Sperm morphology and fertilisation success in the zebra finch

Taeniopygia guttata

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Contents

Appendices

Summary

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The aim of this study was to understand how variation in sperm morphology influences sperm swimming velocity, and consequently, the fertilisation success of males in a competitive context.

Chapter 2 provides the methods behind the three key aspects underpinning the work described in this thesis. The set-up and maintenance of the selective breeding regime producing the population of zebra finches is described. The procedures involved in the collection of sperm, and assessment of sperm quality are then given in detail.

Chapter 3 describes the artificial selective breeding regime, involving the three lines (long, intermediate and short) used to investigate how sperm morphology responds to selection on sperm total length. The tail and total length of sperm respond similarly to artificial selection, with decreasing and increasing lengths observed in the short and long selection lines, respectively. There were differences in the relationships between various sperm components in the three lines, for example midpiece and tail length were positively associated in the short selection line, yet generally, there was a negative relationship between these traits observed across the long and intermediate selection lines.

Chapter 4 focuses on the genetic relationships that underpin the phenotypic associations evident in Chapter 3, and how these genetic relationships may determine the evolutionary trajectory of sperm morphology in response to selection. The difference in phenotypic relationships in the short selection line compared to the long and intermediate lines originate from a difference in genetic covariance between the sperm components across the lines.

Chapter 5 uncovers the possibility that phenotypic changes in sperm morphology may be constrained by stabilising selection, based on the evidence that increased sperm swimming velocity is associated with absolutely larger component dimensions, but only up to certain values, beyond which swimming velocity declines.

Chapter 6 draws together the work carried out through this thesis, and tests the hypothesis that longer, faster swimming sperm have a fertilisation advantage compared to shorter and slower swimming sperm in a competitive situation. There was a strong effect of sperm length on the competitive ability of sperm, but this advantage was mediated by complex interactions between the male and female selection history, and the different relationships that these male – female mating combinations have on the proportions of sperm that finally reach the ovum.

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Abbreviations

Acps	Accessory gland proteins
AI	Artificial insemination
AIC	Akaike information criterion
ATP	Adenosine triphosphate
CASA	Computer Assisted Semen Analysis
CI	Confidence interval
CV	Coefficient of variation
DMSO	Dimethyl sulphoxide
d.f	Degrees of freedom
DNA	Deoxyribonucleic acid
EBV	Estimated breeding value
FAM	Fluorescein
G	Genetic variance covariance matrix
GD	Germinal disc
G_I	G matrix (intermediate line)
G_L	G matrix (long line)
G_{MAX}	Eigenvector with greatest genetic variation
G_S	G matrix (short line)
GLM	Generalised linear model
GLMM	Generalised linear mixed effects model
h^2	Narrow sense heritability
HEX	Hexachloro-Fluorescein
HPD	Highest posterior density
I	Intermediate
I-line	Intermediate selection line
IPVL	Inner perivitelline layer
L	Long
L-line	Long selection line
LIN	Linearity
LM	Linear model
LMM	Linear mixed effects model
lowTE	Buffer solution
MCMC	Markov Chain Monte Carlo
MHC	Major Histocompatibility Complex
OPVL	Outer perivitelline layer
OXPHOS	Oxidative phosphorylation
P2	Proportion of paternity sired by the second male
PBS	Phosphate buffered saline
PC1	Principle Component 1
PCA	Principle Component Analysis
PC	Principle Component
PCR	Polymerase chain reaction
PCSS	Postcopulatory sexual selection
P_E	Permanent environment effect
PI	Propidium iodide
PVL	Perivitelline layer
S	Short
SCA	Sperm Class Analyzer
S.D	Standard deviation
S.E	Standard error
SG	Seminal glomerus
S-line	Short selection line
SML	Straight midpiece length
SYBER14	Cyanine nuclei acid stain
SST	Sperm storage tubules
STR	Straightness
UVJ	Uterovaginal junction
V_A	Additive genetic variance
V_R	Residual variance
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straightline velocity

Units

^oC	Degrees Celsius
µl	Microlitre
µm	Micrometre
µM	Micromoles
cm	Centimetre
d	Day
fps	Frames per second
g	Grams
GMT	Greenwich Mean Time
h	Hours
m	Meter
mg	Milligrams
ml	Millilitre
mm	Millimetre
mM	Millimoles
ng	Nanograms
pH	Hydrogen ion concentration
s	Seconds

Chapter 1

General introduction

1.1: Introduction

Sexual selection

Across individuals of a species there is widespread phenotypic variation. If phenotypic variation is underpinned by heritable genetic variation, and those phenotypes confer a survival advantage to that individual, gradually populations evolve to become better adapted to its environment (natural selection; Darwin 1859). However, some extreme phenotypic traits, such as bright coloration in guppies *Poecilia reticulata,* may be detrimental to survival (Godin & MacDonough 2003). Because these phenotypes persist despite reducing survival, Darwin proposed that they were maintained via sexual selection, because the costs of reduced survival are offset by reproductive benefits (Darwin 1871).

Sexual selection promotes the evolution and maintenance of extreme phenotypic traits via two mechanisms: (i) intra sexual selection, which occurs (usually) between males of the same species, where fighting and competition for access to females select for strength and weaponry, and (ii) inter sexual selection between males and females of a species, where females (although sometimes males) choose mates on the basis of ornamentation or displays (Andersson 1994), assuming that the phenotype honestly advertises the health and resources of the male. Assuming the trait has an additive genetic basis and is heritable, the female gains indirect genetic benefits from copulating with that particular male through the increased health and fitness of the offspring (Zahavi 1975).

Postcopulatory sexual selection

Until the 1970s, female animals were thought to be sexually monogamous and copulate only with a single male. A wealth of evidence demonstrates that female promiscuity is extremely widespread (Birkhead & Møller 1998), and that this promiscuity is beneficial for female reproductive success, for example for the acquisition of additional parental care, fertility assurance, genetic benefits and ensuring the male with the most competitive sperm sires the offspring (Hartley et al. 1995; Yasui 1997; for reviews see Zeh & Zeh 1997; Jennions & Petrie 2000). Female promiscuity means that sexual selection does not end at copulation; instead, postcopulatory sexual selection (PCSS) continues to influence the fertilisation success of rival males by two processes: (i) sperm competition, where the sperm from two or more males compete to fertilise a given set of ova (Parker 1970), and (ii) cryptic female choice, where male

fertilisation success is biased by the female (Eberhard 1996; Thornhill 1983). Both processes have resulted in a suite of adaptations observed across both sexes (see below).

Adaptations to postcopulatory sexual selection: males

Males employ a range of strategies to maximise their fertilisation success. When fertilisation is internal, males may (i) guard the female following copulation (e.g. Birkhead 1979; Møller & Birkhead 1991), (ii) plug the female reproductive tract after insemination (e.g. Devine 1977; Barker 1994), or (iii) remove or displace rival males sperm using elaborate genitalia (e.g. Waage 1979). In mammals, fertilisation success is dependent on which male's sperm are capacitated and capable of fertilising ova at ovulation. Mating order can also affect fertilisation success; males may achieve the greatest paternity share when mated first (e.g. in stalk eyed flies *Cyrtodiopsis whitei;* Lorch et al. 1993), whereas in other species the last male to copulate achieves the greatest paternity share, i.e. last male sperm precedence occurs (Parker 1970; Birkhead et al. 1988b; Xu & Wang 2010 but see Zeh & Zeh 1994). The paternity share is also affected by the time interval between the copulations, where a longer interval results in sperm from the first male being numerically disadvantaged (Colegrave et al. 1995).

Males may also perform frequent copulations, so that their sperm outnumber the sperm of rival males (Crowe et al. 2009). Frequent copulations, however, require additional adaptations, such as larger testes, to avoid male sperm depletion (e.g. Lüpold et al 2009c, 2011; Rowe & Pruett-Jones 2011). Therefore, relative testes size and risk of sperm competition positively covary across species (e.g. Hardcourt et al. 1981; Gage 1994; Stockley et al. 1997; Balshine et al 2001; Bryne et al. 2002), and within species when postcopulatory sexual selection is experimentally enforced (Pitnick 1996; Hosken & Ward 2001).

It was once thought that sperm were energetically 'cheap' to produce (Trivers 1972), but it now seems likely that there are significant costs to sperm production (Pitnick & Markow 1994; Olsson et al. 1997; LaMunyon & Ward 1998), which may reduce male survival (van Voorhies 1992) and immune function (Dowling & Simmons 2012). High costs of making sperm are also likely because species with low risk of sperm competition, such as the bullfinch *Pyrrhula pyrrhula* (Birkhead et al. 2006a), produce ejaculates that have been subject to minimal quality control, resulting in increased morphological variation (Calhim et al. 2007) and greater proportions of defective sperm (Birkhead & Immler 2007). Conversely, when competition is intense, each ejaculate should be the highest possible quality to maximise male fertilisation

success. This may be achieved by (i) adjusting the chemical constituents of the seminal fluid, for example to affect the female's reproductive behaviour (Chapman 2001; Gillott 2003) or by creating the optimum environment for sperm function (Poiani 2006), (ii) improving the overall quality and competitiveness of sperm (e.g. swimming velocity, viability and morphology) in the ejaculate (discussed further below) (Firman & Simmons 2011), and (iii) being prudent in allocation of ejaculates to females, based on the perceived risk of sperm competition (Pizzari et al. 2003), social status (Cornwallis & Birkhead 2006) and female quality (Pizzari et al. 2003; Cornwallis & Birkhead 2007). Production of 'cheaper' apyrene (non-fertilising) sperm occurs in some species (e.g. Lepidoptera) and may also be used to influence female re-mating behaviour by filling the spermathecae (sperm storage organs) of females (Cook & Wedell 1999).

Adaptations to post copulatory sexual selection: females

The ova produced by females are numerically fewer and larger than sperm; consequently, female investment in individual gametes is greater (Trivers 1972). Therefore, the function of female adaptations in response to PCSS is centred on retaining control over paternity by influencing which of several males' sperm fertilise her ova. Adaptations may be: (i) behavioural; such that females eject sperm to limit male success (Pizzari & Birkhead 2000; Peretti & Eberhard 2010), (ii) anatomical; where the physical structure of the female reproductive tract makes forced copulation and strategic placement of sperm difficult (Brennan et al. 2007; Brennan et al. 2010), and (iii) physiochemical; as the female reproductive tract is a hostile environment reducing sperm survival due to low pH, viscous mucus secretions and a targeted immune response (Birkhead & Brillard 2007). The additional role of the female reproductive tract (besides transporting and nurturing the fertilised ovum) involves selecting the fertilising set of sperm. This occurs via chemical and physical barriers, for example, where viscous mucus prevents low motility sperm from progressing higher up the tract, thus preventing fertilisation by these sperm (Birkhead et al. 1993).

