the AB karyotype on the probability of eggs developing past 3 days of incubation. A negative effect would indicate that AA karyotpes are associated with an increase in the probability that eggs will not survive late development relative to AB karyotypes (and vice versa for positive effects). PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

## S4) Offspring sex ratio

### S4A - E) Model investigating the effects of **female** haplotype and haplotype sharing on offspring sex ratio

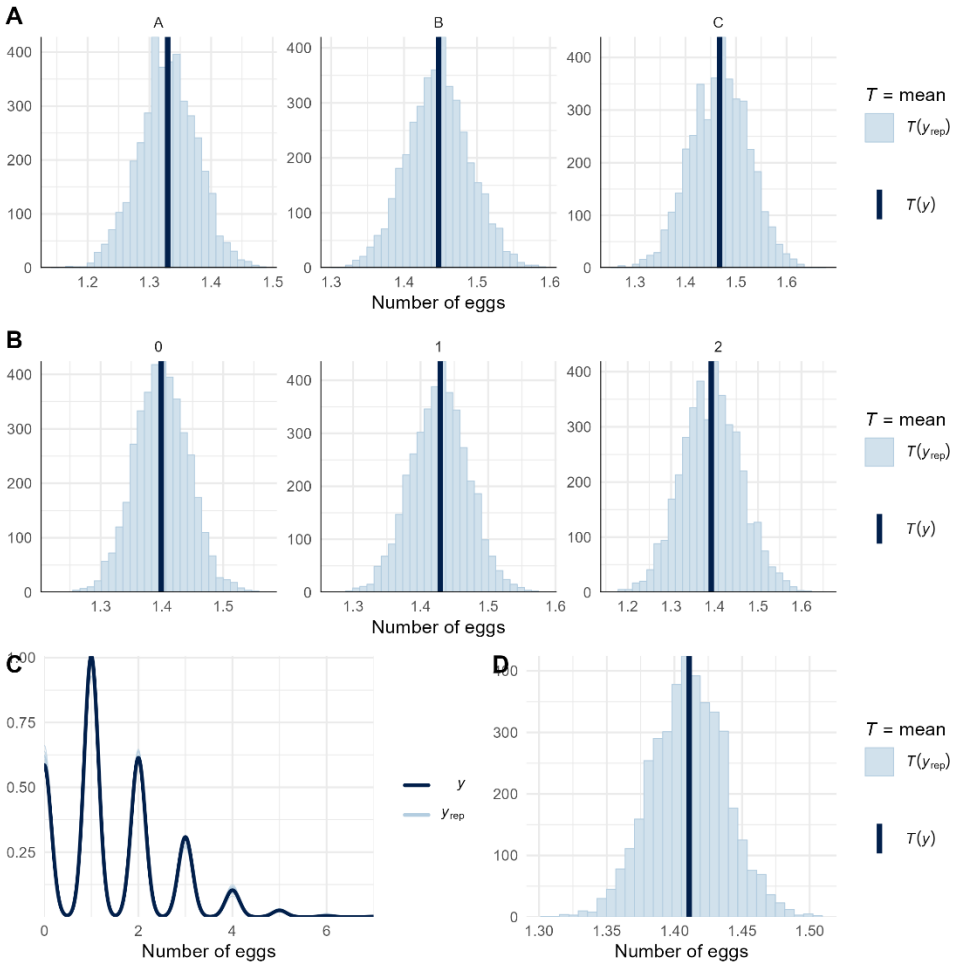


**Figure S4A:** Posterior predictive model checks for model investigating the effects of female haplotype on offspring sex ratio. **A (top):** A check of central tendency within groups of female haplotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (middle):** A check of central tendency within groups of haplotypes shared, comparing the mean of the observed data to the distribution of posterior means. **C (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **D (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