Despite the drastic initial reduction of sperm numbers, the female reproductive tract must also protect and nourish the selected set of sperm, possibly using secretions from the epithelial cells lining the tract (Pollard et al. 1991) or from the sperm storage organs (Alumot et al. 1969). In mammals, sperm are unable to fertilise ova without the female producing sterol binding proteins, which initiate capacitation by cholesterol efflux (Therien et al. 1998). Females of externally fertilising species may also influence male fertilisation success by producing ovarian fluid which bathes the eggs and, in some species, influences sperm swimming velocity (Urbach

et al. 2005; Rosengrave et al 2008; Gasparini et al. 2012) and prolongs sperm viability (Gasparini & Evans 2013).

Interacting effects of male and female on the outcome of PCSS

Because sperm competition and cryptic female choice are not mutually exclusive (and therefore difficult to disentangle (Birkhead 1998; 2000; Eberhard 2000)), the final outcome of PCSS is likely to be an interaction between the male and female adaptations, and not predictable across males (Birkhead et al. 2004). These interactions may originate from the unique genetic combination of each mating male and female (Clark et al. 1999). Males that are related to the females may have reduced competitive success if mechanisms exist to prevent inbreeding (Stockley 1999, but see Denk et al. 2005), which may increase the quality of the offspring. Male fowl *Gallus domesticus* allocate fewer sperm to females with similar major histocompatibility complex (MHC) genotypes (Gillingham et al. 2009), because females may be less likely to choose the sperm of males with genotypes that could result in offspring with suboptimal immune systems (Løvlie et al. 2013). Similarly in guppies *Poecilia reticulata* (Gasparini & Pilastro 2011), sperm swimming velocity is also lowered in the presence of ovarian fluid from related females, which may reduce fertilisation success of males that are relatives of that female (Gasparini et al. 2010b).

Differential fertilisation success across male-female combinations may also be mediated by female sperm storage structures. Sperm and sperm storage structures have coevolved across some species (Briskie & Montgomerie 1992; Pitnick et al. 1999). Within single species, such as Drosophila (Miller & Pitnick 2002), it appears that matching dimensions of sperm and storage structures confers a fertilisation advantage to males producing longer sperm, possibly due to these sperm occupying the optimal position in storage prior to fertilisation (Miller & Pitnick 2002).

Many of the processes governing male-female interactions are dynamic and undergo rapid diversification, such that an evolutionary arms race of reproductive control may occur. The genes determining a wide range of accessory gland proteins (Acps) in Drosophila have been particularly well studied and many, although not all, show rapid evolutionary change, especially when compared to genes coding for non-reproductive proteins (Swanson et al. 2001). Although Acps and other seminal fluid proteins can influence many aspects of female reproductive behaviour (reviewed in Gillott 2003) and elicit change in gene expression in the

female (Mack et al. 2006), it is less well understood if the female can modulate and control these change in gene expression to stay one step ahead in the arms race.

How do sperm influence male fertilisation success?

In this section the way that sperm quality may influence male fertilisation success is considered in more detail.

Sperm number

An increase in the number of sperm inseminated in response to intense sperm competition is widespread across species (see above). Greater sperm numbers can have a positive influence on fertilisation success in non-competitive situations (Taneja & Gowe 1960; Casselman et al. 2006) and when males are in competition for fertilisations (Martin et al. 1974; Gage & Morrow 2003). When sperm competition is akin to a raffle, the probability of success per male, i.e. fertilising eggs, is proportional to the number of sperm inseminated by each male, when everything else is equal (Parker 1990).

However, the fertilising capacity across males is often not equal, as a result of differences in sperm quality between males (see below) (Martin & Dziuk 1977). Sperm quality (the difference in fertilisation capacity when sperm numbers are equal) can be quantified by measuring the following ejaculate traits: (i) viability (the proportion of sperm in the ejaculate able to fertilise eggs, where the sperm membrane is intact), (ii) longevity (the length of time sperm are viable and/or motile), (iii) normal sperm morphology (the dimensions of sperm component parts and the proportion of sperm with no morphological abnormalities), and (iv) sperm velocity (the swimming speed of sperm) (Snook 2005).

Sperm quality: viability and longevity

In species where PCSS is intense, males produce ejaculates with greater proportions of viable sperm (Hunter & Birkhead 2002; Rowe & Pruett-Jones 2011). The proportion of viable sperm also predicts competitive success in individual species (Garcia-Gonzalez & Simmons 2005). However, the impact of sperm longevity on fertilisation success is more complex. In external fertilisers (e.g. fish), where male and female gametes are often released simultaneously, fertilisation is characterised by a ‘race to the egg’, rendering sperm longevity less important

than velocity. When insemination and fertilisation are temporally separated (e.g. in species with internal sperm storage), producing sperm that are viable for longer periods of time is advantageous. Hence, males with sperm that are viable for longer could gain paternity until the end of the female's fertile period (in birds, this advantage may be mediated via swimming velocity – see below) (Pizzari et al. 2008). The effect of longevity on fertilisation success is complicated by differences in quantification methods. For example, longevity may be recorded as the time taken for progressive motility to drop below a certain given threshold (e.g. Gage et al. 2004), or the decline in the proportion of viable sperm over time (e.g. Gasparini & Evans 2013). For maximum insight, both measures may need to be made on each sample, since sperm may be motile but not viable, or viable yet immotile (pers. obs). Longevity – when considered as the length of time that sperm are motile – may also be influenced by both the morphology and velocity of the sperm (Stockley et al. 1997; Levitan 2000; Gage et al. 2002; Helfenstein et al. 2010) (see below).

Sperm quality: morphology and velocity

Between-male variation in sperm morphology is lower in species with intense PCSS compared to those with limited or zero PCSS (Calhim et al. 2007; Kleven et al. 2008; Lüpold et al. 2009b; Fitzpatrick & Baer 2011), and results in species specific optimal sperm designs. This suggests that greater quality control during spermatogenesis may be advantageous for increased sperm competitive ability. Because longer sperm are associated with increasing PCSS intensity across species (Gomendio & Rolden 1991; Lüpold et al. 2009b; but see Stockley et al. 1997), it is often assumed that an increase in sperm length will be advantageous in competition between males of the same species. In mites *Rhizoglyphus robini* (Radwan 1996), which have amoeboid sperm, larger sperm fertilised more eggs, whereas other studies (using species with flagellate sperm) observed greater fertilisation success when males produced shorter sperm (Gage & Morrow 2003; Garcia-Gonzalez & Simmons 2007). The lack of clarity in the relationship between morphology and fertilisation success is also emphasized by studies using fish, where individuals with different reproductive strategies, and consequently experiencing different levels of PCSS, have comparable sperm morphology (Gage et al. 1995; Fitzpatrick et al. 2007) but variable fertilisation success. These inconsistencies found when studying single species may reflect the co-variation of a range of factors, for example when swimming velocity, sperm size and longevity are associated, and combined with the role of sperm storage in fertilisation success (Immler & Birkhead 2007).

As both sperm length and swimming speed are positively associated with increasing sperm competition risk in some taxa (e.g. Gomendio & Rolden 1991; Lüpold et al. 2009b), both variables are often assumed to enhance fertilisation success. The reasoning behind this assumption is that longer sperm swim faster, and faster sperm reach and fertilise the ova before slower swimming sperm. Although comparative studies link sperm morphology and swimming velocity (e.g. Gomendio & Rolden 2008; Lüpold et al. 2009a), within species associations have been more difficult to detect. Longer sperm do indeed swim faster in some species (Mossman et al. 2009; Fitzpatrick et al. 2010) but not in others (Gage et al. 2002). Three key aspects of sperm morphology are used to explain why morphology and swimming velocity should be positively related. First, increased sperm length is generally a consequence of longer flagella, which may exert greater propulsive forces (Cardullo & Baltz 1991). Second, sperm with a longer or larger midpiece could swim faster, due to increased adenosine triphosphate (ATP) production via a greater volume of midpiece mitochondria (Vladic et al. 2002; Rowe et al. 2013). However, there have been negative (Malo et al. 2006) or no associations (Mossman et al. 2009) observed between midpiece length and swimming velocity, suggesting that the relationship between midpiece length and velocity is far from resolved. Third, the relative lengths of different sperm components may influence swimming velocity, for example where a relatively longer midpiece could provide more energy for swimming per unit of sperm length (Laskemoen et al. 2010), or where a shorter head relative to the flagellum length may increase the hydrodynamics of sperm and reduce the negative effects of drag (Humphries et al. 2008; Mossman et al. 2009; Helfenstein et al. 2010).

In contrast to the influence of sperm morphology on fertilisation success, the relationship between sperm velocity and competitive success is well established. Fertilisation success is determined by increased swimming velocity in multiple species, both in non-competitive (Froman & Feltman 1998; Levitan 2000; Malo et al. 2005) and competitive contexts (Birkhead et al. 1999b; Gage et al. 2004; Gasparini et al. 2010b; Borschetto et al. 2011). In the frog *Crinia georgiana* (Dziminski et al. 2009), males with high velocity sperm did not actually achieve greater success. Interestingly, but not unexpectedly, the overall motility (the proportion of sperm that can move progressively forward) of the ejaculate was actually the primary determinant of competitive success, due to slower sperm swimming for longer. This study demonstrates a trade-off between longevity and velocity, which has also been observed elsewhere (e.g. Levitan 2000); fast swimming sperm appeared to be motile for less time than slower swimming sperm, indicating there may be a finite energy budget for sperm motility, managed by adopting alternative swimming strategies. Sperm morphology has also been

linked to sperm longevity, where shorter sperm live for longer (Stockley et al. 1997; Gage et al. 2002). Although morphology and velocity were unrelated in the salmon *Salmo salar*, the longer flagella of the sperm were suggested to deplete the energy reserves, due to increased hydrostatic forces (Gage et al. 2002). Taken together, the above examples illustrate the links between two major influences of fertilisation success; sperm morphology and swimming velocity. Although morphology and velocity have independently been reported to be associated with fertilisation success (see Figure 1 for a summary), currently there is limited direct empirical evidence from individual species that sperm morphology affects fertilisation success via differences in swimming velocity.

In the zebra finch *Taeniopygia guttata* (see Section 1.2 for general species information) patterns of sperm morphology are well studied, and extensive but repeatable variation exists between males (Birkhead et al. 2005). The relationship between morphology and velocity has been established, such that sperm with absolutely longer tails and greater total length, and relatively smaller heads compared to the rest of the sperm achieve greater swimming speeds (Mossman et al. 2009). Although the incidence of sperm competition is low in this species (Birkhead et al. 1988b; Birkhead et al. 1990), virtually nothing is known about the effect of natural variation in sperm morphology on male competitive success. The zebra finch reproductive biology makes birds suitable subjects for the study of PCSS, because of the extended fertile period and sperm storage. The specific details of the reproductive biology of birds are outlined below.

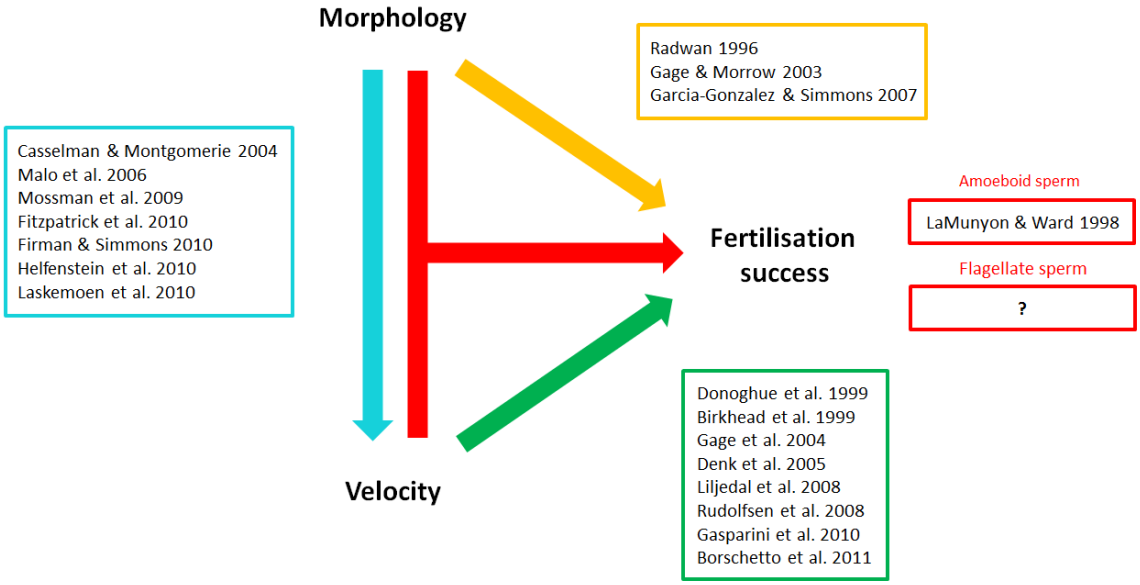


Figure 1. Schematic illustrating the significant associations between sperm morphology, swimming velocity and fertilisation success across taxa documented in the literature. Each coloured box contains examples of research relating to the variables linked by the arrow of the same colour. The link between all three variables is in red. Note that the references are not exhaustive. Literature cited with regard to fertilisation success refers to studies where competitive fertilisations were carried out, either by natural copulations or via artificial insemination.

Postcopulatory sexual selection in birds

Initial selection of sperm

Generally in birds, copulation comprises a brief 'cloacal kiss' resulting in sperm transfer from male to female (for exceptions see Briskie & Montgomerie 1997; Winterbottom et al. 2001). Sperm are placed just inside the cloaca and are moved into female reproductive tract (oviduct) by cloacal contractions. As many more sperm are inseminated than are required for fertilisation, the vagina has an important role in reducing the number of sperm. Oviductal mucus secretions may prevent sperm with suboptimal motility from progressing up the tract, by trapping sperm or by altering the environment of the oviduct, for example the pH (Holm & Wishart 1998). This sperm selection means that only around 1-2% of sperm inseminated reach the uterovaginal junction (UVJ) (Bakst et al. 1994), the site of sperm storage (Figure 2).

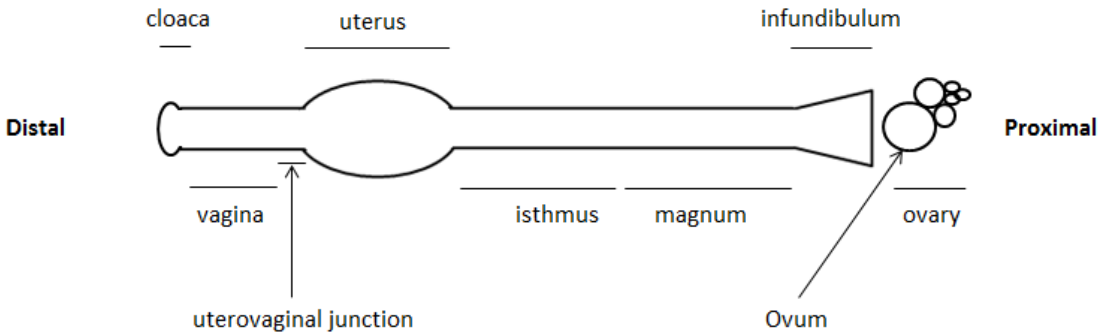


Figure 2. Schematic of the general structure of the oviduct in female birds from the cloaca (left) to the end of the infundibulum (right). The ovum is released from the ovary and is captured by the infundibulum, where fertilisation takes place by the waiting population of sperm. The fertilised ovum then moves down the oviduct (from right to left in this image) where successive processes occur before the egg is ready to be laid; around 24 h post-ovulation in species that lay one egg per day. The relative sizes of each part of the oviduct are not drawn to scale.

Sperm storage in birds

Sperm storage is ubiquitous across all studied bird species (e.g. Birkhead & Møller 1992; Birkhead & Møller 1993), with the duration of storage across species varying widely from days to months (Birkhead and Møller 1993), resulting in temporal separation of insemination and fertilisation. Sperm are stored in sperm storage tubules (SSTs), which are tubular invaginations of the UVJ epithelium. Each SST has a single opening into the UVJ lumen by which sperm must enter and exit. Although the processes governing the uptake of sperm into the SSTs are unclear, only sperm with normal morphology have been observed inside SSTs (Allen & Grigg 1957). One hypothesis explaining sperm loss from the SSTs involves the sperm swimming velocity (Froman 2003). Sperm are orientated with the sperm head facing the blind end of the SST, i.e. facing away from the SST exit, and appear to be swimming against a current, potentially generated from the presence of aquaporins (Zanziboni & Bakst 2004). Sperm are suggested to remain in the SSTs by positive rheotaxis, until the sperm membrane degradation causes motility to fall below a threshold level (<25µm/s in domestic fowl: Froman 2003), after which sperm are flushed back out into the oviduct (Froman 2003). Zanziboni & Bakst (2004) suggest that the fluid flow through aquaporins may be under hormonal control, partly explaining why differential sperm retention occurs through the breeding cycle of female turkeys *Meleagris gallopavo* (Bakst et al. 1994). Hormones such as progesterone have also been implicated in sperm loss from the SSTs in Japanese quail *Coturnix japonica* (Ito et al. 2011).

Transport of sperm to the ovum and fertilisation

Once sperm are released from storage, passive transport by cilia and oviductal contractions move sperm through the uterus, magnum and isthmus towards the infundibulum (Allen & Grigg 1957). The infundibulum is the site of fertilisation and receives the ovum immediately following ovulation. The recently ovulated ovum consists of yolk bound by a glycoprotein matrix called the perivitelline layer (PVL). The population of sperm in the infundibulum results in fertilisation occurring within approximately 15 minutes of ovulation. The sperm reach the germinal disc (from where the embryo develops), probably by chemotaxis (Howarth & Digby 1973), and undergo the acrosome reaction on contact with the PVL producing a hole which they pass through to the inside of the ovum. In birds, multiple sperm can pass through the PVL (polyspermy) without disrupting development (physiological polyspermy: Snook et al. 2011)

although only one sperm fuses with the female pronucleus situated at the centre of the germinal disc. A second glycoprotein layer, the outer perivitelline layer (OPVL) is then laid down around the original PVL (now called the inner perivitelline layer - IPVL). Additional sperm around the ovum at this time are embedded in the OPVL. Together, the IPVL and the OPVL provide an accurate representation of the number of sperm that reached the ovum: the holes in the IPVL plus the number of sperm in the OPVL are proportional to the number of sperm reaching the SSTs (Brillard & Antoine 1990; Brillard & Bakst 1990). After fertilisation, the ovum then spends approximately 2 h moving down the tract though the magnum and isthmus, where proteins are secreted and albumen enlarges the ovum. The shell is added in the uterus during the next 18 - 20 h before the egg is laid via the vagina. The whole process (ovulation to oviposition) takes approximately 24 h in the majority of bird species (for some exceptions see Birkhead et al. 2011).

1.2: Study species

The zebra finch is a small (approximately 17g in captivity), sexually dichromatic finch native to Australia and the Lesser Sunda Islands (Zann 1996). Since domestication in the 1870s (Zann 1996), zebra finches have been popular pets, in addition to being reared in laboratories for scientific research. They are relatively cheap to maintain and will breed all year round when provided with suitable conditions (Zann 1996).

The zebra finch is a model species for a variety of scientific disciplines including neurobiology, song development and physiology (Nordeen et al. 1986; Burek et al 1991; Zann & Cash 2008) and is extensively used in behavioural ecology, including postcopulatory sexual selection (Birkhead et al. 1988a; Birkhead & Fletcher 1995b; Birkhead et al. 1998; Forstmeier & Birkhead 2004). The low incidence of extra-pair copulations in zebra finches (Birkhead et al. 1990; Griffith et al. 2010) indicates they experience low risk of sperm competition. Minimal sperm competition may explain high variation in sperm phenotypes, which means zebra finches are a useful model to understand how differences in sperm phenotypes influence fertilisation success. More recently, following the completion of the zebra finch genome project (Warren et al. 2010), and several other avian genomic resources (Stapley et al. 2008; 2010; Dawson et al. 2010), the zebra finch has become a useful species for genetic research, with the aim of understanding the genetic basis of phenotypic variation.

1.3: Thesis outline

The primary aim of this thesis was to establish how sperm morphology influences fertilisation success in a competitive context in a passerine bird, the zebra finch. Figure 3 illustrates how the chapters and appendices link together.

In Chapter 2, three important procedures underpinning the work carried out for this thesis are described in detail.

In Chapter 3, I describe how I established selection lines to artificially select for a divergence in the total length of sperm in a population of zebra finches. I then describe the changes in the dimensions of the following sperm components: (i) head, (ii) midpiece, (iii) tail, and (iv) total length. The phenotypic relationships between the individual components of sperm morphology (head, midpiece and tail) are then described to understand if different sperm designs exist between the selection lines.

In Chapter 4, the phenotypic patterns observed in the previous chapter are further investigated by characterising the underlying genetic relationships between the three components of sperm morphology: (i) head, (ii) midpiece, and (iii) tail. Using the additive genetic variance covariance matrix of the three sperm components for each selection line, differences in genetic variance are statistically described and visualised to understand how sperm design and future adaptive change may be constrained.

In Chapter 5, I examine how variation in sperm morphology influences sperm swimming velocity, primarily using four measures of absolute sperm morphology (see above), and then by using relative measures of sperm morphology in the form of ratios between specific sperm components.