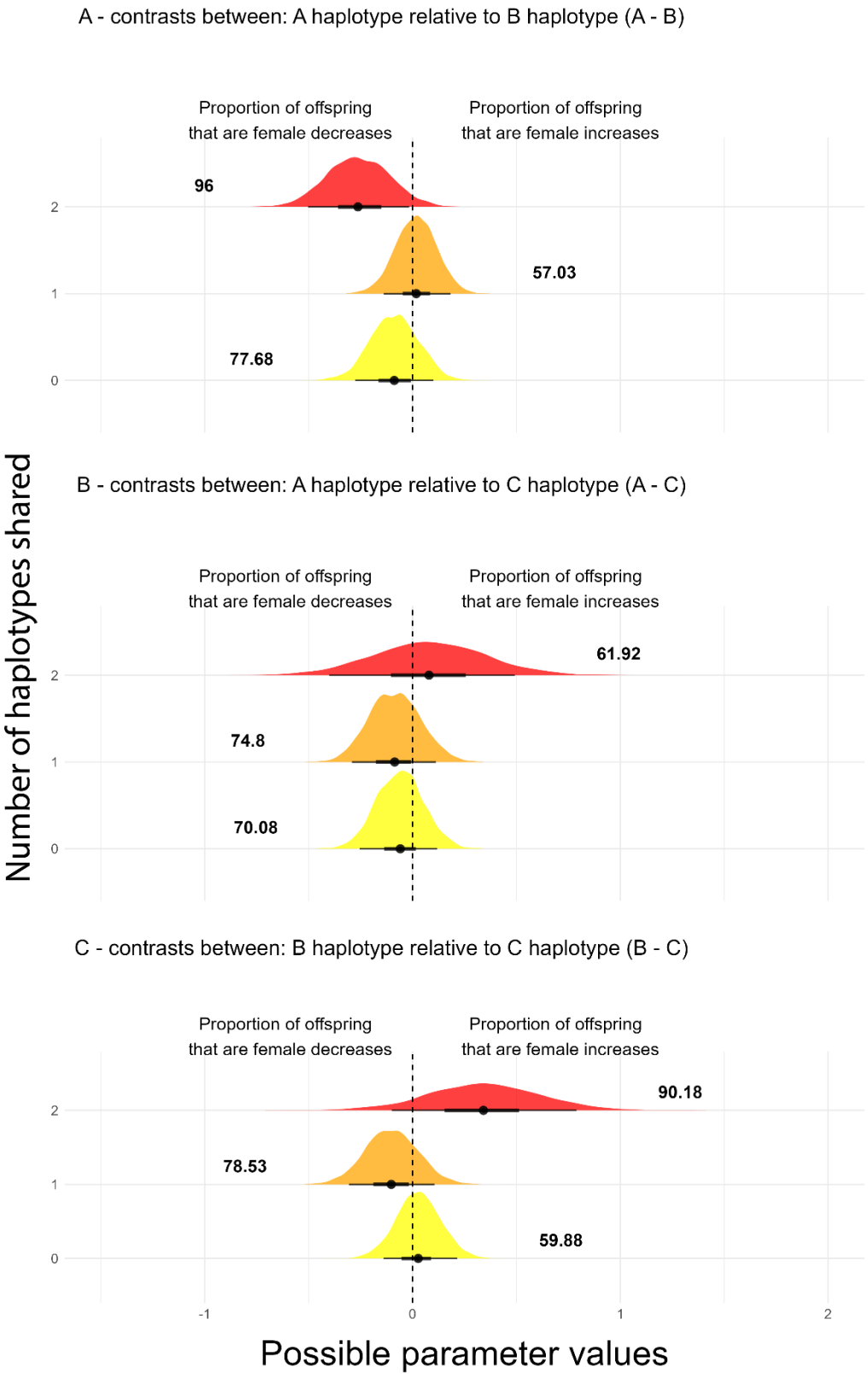


**Figure S4B:** Probability of direction (PD) plot showing the effect of female haplotype on the probability of offspring being female (relative to male), where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution.

Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts between the A haplotype relative to the B haplotype (E.g., A0 – B0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will be male relative to the B haplotype (and vice versa for positive effects). **B:** Contrasts between the A haplotype relative to the C haplotype (E.g., A0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the A haplotype is associated with an increase in the probability that eggs will be male relative to the C haplotype (and vice versa for positive effects). **C:** Contrasts between the B haplotype relative to the C haplotype (E.g., B0 – C0). Separate comparisons are made for each number of haplotypes shared (0, 1 and 2). Negative effects would indicate that the B haplotype is associated with an increase in the probability that eggs will be male relative to the C haplotype (and vice versa for positive effects).

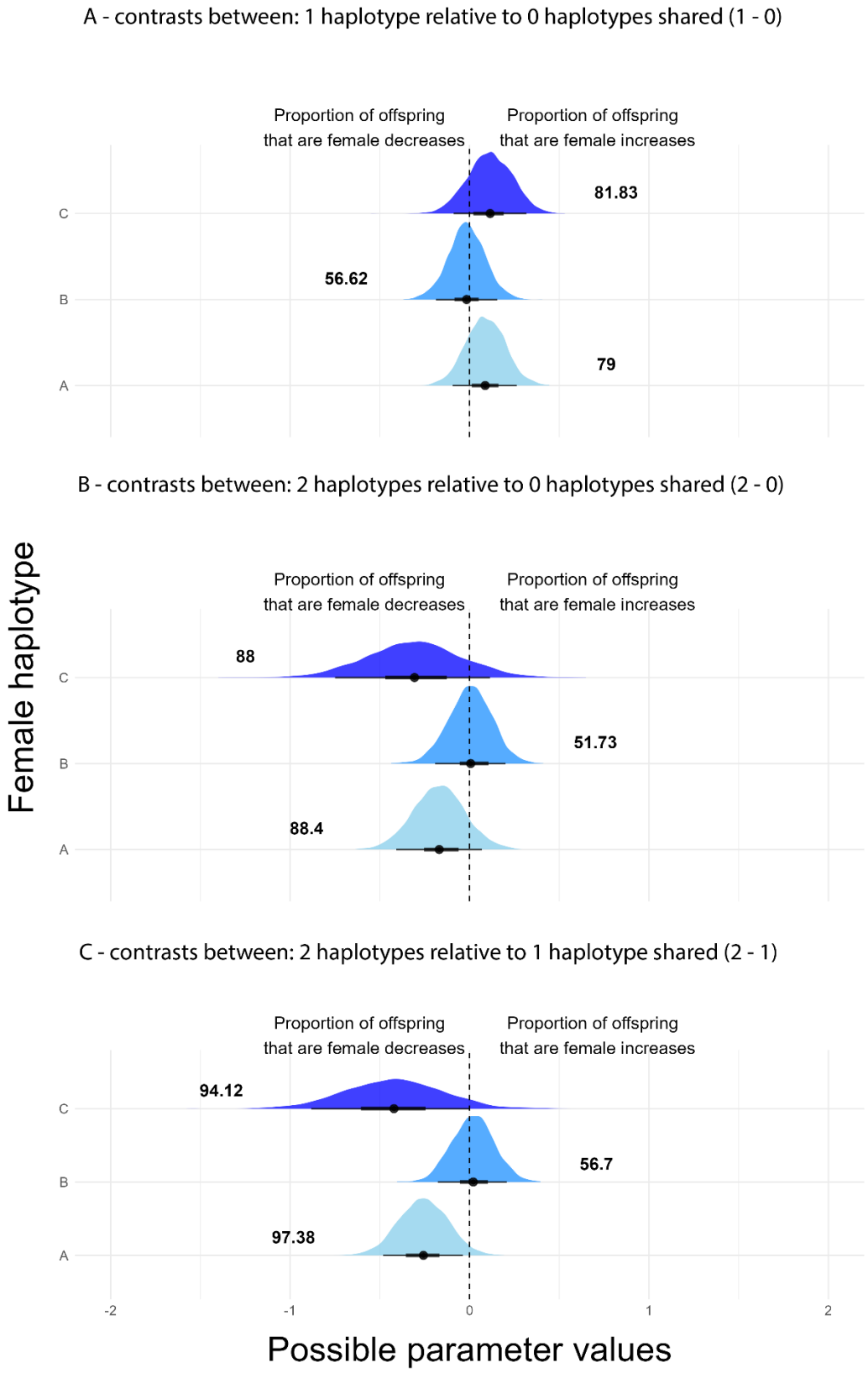


**Figure S4C:** Probability of direction (PD) plot showing the effect of haplotype sharing between males and females on the probability of offspring being female, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each

probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines). **A:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 1 haplotype shared relative to no haplotypes shared (E.g., A1 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 1 haplotype with the female is associated with an increase in the probability that eggs will be male, relative to sharing no haplotypes (and vice versa for positive effects). **B:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to no haplotypes shared (E.g., A2 – A0). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing 2 haplotypes with the female is associated with an increase in the probability that eggs will be male, relative to sharing no haplotypes (and vice versa for positive effects). **C:** Contrasts (from the interaction term between female haplotype and haplotype sharing) between 2 haplotypes shared relative to 1 haplotype shared (E.g., A2 – A1). Separate comparisons are made for each female haplotype (A, B and C). Negative effects would indicate that a male sharing both haplotypes with the female is associated with an increase in the probability that eggs will be male, relative to sharing 1 haplotype (and vice versa for positive effects).