Chapter 6 then experimentally tests how sperm morphology determines fertilisation success in the zebra finch. This is carried out by competing long sperm males against short sperm males in a series of controlled sperm competition trials. Fertilisation success is determined in two ways: (i) by counting the number of sperm from each male that reached the ovum, and (ii) assigning paternity to each resultant embryo.

Chapter 7 summarises the main findings above, and highlights directions for future research.

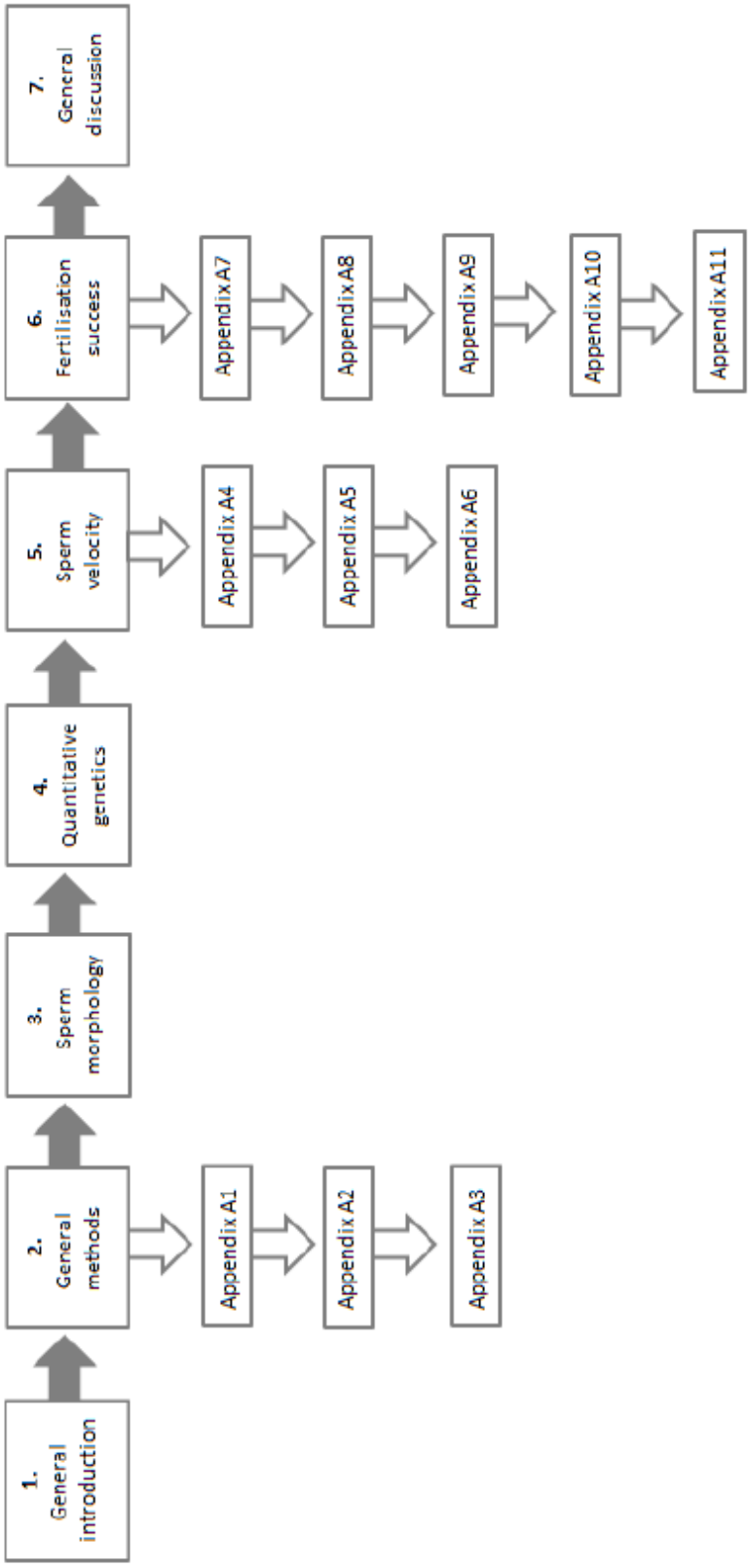


Figure 3. A flow diagram to illustrate how the chapters and appendices are related. The number of the appendix (e.g. A1) relates to the order in which that appendix is referred to in the thesis. The data presented in each appendix is more relevant to the chapter that it appears below in the diagram, although some appendices may be referred to in other chapters as appropriate.

1.4: Terminology

Cohort

A cohort describes the group of zebra finches bred in a defined period of time, i.e. cohort 1 was bred in the first year of the selective breeding regime and comprises birds from all three selection lines (long, intermediate and short). Because the parent birds for breeding each new cohort were chosen on the basis of estimated breeding values, some birds contributed offspring to more than one cohort. Therefore, the term 'generation' was deemed unsuitable to describe each cohort of birds.

Estimated breeding value

This is a measure of the breeding potential of an individual with regards to a specific trait and is vital for use in selective breeding programmes. Values are obtained by complex models, such as a special type of linear mixed model called the animal model (Kruuk 2004), and require a pedigree combined with phenotypic measurement for the specific trait under selection.

Sperm component

The individual parts that make up a sperm; in passerine birds, a typical sperm comprises the head, midpiece and tail.

Sperm design

Sperm design is a non-specific term used to encompass sperm size, shape and associations between the component parts.

Sperm morphology

This is the absolute dimensions of the sperm components, and their combined measures of flagellum and total length.

Sperm morphotype

This term is used to describe and separate individual sperm into one of two groups according to the different associations between sperm components. In this thesis, the relationship between midpiece and total length is used to make this division.

Sperm motility

Sperm motility is a general term, which describes when sperm are able to swim independently by moving the flagellum.

Sperm velocity

Sperm velocity is used to describe sperm that swim with direction (forward) and measureable speeds, and is measured by various kinematic parameters, each describing specific aspects of sperm motility.

Chapter 2

General Methods

2: Introduction

There are three key aspects underpinning the work described in Chapters 3 to 6: (i) the zebra finch selective breeding regime, (ii) obtaining sperm from male zebra finches, and (iii) sperm quality assays. The methods for each of these are described in detail in this chapter to avoid unnecessary repetition. In Chapters 3 to 6, these methods will be mentioned briefly and the reader will be directed back to this section for further details.

2.1: Zebra finches

All zebra finches used in this study were from a domesticated, outbred, pedigreed population that has been maintained at the University of Sheffield since 1985. The birds were housed at 18-24^{o}C on 14:10 light: dark cycle using Osram L 36W/965 Deluxe Cool Daylight light bulbs controlled by a Lutron Grafik integrale GXI-3000 system. The birds receive a standard zebra finch diet with ratios of 4:4:2:2:2:1 of white, panicum and yellow millet, canary seed, Japanese and red millet (purchased from Haiths, UK). Water, grit and cuttlefish were available *ad libitum*, supplemented weekly by lettuce, orange, millet spray and Abidec® liquid vitamins (approximately 3 drops per litre of water). Breeding pairs with chicks received egg-food (soaked seed and boiled egg) daily.

2.2: Zebra finch selective breeding regime

The selective breeding regime described here produced all of the zebra finches used in Chapters 3 to 6.

Three selection lines were created in October 2009 from the available population of zebra finches; long (L-line), intermediate (I-line) and short (S-line). The breeding regime aimed to increase the existing variation observed in sperm total length (Birkhead et al. 2005). The estimated breeding values (EBVs) for all zebra finches were originally computed using the Animal model (Kruuk 2004) in AsREML v2.0 (Gilmour et al. 2006), which used an extensive pedigree of the whole population (n > 8000 birds) and the available sperm total length data obtained for each male (see Section 2.4.2). Although females do not produce sperm, the Animal model still computes EBVs for sperm total length for each female using the phenotypic

data from her male relatives. Thus females that are expected to produce sons with long or short sperm can be distinguished.

The EBVs were imported into a custom-made database for the zebra finch long term study (Sunadal Data Solutions), which produced a list of male-female pair combinations and associated pair-average EBVs. Pairings were made between birds only if the EBVs of both birds were within ± 1.0 unit of each other. The list of pairs was sorted in numeric order by pair-average EBV and from this, 90 unique pairs were selected for the selection lines (30 in each line). Thirty pairs were selected from both extremes of the pair average EBV distribution for the L- and S-line (positive and negative values of EBV respectively). The 30 pairs for the I-line were chosen from the centre of the distribution where the pair average EBV was nearest to zero (each male and female EBV was around zero), to produce males with sperm phenotype midway between sperm in the L- and S-line.

The breeding pairs were housed in cages (dimensions: 0.6 x 0.5 x 0.4m) and provided with a nest box half filled with sterile hay. The nest boxes were checked daily, and egg lay date, and subsequently chick hatching date (incubation length is 14 d) were recorded. Chicks were weighed on the morning of hatching (to the nearest 0.01g) using a Sartorius Acculab balance. Each chick was marked using permanent non-toxic marker pens to enable identification until ringing and weights were recorded on 5, 10, 20, 30 and 40 d post hatching. Chicks were ringed with a closed metal identification ring on the right leg at 10 d old. Juveniles were separated from the parents when the youngest chick reached 40 d old and housed in mixed sex groups of multiple families until sexual maturity at around 100 d, after which they were housed as described in Section 2.3.1.

The following morphological measurements were recorded for all birds at 100 d old: mass (to the nearest 0.01g), left tarsus length (to the nearest 0.01mm using digital callipers (Mitutoyo (UK) Ltd), which is used as a measure of skeletal size (Birkhead et al. 2006b), and bill colour. Bill colour was assigned using colour cards on an ordinal scale (0-6), where 0 is light orange and 6 is dark red. The observer consistency was high for both tarsus length (tarsus; $r = 0.98$, $F_{49, 50} = 129.44$, $p < 0.0001$) and bill colour (Spearman rank correlation, $r_s = 0.97$, d.f = 48, $p < 0.0001$). A similar method of scoring bill colour using Munsell colour chips was previously shown to be consistent both across observers (Birkhead et al. 1998) and against a spectrophotometer (Birkhead et al. 1998). All body mass data were collected between 1400-1600 h GMT to avoid diurnal effects (Metcalfe & Ure 1995). All morphological data were collected by the same person to minimise observer error. These data are presented in Appendix A1.

A sperm sample was also collected from each male zebra finch at 100 d (Section 2.3.3). Five sperm with a normal morphology, i.e. undamaged and no developmental abnormalities, were measured (Section 2.4.2) to give the sperm phenotype of each male. Five sperm are sufficient to capture nearly all of the variation in sperm length in zebra finches (see Appendix A2). The sperm measurement data were used to re-calculate new EBVs and select the next set of 90 pairs to breed the next cohort of each selection line.