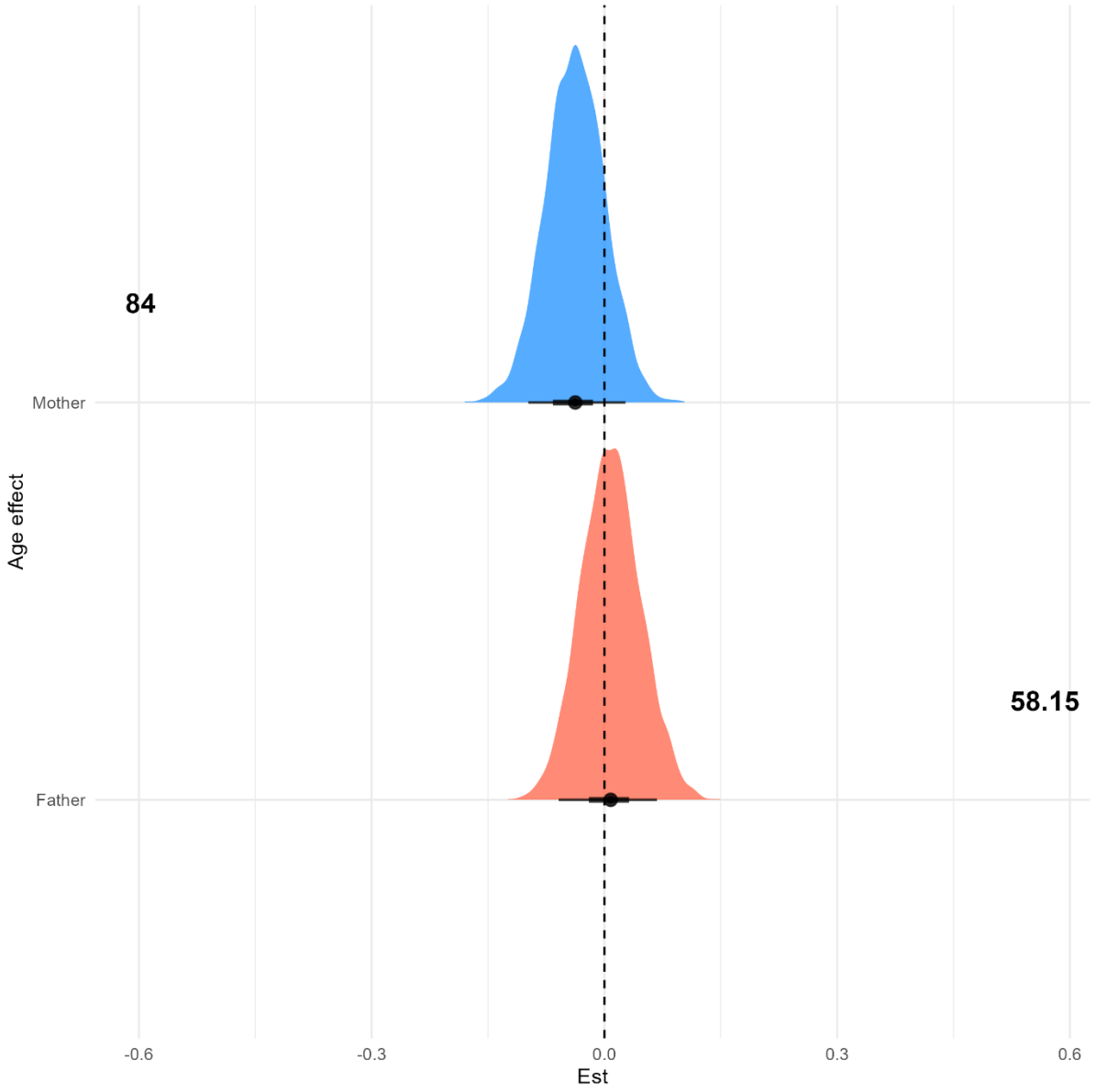


**Figure S4D:** Probability of direction (PD) plot showing the effect of male and female age on offspring sex ratio, where PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

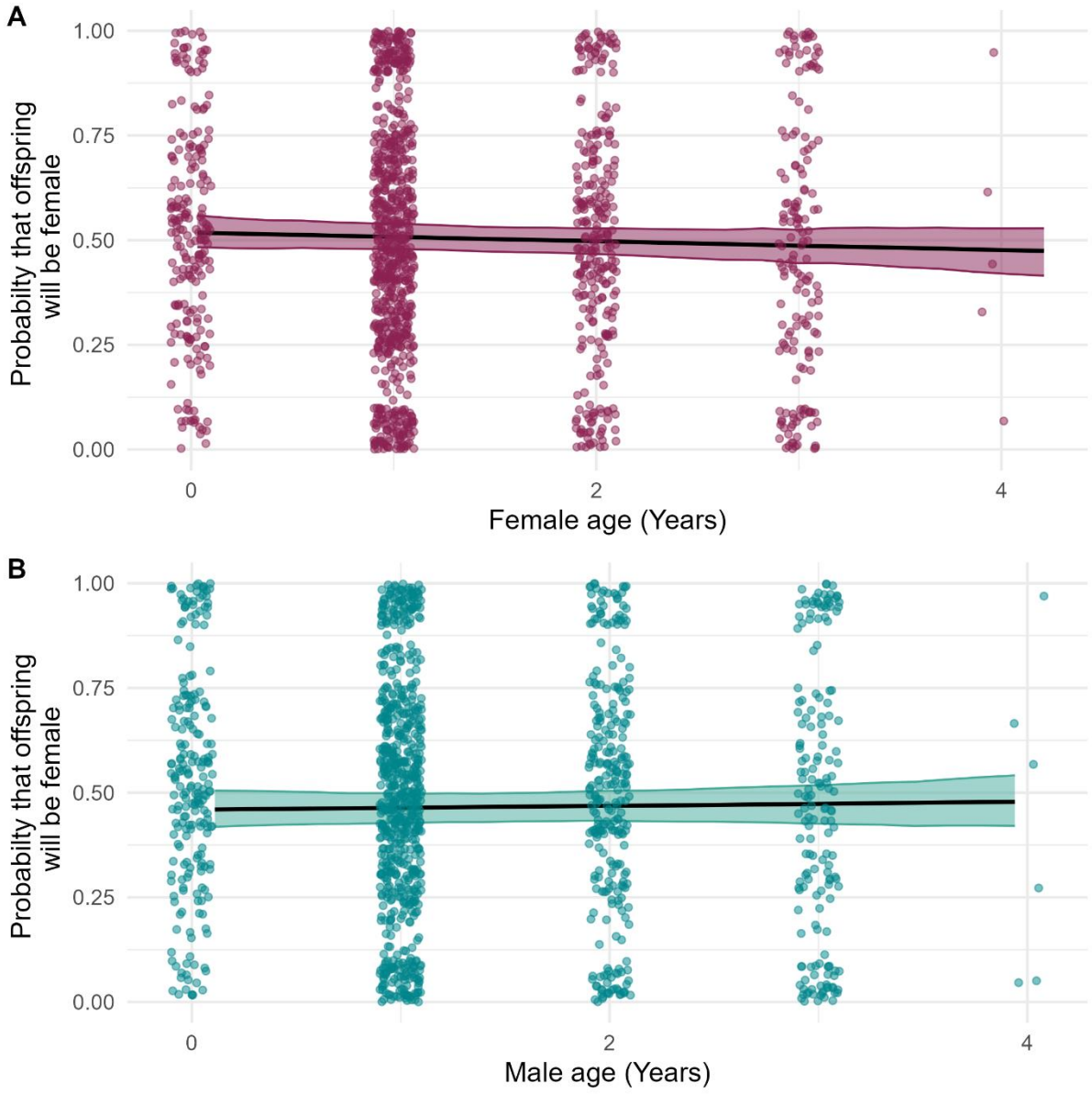


**Figure S4E:** The effect of female age (**A,** PD = 84) and male age (**B**, PD = 58.15) on the probability of offspring being female (relative to male). Lines are the posterior medians and coloured ribbons represent the 90% highest density intervals (HDIs) for the average individual. Points show the raw observed data and have been jittered to aid visualisation and prevent overlaying points.

S4F - G) Model investigating the effect of male karyotype on offspring sex ratio

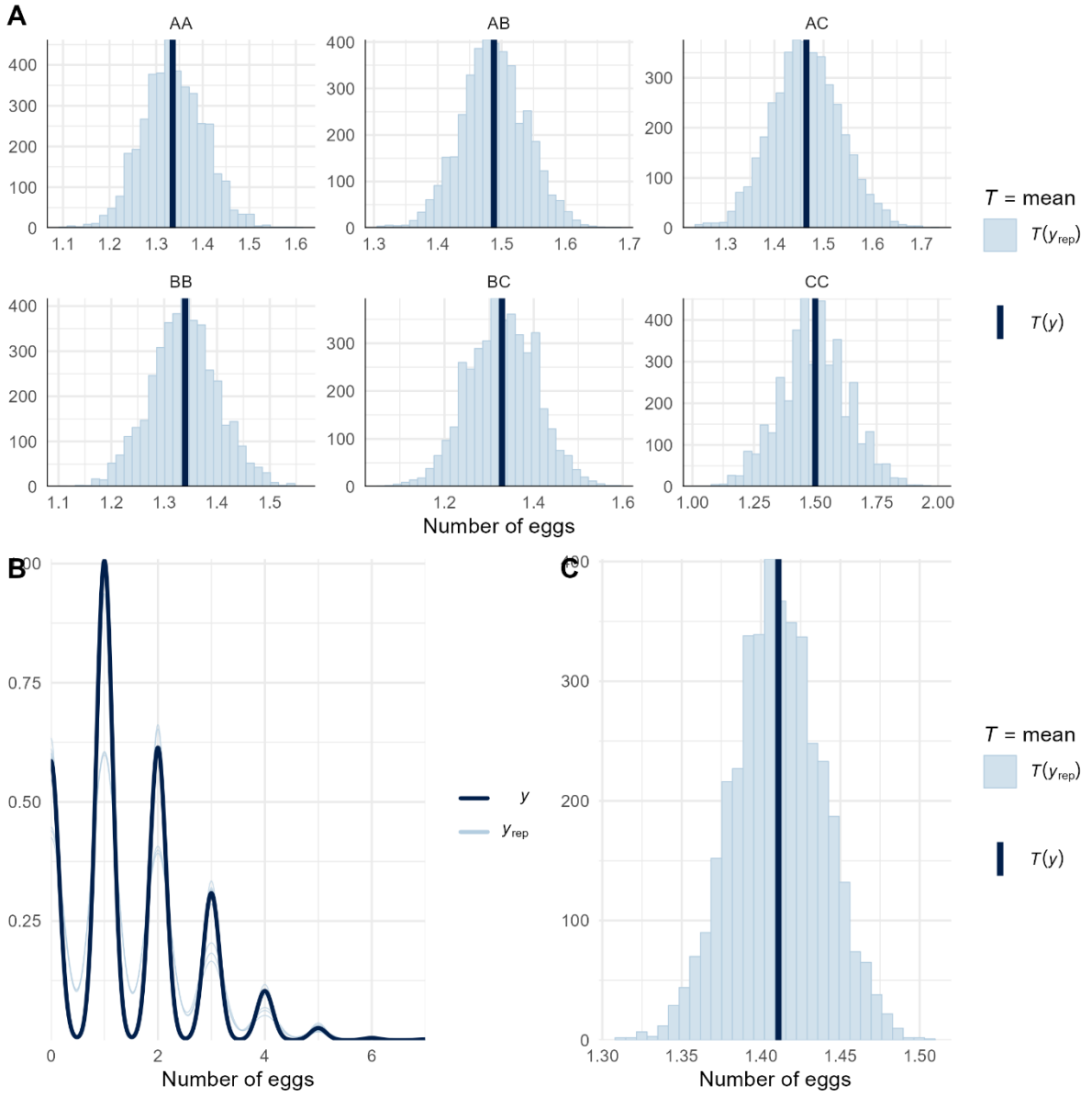


**Figure S4F:** Posterior predictive model checks for model investigating the effects of male karyotype on offspring sex ratio. **A (top):** A check of central tendency within groups of male karyotype, comparing the mean of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (bottom left):** A density overlay plot, comparing the distributions of the observed data to the distribution of posterior data. **C (bottom right):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.