Within each selection line, three cohorts of zebra finches were produced as described above. In this thesis, a cohort refers to all birds that hatched from all three selection lines in a given breeding season. The EBVs were originally produced using AsREML (as discussed above). Subsequently, the EBVs were produced using MCMCglmm (Hadfield 2010) in R (R Core Team 2012). The EBVs produced by AsREML and MCMCglmm were significantly positively correlated (Pearson's correlation; $r = 0.44$, $d.f = 446$, $p < 0.0001$).

2.3: Sperm collection

2.3.1: Experimental male zebra finches

All zebra finches used in Chapters 4-6 were housed in groups of 20 birds, with a group of 10 males separated from 10 females by a wire divider in a row of 4 cages (dimensions: 0.6 x 0.5 x 0.4m per cage). This arrangement allowed males visual and acoustic (but not physical) contact with females, encouraging them to produce sperm while avoiding the risk of sperm depletion that would occur if males and females were housed together and allowed to copulate (Birkhead & Fletcher 1995a). Although increased sperm production by close contact has not been tested in detail this study, preliminary trials demonstrated that the seminal glomera (SG) of male zebra finches housed in this way contained greater amounts of mature sperm than males housed without visual access to females (pers. obs).

2.3.2: Obtaining sperm from male zebra finches

There are two reliable methods to obtain sperm from male zebra finches: (i) dead sperm, from the liquid part of faeces (Immler & Birkhead 2005), and (ii) live sperm, by dissection of the SG. Although natural ejaculates can be obtained using a dummy female (Pellat & Birkhead 1994), this method is only successful for few males (Birkhead & Fletcher 1995b), and was not suitable

for use in the present study as sperm samples were required from specific males at specific times.

2.3.3: Sperm collection from faeces

This technique collects dead sperm from males to analyse sperm morphology. Male zebra finches were placed in a cage with a non-absorbent cage liner. After defecation the liquid part of the faeces (which contains sperm released from the SG) was collected using a Gilson pipette and fixed in 500µl of 5% formalin. There is no morphological difference between sperm collected using this technique and from natural ejaculates (Immler & Birkhead 2005).

2.3.4: Sperm collection by dissection

All male zebra finches dissected to obtain live sperm were housed as described in Section 2.3.1. Body mass (to the nearest 0.01g), the left tarsus length (to the nearest 0.01mm) and bill colour (ordinal scale 0-6) were recorded. Males were humanely killed by cervical dislocation and the dissection was carried out immediately as follows. The male reproductive tract was exposed by removing the abdominal organs, and the left SG (Figure 1) was removed and placed in a small petri dish containing phosphate buffered saline solution (PBS). The SG was dissected free of the ureter and any excess tissue, and washed repeatedly in clean PBS to remove fat residue.

Mature sperm were collected from the distal portion of the SG (nearest to the cloaca; Figure 2) by holding the proximal end of the SG with fine forceps and squeezing the sperm from the distal end of the SG into a small petri dish containing Hams F10 media (Life Technologies™) warmed to 38^{o}C. The sperm resemble a pink sausage-like bundle. The sperm are activated by the warm media, and start to swim out from the sperm bundle into the surrounding media in a cloud. After waiting around 10 s, sperm were collected from the edge of the cloud (Figure 3) using a Gilson pipette and these sperm samples were used for the sperm quality assays (Section 2.4 of this Chapter).

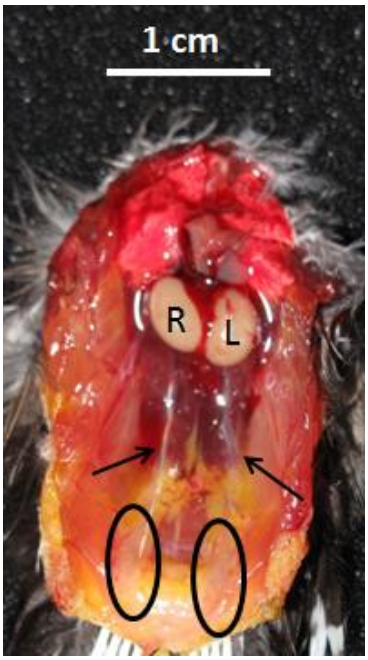


Figure 1. The organisation of the male reproductive system. The left (L) and right (R) testes produce sperm that are carried to the corresponding SG via the vas deferens (indicated by arrows). The positions of the left and right SG are shown by the black circles. The scale bar represents 1cm. Sperm were obtained from the left SG in all experiments conducted in this thesis.

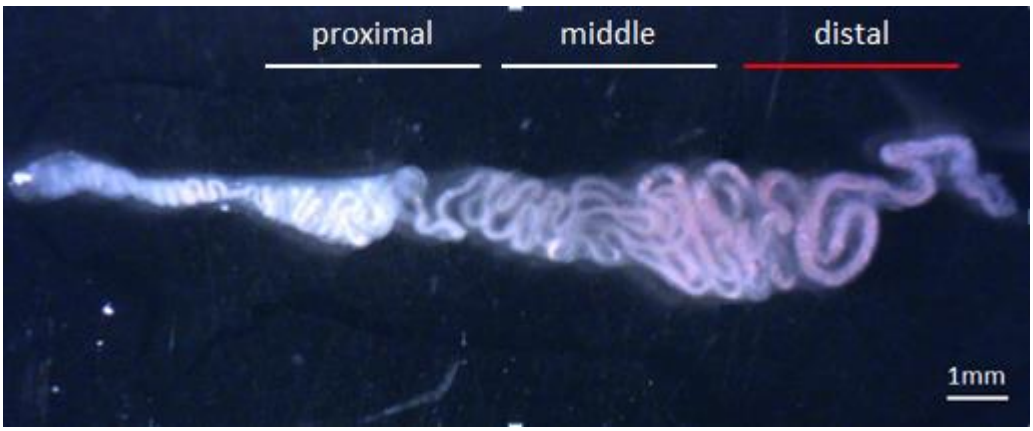


Figure 2. A dissected left seminal glomerus (SG) from a male zebra finch. Bars represent the proximal, middle and distal portions of the SG. The red bar indicates the portion of the SG containing mature sperm that was used for sperm quality assays. Only the distal portion was sampled because sperm velocity varies between the proximal and distal portions of the SG (Birkhead et al. 1995).

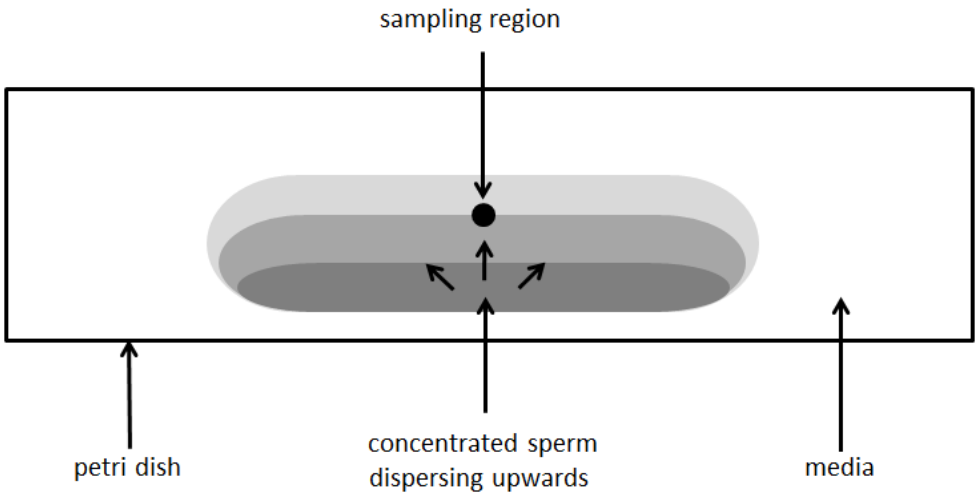


Figure 3. Schematic illustrating sperm sampling using the 'swim out' technique. The sperm are squeezed out of the SG into the warm media and start to swim. The sampling region indicates where sperm were collected from. Note that when the sperm concentration was low, a sperm cloud was not observed and sampling was carried out immediately without delay to avoid the loss of sperm into the surrounding media.

2.4: Sperm quality assays

2.4.1: Sperm motility

Sperm motility was assessed by Computer Assisted Sperm Analysis (CASA) which involves recording and analysing videos of swimming sperm. Video clips of sperm were captured using the Sperm Class Analyzer® (SCA) (Microptic, Barcelona, Spain) with a Basler acA780-75gc camera connected to an Olympus BX41 microscope (Figure 4). Each 1 s video clip was captured using pseudo negative phase at x200 magnification with the 0.5x c-mount and the capture settings listed in Table 1. Successive clips were recorded from different areas of the sample in a systematic manner.

Sperm are recognised by the CASA software based on differences in contrast between the sperm head and the background of the sample, allowing the software to track the movement of each sperm. The captured sperm track can be subdivided into various kinematic (motion) parameters for analysis of overall sperm motility and velocity (swimming speed). Five kinematic parameters (Figure 5; Table 2) were used to describe sperm swimming motion: (i)

average path velocity (VAP), (ii) curvilinear velocity (VCL), (iii) straight line velocity (VSL), (iv) straightness (STR), and (v) linearity (LIN).

Four microlitres of sperm solution was collected using a Gilson pipette and warm tips. The sample was loaded into a 20µm slide chamber (Leja®, Netherlands) and allowed to equilibrate on the microscope heated stage (heated to 38°C) for 30 s. Initial trials were carried out using 40°C (mean avian body temperature: Prinzinger et al. 2001); however, this higher temperature increased evaporation and sample drift. The number of videos clips recorded per male varied depending on the concentration of the sperm sample. Five individual kinematic parameters for each sperm were then extracted (i) VAP, (ii) VCL, (iii) VSL, (iv) STR, and (v) LIN. At least 100 sperm were tracked per male. A cumulative means analysis was performed on the sperm samples of 3 males and showed that analysing a minimum of 100 sperm resulted in consistent estimates of the mean for each sample for VAP, VCL and VSL. Figure 6 shows the cumulative mean plots for VAP, VCL and VSL from the sperm sample of a single male, representative of the males investigated.

CASA systems 'recognise' the sperm head based on the internal settings of the software, e.g. the particle size range and contrast levels. Therefore, cell debris of similar size to sperm heads may be incorrectly tracked as sperm (Mossman et al. 2009). Cell debris and dead sperm may also move slowly by drift or by Brownian motion, falsely inflating the number of motile sperm, but also reducing the mean kinematic value of the sample. Cell debris was removed manually using the software before analysis. However, because deciding whether a sperm was drifting or swimming slowly was more subjective the following procedure was used to identify the kinematic profile of drifting sperm. Three samples of dead sperm were videoed using the same capture settings (Table 1) and the individual kinematic parameters (VAP, VCL and VSL) extracted for each dead sperm. Biplots of VAP, VCL and VSL (Figure 7) revealed the kinematic profile of sperm drifting or moving by Brownian motion. The biplots were used to identify threshold values of VAP, VCL and VSL to use to filter the real data set. Sperm with kinematic values below this threshold are presumed to be immotile. The following threshold values were selected: VAP = 7.5µm/s; VCL = 14.0µm/s and VSL = 2.5µm/s. These values encompassed over 95% of the dead sperm shown in Figure 7, but were not increased to include all the dead sperm as this may remove sperm that swam extremely slowly in the actual dataset (pers. obs). In the actual data set, sperm with values below the threshold for all three parameters were classified as immotile.