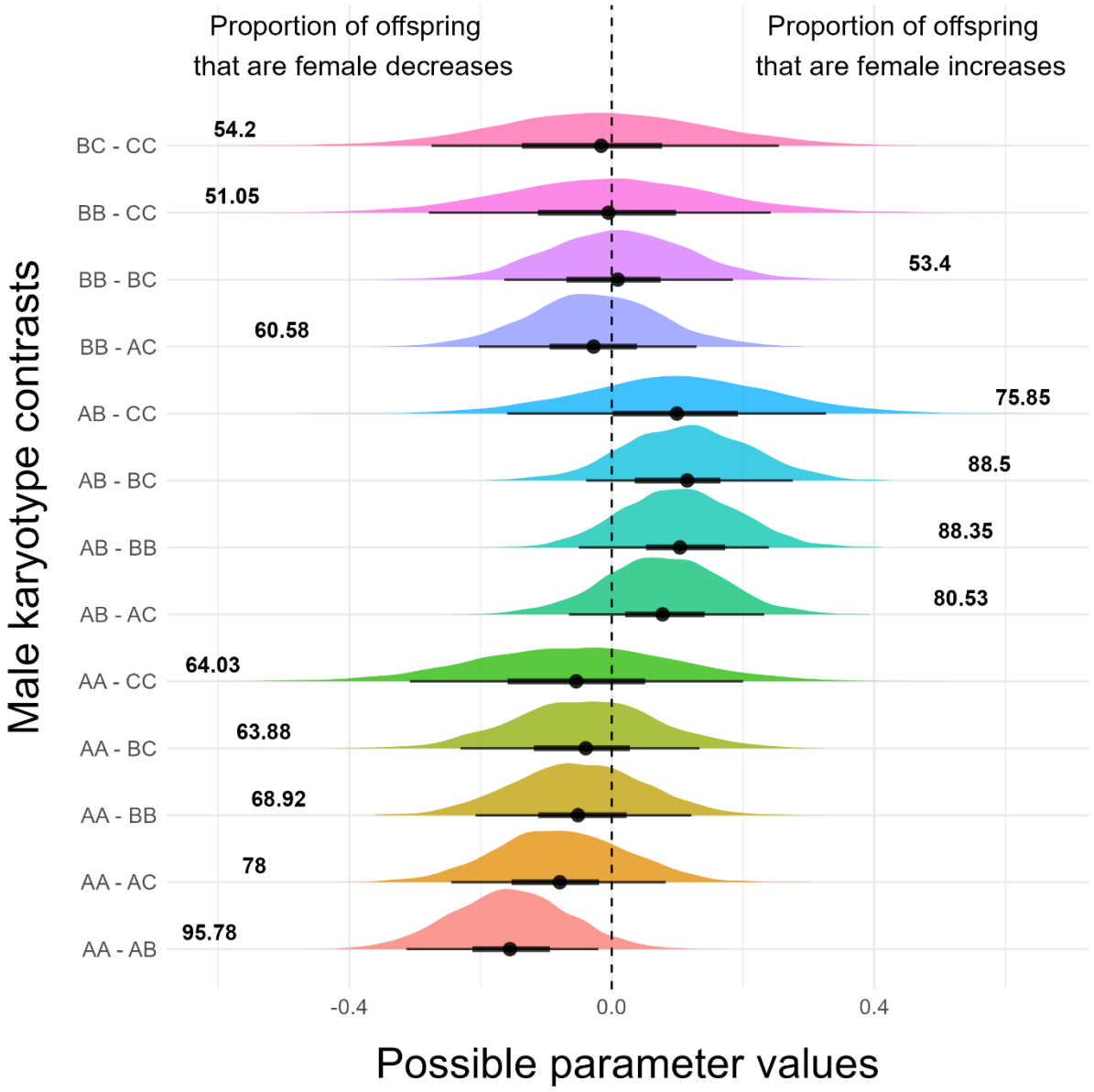


**Figure S3G:** Pobability of direction (PD) plot showing the effect of male karyotype on the probability of offspring being female. The Y axis shows contrasts between male karyotypes, where, for example, AA – AB represents the effect of the AA karyotype relative to the AB karyotype on the probability of eggs being female. A negative effect would indicate that AA karyotpes are associated with an increase in the probability that eggs will be male relative to AB karyotypes (and vice versa for positive effects). PD values are given to the left (if the median is negative) or right (if the median is positive) of each probability distribution. Medians are represented (black circles) along side 90% highest density intervals (90% HDI) (black lines).

## S5) Previous modelling attempts and the cRatio family

Prior to the adoption of a Bayesian framework, modelling was attempted using the generalised linear mixed model framework using the "lme4" package. Attempts to model egg production data initially included: a Poisson distribution (abandoned due to singular fit, and under dispersion); a Conway-Maxwell Poisson (abandoned due to under-dispersion and convergence failure); a Generalised Poisson (also abandoned due to under-dispersion and convergence failure); modelling egg production as a binary variable (abandoned due to convergence failure, one-inflation, and under-dispersion). Attempts to model hatching success proved difficult as well, with a proportional model using a binomial family resulting in within-group deviations from uniformity and one-inflation.

After moving on to a Bayesian framework, we still had trouble modelling egg production (Figure S5A). This was due largely to one-inflation, but also given the peak at around 5 eggs and the bounded nature of the data. The fit was not improved by using more flexible modelling approaches such as negative binomial (Figure S5B) or hurdle models (Figure S5C). A negative binomial hurdle model was the least poorly fitting of these attempts but was still deemed inappropriate.

Consequently, we approached the modelling of egg production using a continuation ratio family (cRatio), which had superior model fit (Figure S1A). This model considers the response (egg count) as an ordinal variable, and can be parametrised as the result of a sequential process where higher counts of eggs are only realised after the preceding counts have been laid. This model assumes that for every count of eggs $n$ there is a latent continuous variable $\bar{Y}_n$ that determines the transition between the $n^{\text{th}}$ and the $n + 1^{\text{th}}$ category. $\bar{Y}_n$ represents all the factors contributing to the probability of an additional egg being laid beyond count $n$ and could be broadly interpreted as an unobserved variable describing laying potential. Consecutive egg counts are separated by thresholds $\tau_n$. If $\bar{Y}_n$ is greater than the threshold $\tau_n$, the sequential process (egg laying) continues otherwise it stops at count $n$.

S5A - C) Examples of the posterior predictive checks for prior failed attempts to model the egg production analysis using traditional approaches. Examples included here: Poisson, negative binomial, and hurdle negative binomial models.

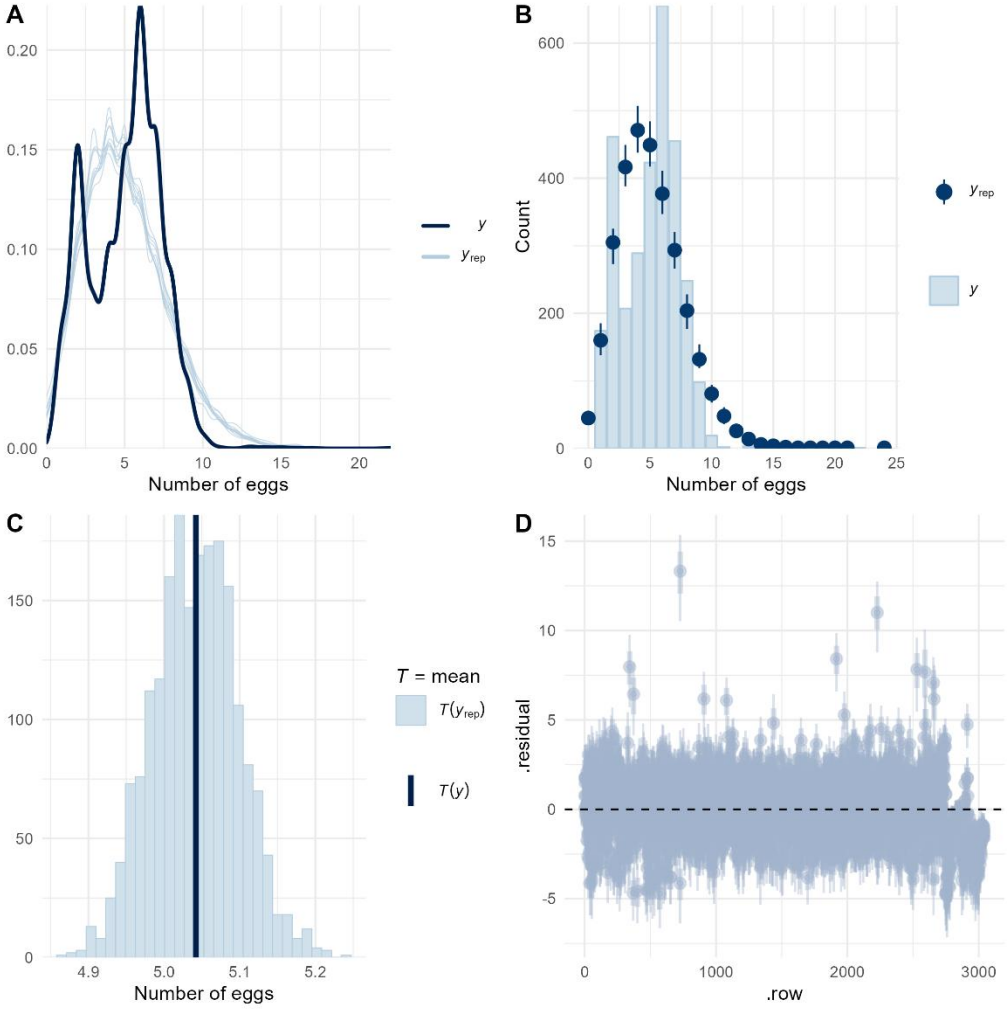


**Figure S5A:** Posterior predictive checks for a Poisson model investigating the effect of female haplotype and haplotype sharing on egg production. We deemed this model a poor fit. **A (top left):** A density overlay plot, comparing the distributions of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (top right):** Compares the counts (egg number) of observed data with the distribution of posterior estimated counts. **C (bottom left):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.**D (bottom right):** A check of the overall distribution of residual draws.

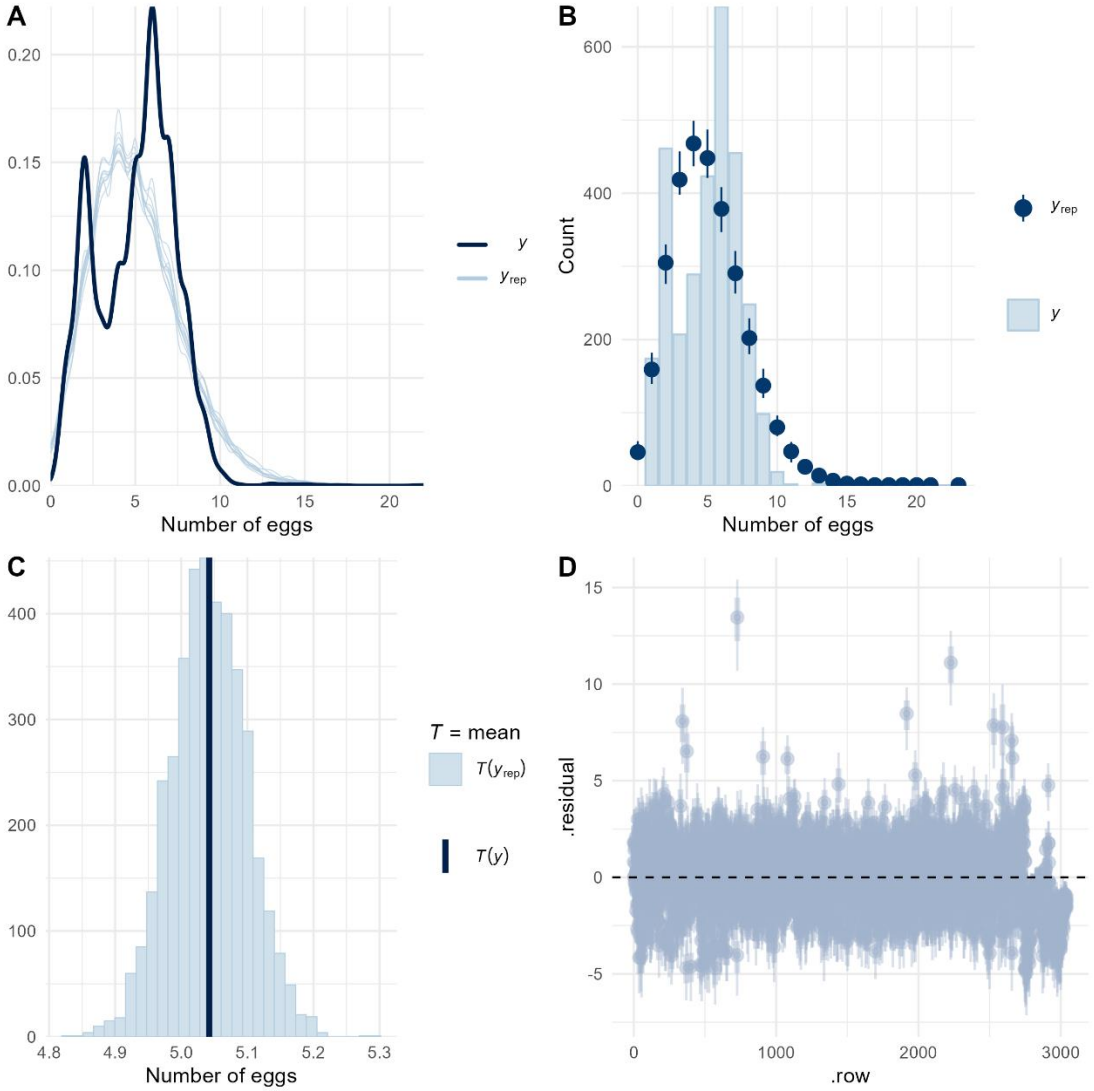


**Figure S5B:** Posterior predictive checks for a negative binomial model investigating the effect of female haplotype and haplotype sharing on egg production. We still deemed this model a poor fit. **A (top left):** A density overlay plot, comparing the distributions of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (top right):** Compares the counts (egg number) of observed data with the distribution of posterior estimated counts. **C (bottom left):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.**D (bottom right):** A check of the overall distribution of residual draws.