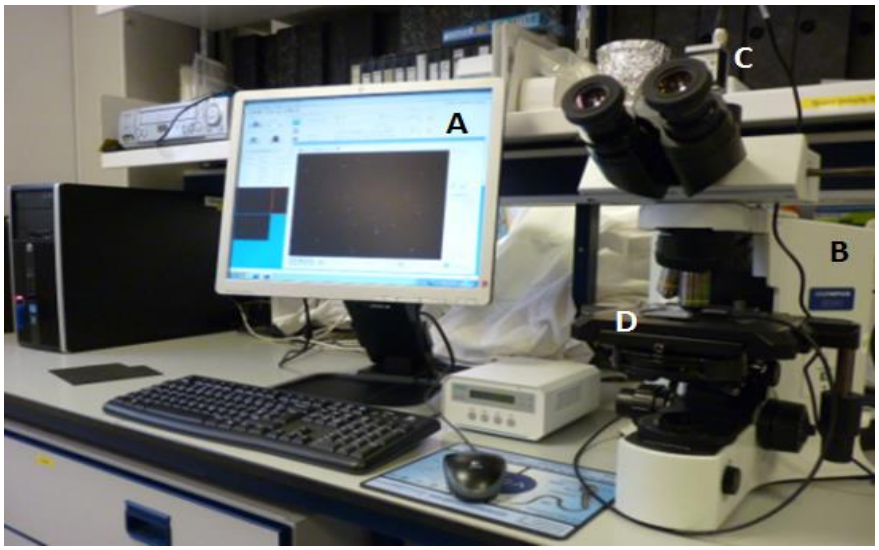


Figure 4. The CASA system used in the motility assay. (A) The computer loaded with SCA software is connected to a microscope (B) and microscope camera (C). A heated microscope stage (D) is essential to maintain sperm samples at 38^{o}C during analyses.

Table 1. The settings used in video capture and analysis of the motility data obtained from male zebra finches.

Setting/requirement	**Value**
Heated stage temperature (^{o}C)	38
Slide chamber depth (µm)	20
Frame rate (fps^{-1})	50[1]
Length of capture (s^{-1})	1
Aspect ratio	1:1
Brightness/contrast	Adjusted for each sample
Minimum track time (frames)	10
Connectivity	18
VAP points	5
Particle size (µm^{2})	2-200
Ideal sperm concentration (x10^{6}/ml)	4-8

[1]Motility data for a subset of males were recorded at 37fps due to an internal error between the software and microscope camera. As the difference in frame rate did not affect the values of the kinematic parameters (Appendix A3), the data obtained from males using 37fps (n = 42) were included in the large data set and analysed in Chapter 5.

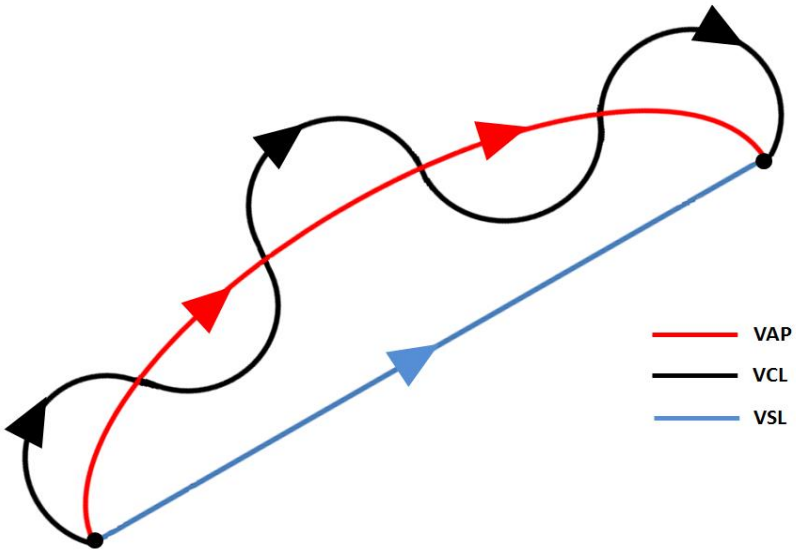


Figure 5. A singe sperm track illustrating three kinematic parameters (VAP, VCL and VSL) measured by CASA. Two additional parameters STR and LIN are calculated by VSL/VAP and VSL/VCL respectively.

Table 2. Description of five kinematic parameters used in CASA. All parameters are measured in two dimensions.

Kinematic parameter	**Description**
VAP (μm s^{-1})	Time average velocity of the sperm head along the average trajectory. The curvilinear path is smoothed by program specific algorithms to achieve this
VCL (μm s^{-1})	Time averaged velocity of the sperm head along the actual two dimensional curvilinear path
VSL (μm s^{-1})	Time averaged velocity of the sperm head along the straight path between the first and last detected position
STR	The linearity of the average path (VSL/VAP)
LIN	The linearity of the curvilinear path (VSL/VCL)

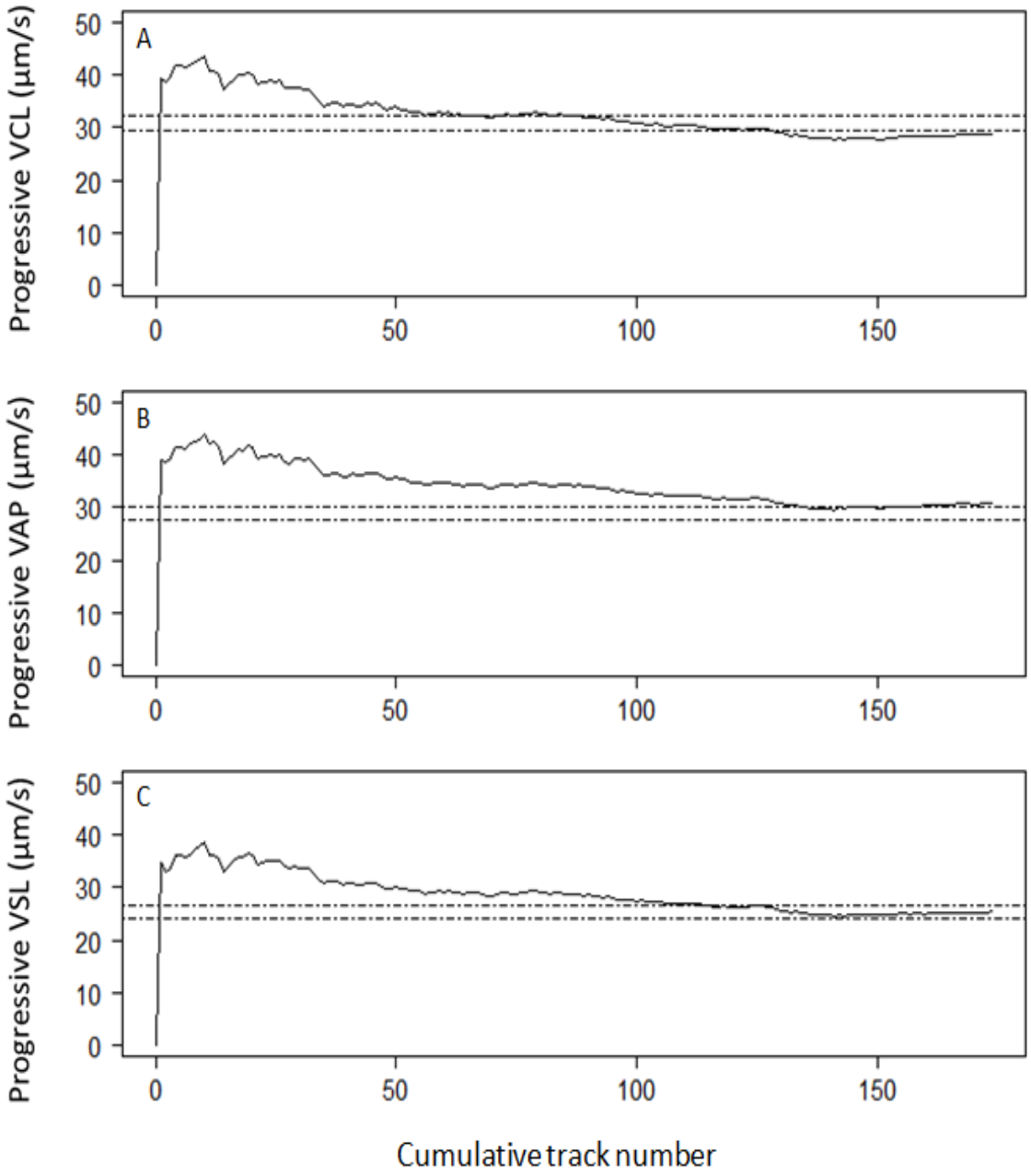


Figure 6. Cumulative sampling plots for (A) VAP, (B) VCL and (C) VSL for a single male zebra finch. As the number of sperm tracked increases, the cumulative mean (solid line) gradually stabilises. When over 100 sperm are tracked, the cumulative mean varies within 1 standard error of the total sample mean (dashed lines). Two additional males were analysed in this way (data not shown). This male was representative of the general pattern across these males.