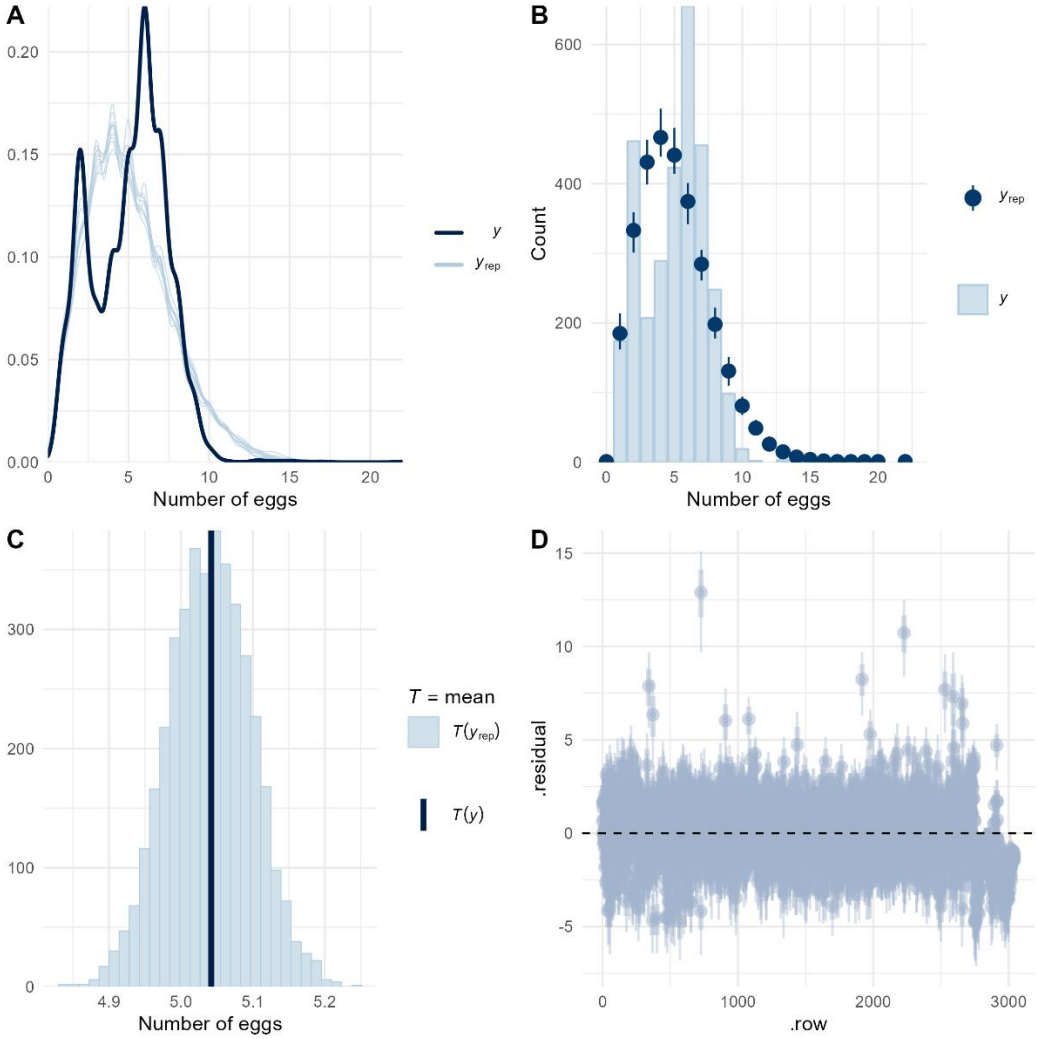


**Figure S5C:** Posterior predictive checks for a hurdle negative binomial model investigating the effect of female haplotype and haplotype sharing on egg production. This model was not an improvement and still deemed a poor fit. **A (top left):** A density overlay plot, comparing the distributions of $y$ (observed data) to the distribution of $y_{rep}$ (posterior predictions). **B (top right):** Compares the counts (egg number) of observed data with the distribution of posterior estimated counts. **C (bottom left):** A check of overall central tendency, comparing the mean of the observed data to the distribution of posterior means.**D (bottom right):** A check of the overall distribution of residual draws.

## S6) Variance decomposition analysis (Table S1)

A variance decomposition analysis allowed us to determine the relative contribution of the component structures of our models to the total variance associated with each reproductive trait. For the sex ratio analysis, almost all the variance was explained by the fixed effect structure. For the other measures; egg production, fertility and early development; and late development, the fixed effects still recovered most of the variance, but individual and (to a lesser extent) temporal differences had a larger role in determining reproductive outcomes (Table S1). This suggests that individual differences – specifically those not explained by differences in inversion genotype (or any other measure we were able to account for) - are important in generating variance in these traits.

**Table S1.** Summary of variance decomposition analyses to determine the relative contribution of the component structures of each model to the total variance associated with each reproductive trait. Variance was conditioned on the fixed effects (mother and father genotype and Z-scored age), the random effects (mother and father ID and mother birth year), and the fixed effects and individual ID.

| | Variance conditioned on fixed effects only | | Variance conditioned on random effects only | | Variance conditioned on fixed effects + individual ID | |
|---|---|---|---|---|---|---|
| | Variance | 90% CI | Variance | 90% CI | Variance | 90% CI |
| Egg production | 0.55 | 0.49 - 0.61 | 0.45 | 0.39 - 0.51 | 0.84 | 0.75 - 0.93 |
| Egg fertility & embryo development | 0.87 | 0.77 - 0.97 | 0.13 | 0.03 - 0.23 | 0.97 | 0.89 - 1.05 |
| Hatching success of developing eggs | 0.9 | 0.8 - 0.99 | 0.1 | -0.01 - 0.2 | 0.99 | 0.90 - 1.07 |
| Offspring sex ratio | 0.98 | 0.9 - 1.06 | 0.02 | -0.06 - 0.09 | 1.00 | 0.92 - 1.07 |

## S7) Summary of genotype sample sizes (Table S2)

**Table S2** Number of individuals genotyped for each parental combination, given for each analysis: 1 - Egg production, 2 - egg fertility and early embryo development, 3 - hatching success of developing eggs, and 4 - offspring sex ratio.