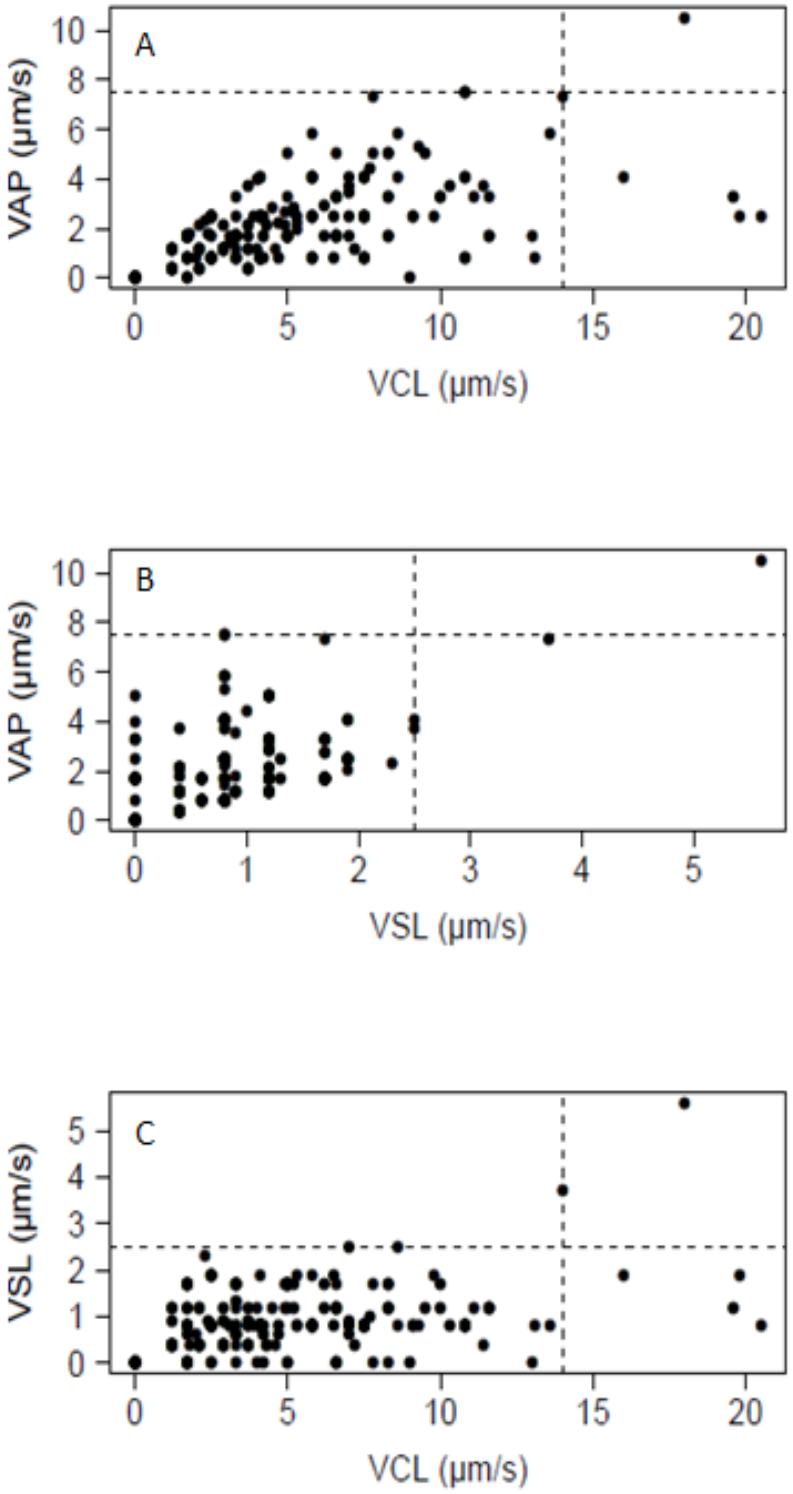


Figure 7. Biplots of the kinematic profiles of dead sperm for (A) VAP/VCL, (B) VAP/VSL and (C) VSL/VCL. Each point is a single dead sperm (n = 236). The dashed lines illustrate the values below which sperm in the actual data set would have all kinematic parameters set as zero, i.e. sperm where VAP ≤ 7.5, VCL ≤ 14 and VSL ≤ 2.5µm/s^{-1} (in the bottom left portion of each plot).

2.4.2: Sperm morphology

Five microlitres of sperm solution were collected by dissection (Section 2.3.4) and fixed in 5% formalin. Ten morphologically normal and undamaged sperm were photographed using light microscopy (Leitz Laborlux S) at x400 using an Infinity 3 camera (Luminera Corporation) and the following sperm components (Figure 8) were measured to the nearest 0.01µm (6.25 pixels per µm): (i) head, (ii) midpiece, and (iii) tail, using ImageJ (Abramoff et al. 2004) by a single researcher. Measurement repeatability (Lessels & Boag 1987) was high (r = 0.97 or greater for all sperm components: Table 3). Sperm morphology was also consistent, both within- and between-ejaculates of individual males (see Table 3 for both estimates; see also Birkhead & Fletcher (1995) for an additional estimate of within-ejaculate consistency). Flagellum and total length were calculated by adding together the values for the relevant component parts (midpiece plus tail, head plus midpiece plus tail respectively). The number of head and midpiece gyres were also counted. Straight midpiece length (the length of the midpiece when the midpiece gyres are stretched flat) was calculated using the formula in Birkhead et al. (2005). Ratios of lengths of particular sperm components were also calculated, for example, flagellum: head ratio (flagellum length divided by head length) and midpiece: tail ratio (midpiece length divided by tail length).

2.4.3: Proportions of sperm with normal morphology

The proportion of sperm with normal morphology was estimated from the same sperm sample collected for morphology analyses (Section 2.4.2). A minimum of 200 sperm were scored as normal or abnormal using a Leitz Laborlux S microscope with dark field optics and x400 magnification, giving the proportion of normal sperm per male. Normal sperm refers to sperm with no apparent damage to any component part and no visible abnormalities, such as a swollen nucleus. The specific abnormality or type of damage was recorded for all non-normal sperm, described in Table 4.

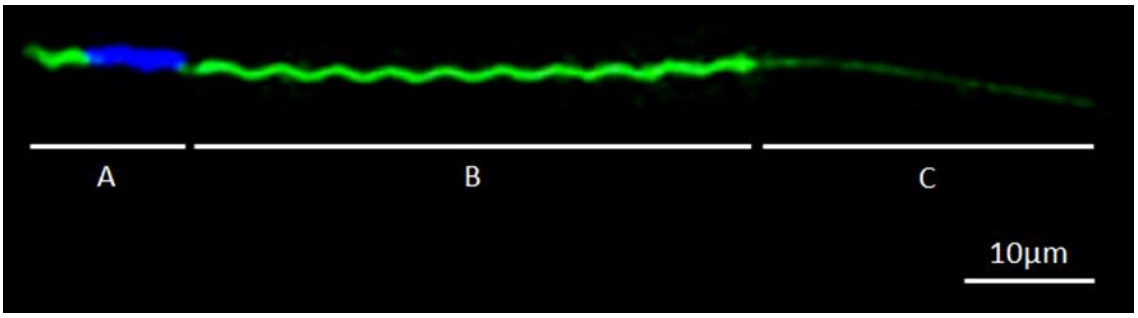


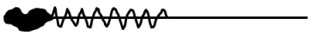
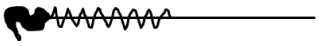
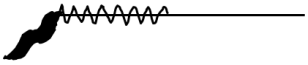
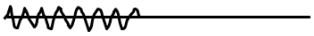
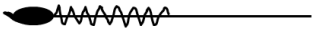
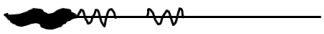
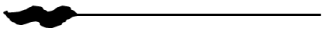
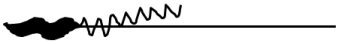
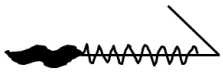
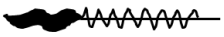
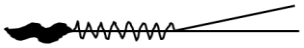
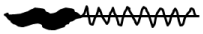
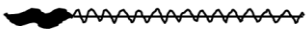
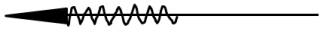
Figure 8. A single zebra finch sperm illustrating the component parts of the sperm. The sperm is counterstained with Hoescht 33342 and Mitotracker Green for ease of viewing. The head (A) is comprised of the nucleus (blue) and the acrosome (green). The midpiece helix (B) and the tail (C) together comprise the flagellum.

Table 3. Estimates of within- and between-ejaculate male consistency (r_c) and observer measurement reliability (r_r) of sperm components, calculated using methods from Lessels & Boag (1987). Significant relationships are in bold and significance is indicated by asterisks. ** indicates where $p < 0.003$ and *** indicates where $p < 0.0001$.

Sperm component (µm)	**Male consistency (r_c)**				**Observer measurement reliability (r_r)[c]**	
	Within ejaculate[a]		**Between ejaculate[b]**			
	$F_{24,350}$	**r_c[a]**	**$F_{19,20}$**	**r_c[b]**	**$F_{49,50}$**	**r_r**
Head	43.0	**0.74*** **	15.9	**0.88*** **	74.9	**0.97*** **
Midpiece	35.9	**0.70*** **	3.67	**0.57** **	1390.1	**0.99*** **
Tail	57.8	**0.79*** **	9.95	**0.82*** **	616.3	**0.98*** **
Total length	71.6	**0.82*** **	15.03	**0.89*** **	142.4	**0.93*** **

[a]Data were obtained by measuring 15 sperm from the left SG sperm samples from 25 males.
[b]Data were obtained by measuring 5 sperm from faecal samples from 20 different males. Sperm were collected from each male on two occasions at least two months apart. Mean values per ejaculate for each male were compared.
[c]Data were obtained by measuring the same 5 sperm from faecal samples from 10 different males twice.

Table 4. Common categories of sperm found in SG sperm samples of male zebra finches. Sperm possessing any of these morphological characteristics were classified as morphologically abnormal.

Component	Characteristic	Illustration of abnormality	Presumed reason for abnormality
Head	Acrosome missing		Aged
	Acrosome reduced/bent		Aged
	Bent at 'neck'		Damage
	Head missing		Damage
	Nucleus swollen		Aged
Midpiece	Bent		Damaged
	Broken		Damaged
	Missing		Damaged
	Unravelled		Damaged
Tail	Bent		Damaged
	Broken		Damaged
	Multiple tails		Developmental
	Missing		Damaged
Other	Immature sperm		Developmental
	Spear shaped sperm		Developmental

2.4.4: Sperm viability

Sperm viability was estimated using a membrane integrity assay: Live/Dead Viability kit (Invitrogen™). The viability dyes were prepared as follows: 1µl of 2.4mM propidium iodide (PI) was diluted using 200µl PBS and 1µl of 1mM SYBR14 was diluted with 10µl of dimethyl sulphoxide (DMSO). A small sample (0.5µl) of sperm was collected (Figure 3) and placed on a microscope slide. Three microlitres each of PI and SYBR14 were pipetted onto the sperm sample and covered with a coverslip, then incubated in the dark for 5 minutes. Using a fluorescent microscope (Leica DMBL) at x200 magnification, a minimum of 200 sperm were scored as having an 'intact membrane' or 'damaged membrane' according to the colour of the nucleus. Differential staining of the nucleus operates on the basis that SYBR14 stains all nuclei green by binding to DNA, whereas PI can only pass through damaged membranes. Damaged nuclei exhibit red fluorescence because PI is dominant over SYBR14.

2.4.5: Sperm longevity

The remaining sperm in the left SG were collected as follows: the SG was visually divided into thirds (Figure 2) and the distal third portion containing the remainder of the mature sperm was unravelled into a long tube. The sperm were carefully squeezed out of the tube using fine forceps into 15µl of Ham's F10 media at room temperature, resulting in a concentrated sperm solution.