**1 Egg Production**

| | A | B | C | Total |
|---|---|---|---|---|
| AA | 96 | 89 | 59 | 244 |
| AB | 150 | 157 | 88 | 395 |
| AC | 82 | 63 | 65 | 210 |
| BB | 75 | 134 | 36 | 245 |
| BC | 70 | 75 | 29 | 174 |
| CC | 15 | 21 | 15 | 51 |
| Total | 488 | 539 | 292 | **1319** |

**2 Egg fertility and early embryo development**

| | A | B | C | Total |
|---|---|---|---|---|
| AA | 30 | 35 | 15 | 80 |
| AB | 49 | 56 | 21 | 126 |
| AC | 27 | 17 | 27 | 71 |
| BB | 25 | 53 | 12 | 90 |
| BC | 21 | 30 | 14 | 65 |
| CC | 6 | 10 | 6 | 22 |
| Total | 158 | 201 | 95 | **454** |

**3 Hatching success of developing eggs**

| | A | B | C | Total |
|---|---|---|---|---|
| AA | 32 | 30 | 14 | 76 |
| AB | 51 | 56 | 27 | 134 |
| AC | 27 | 27 | 28 | 82 |
| BB | 31 | 48 | 14 | 93 |
| BC | 21 | 26 | 14 | 61 |
| CC | 5 | 5 | 4 | 14 |
| Total | 167 | 192 | 101 | **460** |

**4 Offspring sex ratio**

| | A | B | C | Total |
|---|---|---|---|---|
| AA | 55 | 61 | 39 | 155 |
| AB | 100 | 102 | 63 | 265 |
| AC | 54 | 51 | 49 | 154 |
| BB | 45 | 85 | 25 | 155 |
| BC | 46 | 46 | 21 | 113 |
| CC | 12 | 15 | 11 | 38 |
| Total | 312 | 360 | 208 | **880** |

# Appendix 4:

## Supplementary material for ‘The surprising complexity and diversity of sperm storage structures across Galliformes’

# Appendix 4

## Dichotomous key for determining sperm storage tubule morphology according to the categories presented in 'The surprising complexity and diversity of sperm storage structures across Galliformes' (Assersohn et al., 2024).

**1a** The storage structures are clear and well-defined (located in the vagina, distinct and markedly different from the surrounding tissue) *Go to step 2*

**1b** The storage structures are not clear or well-defined (structures are not markedly different from the surrounding tissue) *Go to step 3*

**2a** Structures are very small compared to the largest tubule in the sample (tubules are <30% the size of the largest tubules in the sample) *Go to step 4*

**2b** Structures are **not** very small compared to the largest tubule in the sample (tubules are at least 30% the size of the largest tubules in the sample) *Go to step 6*

***3a*** Structures appear towards the uterus end of the sample (in the vagina, but between the population of 'normal' tubules and the uterus) *Go to step 5*

***3b*** Structures do not appear in the uterus end of the vagina – *UNDEFINED STRUCTURE.*

*Structures may fall outside of this categorisation key for the following reasons:*

- *Structure is not storage or storage-related tissue*
- *Image quality is not sufficient to observe structures*
- *The structure is currently undescribed*

**4a** Structures look like the other tubules in the sample in all but length. They have similar sized entrances, epithelium wall and lumen diameter to 'normal' tubules, but are just much shorter in length - *MINI-TUBULES*

**4b** Structures differ from the other tubules, they are much shorter in length but also have different sized entrances, epithelium walls, and lumen diameter. *Go to step 3*

5a Structures appear as densely concentrated, but very small tubule-like structures. They are much smaller than mini-tubules, and as they become denser, they transition into uterus tissue – *TRANSITIONAL TISSUE*

5b Structures do not appear like densely concentrated, but very small tubule-like structures. They are not smaller than mini-tubules and do not appear to transition into uterus tissue. *Go to step 7*

6a Tubules are simple 'straight' tubes. They may be curved, but not coiled/twisted, globular or agglomerate. *Go to step 8*

6b Tubules are not simple 'straight' tubes. They may be coiled/twisted, globular or agglomerate. *Go to step 9*

7a Structures appear 'fluffy' or 'cloud-like'. They look like poorly defined agglomerate tubules, but are distinct from uterus tissue. They may transition from agglomerate tubules into uterus tissue – *TRANSITIONAL TISSUE*

7b Structures do not appear 'fluffy' or 'cloud-like' *– UNDEFINED STRUCTURE*

*Structures may fall outside of this categorisation key for the following reasons:*

- *Structure is not storage or storage-related tissue*
- *Image quality is not sufficient to observe structures*
- *The structure is currently undescribed*

8a Structures are 'channel-like' in appearance. They may begin at the cloaca-end of the vagina, or further up the UVJ. They may span more than 1 field of view, and lead into tubule tissue. Some may terminate directly into a tubule *– CHANNEL TISSUE*

8b Structures are not 'channel-like' in appearance. *Go to step 10*

9a Structures are agglomerate in appearance – they are highly branched or very dense clusters of indistinguishable tubules. Structure appears somewhat globular. Branches may appear from a larger central 'body'. *– AGGLOMERATE*

9b Structures are not agglomerate in appearance. They do not appear as highly branched or very dense clusters of indistinguishable tubules. *Go to step 11*

10a Structures are branched. They are clear tubules with an obvious split into a clear branch i.e., they are not just overlaying tubules, which will have >1 lumen. *– STRAIGHT BRANCHED*

10b Structures are not branched. They are clear, single branched blind-ended tubes with no splits and only one lumen. *– STRAIGHT UNBRANCHED*

11a Structures are twisted/coiled. They are distinct separate tubules, but they appear coiled or twister. They may be branched or unbranched *- COILED*

11b Structures are not coiled or twisted. *– UNDEFINED STRUCTURE*

*Structures may fall outside of this categorisation key for the following reasons:*

- *Structure is not storage or storage-related tissue*
- *Image quality is not sufficient to observe structures*
- *The structure is currently undescribed*