To assess *in vitro* sperm longevity (quantified as the length of time all sperm remained motile), the concentrated sperm solution was divided into 1µl aliquots and diluted with an additional 3µl of Ham's F10 media. One of the aliquots was saved for concentration analysis (Section 2.4.6). The remaining aliquots were placed in a water bath at 38^{o}C. At 15 minute intervals an individual aliquot was diluted with warm media and loaded onto a slide chamber and at least 5 video clips recorded (Section 2.4.1). The amount of extra media added varied depending on the concentration of the sperm, as the concentration must be low enough to allow successful video capture (G. van der Horst, pers. comm). The assay ended when two successive aliquots contained no motile sperm. The number of motile sperm on each video were counted and divided by the total number of sperm recorded. An average of the fields was taken to give the proportion of motile sperm for each aliquot. The longevity of each male was given by the length of time until all sperm were immotile.

2.4.6: Sperm concentration

The 1µl aliquot saved for concentration analysis was made up to a volume of 100µl (1 in 100 dilution) with 5% formalin. Sperm samples were mixed for 20 s using a vortex (Autovortex mixer SA2) to disperse any sperm clumps and then a sample was immediately loaded onto an Improved Neubauer chamber. All sperm were counted across the whole grid of both sides of the Neubauer chamber and an average of the two grids was calculated giving the number of sperm found in a volume of 0.9mm^3. The sperm concentration per millilitre (ml), corrected for dilution, was calculated using the following formula (Equation 1).

$$\text{Sperm concentration} \cdot 10^6(\text{ml}) = \left(\left(\frac{\text{sperm count}}{0.9}\right) \cdot 1000\right) \cdot 100 \qquad \text{Equation 1}$$

Chapter 3

Sperm morphology and the response to artificial selection

3.1: Introduction

Sperm phenotypes (e.g. head, midpiece and tail) can evolve when (i) differences in the traits result in varying fertilisation success across males, and (ii) if the trait has additive genetic variation (i.e. it is heritable), which is the raw material of evolution. Sperm morphology determines male competitive success in a number of species (Radwan 1996; LaMuynon & Ward 1998; Gage & Morrow 2003; Garcia-Gonzalez & Simmons 2007), and the significant heritability (h^2) of sperm morphology has been repeatedly demonstrated in a range of species including rabbits *Oryctolagus cuniculus* (Napier 1961), bumble bees *Bombus terrestris* (Baer et al. 2006), dung beetles *Onthophagus taurus* (Simmons & Kotiaho 2002) and the zebra finch *Taeniopygia guttata* (Birkhead et al. 2005; Mossman et al. 2009). The heritability estimates of individual sperm components are typically high, (e.g. midpiece length of mice = 0.76 ± 0.02 (mean ± S.E) (Woolley 1971)) when compared to morphological traits (Mousseau & Roff 1987; Simmons & Moore 2009), meaning that sperm morphology is under strong genetic control.

Artificial selection experiments have demonstrated that sperm morphology can respond to selection (Woolley 1971; Morrow & Gage 2001a; Miller & Pitnick 2002; Dobler & Hosken 2010). However, phenotypic traits may not always respond to artificial selection despite significant additive genetic variation, because the genetic architecture may be constrained in some way, thus limiting evolutionary change (Blows & Hoffmann 2005). Strong phenotypic correlations between sperm components (e.g. between the midpiece and flagellum of passerine sperm) may indicate design constraints that limit the potential for phenotypic change, if these associations are underpinned by similar genetic relationships.

Across passerine birds there is a strong positive association between the length of the midpiece and the flagellum of sperm (Immler & Birkhead 2007; Lüpold et al. 2009a). Although these two sperm components are not independent (because the midpiece is part of the flagellum), the relationship between them is important because it may be determined by the energetic demands of the sperm (Immler & Birkhead 2007; Lüpold et al. 2009a), by a longer midpiece providing more energy that results in faster swimming velocity (Laskemoen et al. 2010; but see Malo et al. 2006; Rowe et al. 2013).

Interestingly, previous research on zebra finch sperm morphology did not detect a positive association between midpiece and flagellum length (Birkhead et al. 2005; Figure 1A); instead, there was an odd distribution between the two variables described by a weak negative

relationship, suggesting that midpiece length decreased as sperm length increased. However, after examination of the relationship reported by Birkhead et al. (2005), it seems likely that the negative correlation between midpiece and flagellum length does not describe the data particularly well. Instead, we propose that the distribution of sperm in Figure 1A may comprise at least two sperm morphotypes (an idea that is, at present, untested): (i) sperm with a long midpiece that comprises the majority of the flagellum, and (ii) sperm where the midpiece is relatively short compared to the flagellum. This variable sperm design is unlikely to be a consequence of zebra finch domestication as a very similar distribution has been observed in wild zebra finches (Immler & Birkhead 2012; Figure 1B).

The extreme variation in sperm morphology and sperm design means that the zebra finch is a useful species to explore the relationships between sperm form and function. Given that there is extensive phenotypic variation, and all sperm components are heritable (Birkhead et al. 2005; Mossman et al. 2009), it should be possible to apply artificial selection to experimentally alter sperm total length in this species. This would allow the relationships and constraints between sperm components to be explored, and would provide a population of birds suitable for investigating the impact of changing sperm morphology on sperm function. Care should be taken, however, that the outcomes of analyses are not affected by the odd distribution of associations between sperm components.

Aim

The aim of this study was to conduct bidirectional artificial selection on sperm total length, and then describe the resulting changes in sperm morphology, specifically the changes in the head, midpiece, tail and total length dimensions. Because total length is highly heritable (estimated to be 0.63 ± 0.11 (mean ± S.E), $p < 0.001$; Mossman et al. 2009), it was expected that males in the long selection line would produce significantly longer sperm than males in the short selection line. An additional aim was to establish how artificial selection on total length influenced the relationships between all other sperm components, in particular to obtain insights into the unusual relationship between midpiece and flagellum length in the zebra finch.

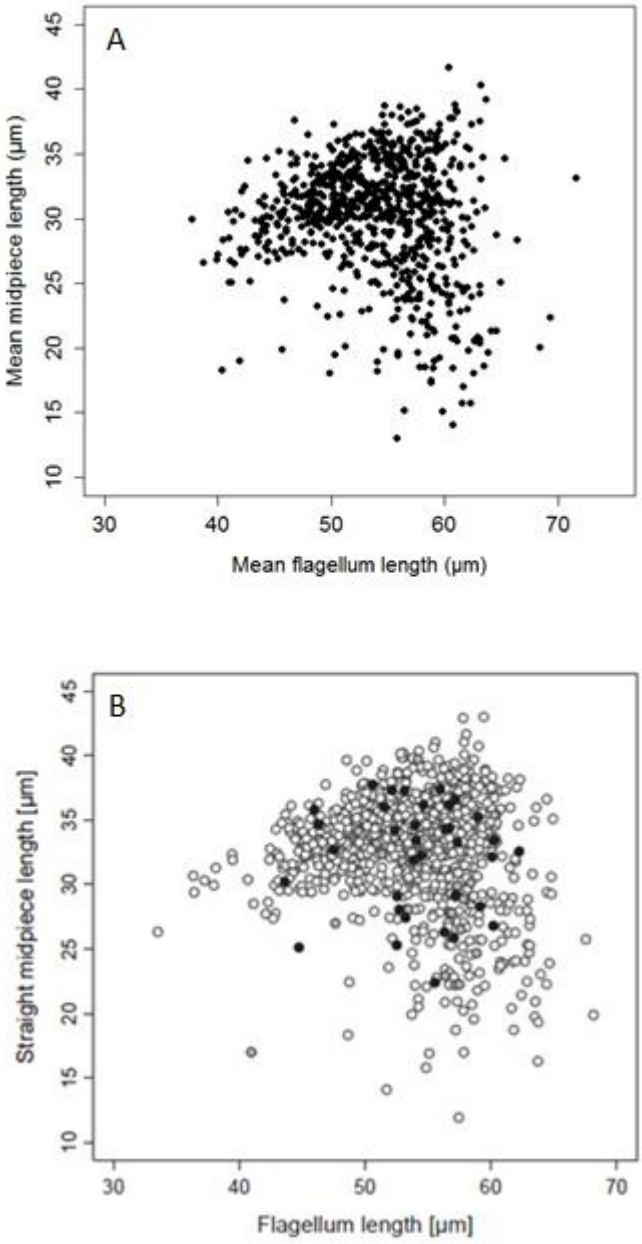


Figure 1. Relationships between midpiece length and flagellum length in two datasets of zebra finch sperm morphology. Each point is the mean value of sperm components for a single bird. Note that flagellum and total length are strongly correlated (r = 0.99) and so can be considered interchangeably. (A) A subset of birds analysed in Birkhead et al. (2005). There was a weak but significant negative correlation (r = -0.085, d.f = 772, p < 0.018) between the sperm components. (B) Three populations of wild zebra finches (n = 914) from Immler & Birkhead (2012). There was a negative correlation between midpiece and flagellum length in all populations. Both distributions indicate that there may be at least two sperm morphotypes occurring across zebra finch males, defined by the variation in midpiece length for a given sperm length.

3.2: Methods

Study population

The zebra finches used in this study were bred from October 2009 until July 2011 using an intensive selective breeding regime (Chapter 2 Section 2.2), which aimed to increase the divergence of sperm total length across the population using bidirectional artificial selection. Three selection lines (long (L-line), intermediate (I-line) and short (S-line)) each comprising thirty pairs of birds produced three cohorts (one cohort per year). Due to space and workload constraints it was not possible to produce replicate lines. Although there was not a true control line, bidirectional selection on the L- and S-line meant that each line acted as a control for the other line (Falconer & Mackay 1996). The I-line, producing males with values of sperm total length approximately mid-way between the males from the L- and S-line, could also be used for comparisons.

Sperm sampling

To create the three selection lines, pairs of male and female zebra finches were selected using pair-averaged estimated breeding values (EBVs) (Chapter 2 Section 2.2), such that positive or negative EBVs indicated that male offspring produced by that pair would have either long or short sperm respectively.

The sperm phenotype was assessed for each cohort of male birds by obtaining sperm samples from each bird at sexual maturity (around 100 d old (Sossinka 1980)) (see Chapter 2 Section 2.3.3). Briefly, dead sperm were collected from the faeces of each male and five morphologically normal sperm per male were measured to the nearest 0.01µm using ImageJ (Abramoff et al. 2004) for the following sperm components (see Chapter 2 Section 2.4.2): (i) head, (ii) midpiece, and (iii) tail (Figure 2). Flagellum and total length were calculated by adding together the values for the relevant component parts (midpiece plus tail, head plus midpiece plus tail respectively). Straight midpiece length was calculated from the formula in Birkhead et al. (2005). Five sperm were sufficient to capture 95% of the morphological variation for each sperm component (Birkhead et al. 2005; Appendix 2), because sperm morphology is consistent within the ejaculate of a given male (Chapter 2 Section 2.4.2).

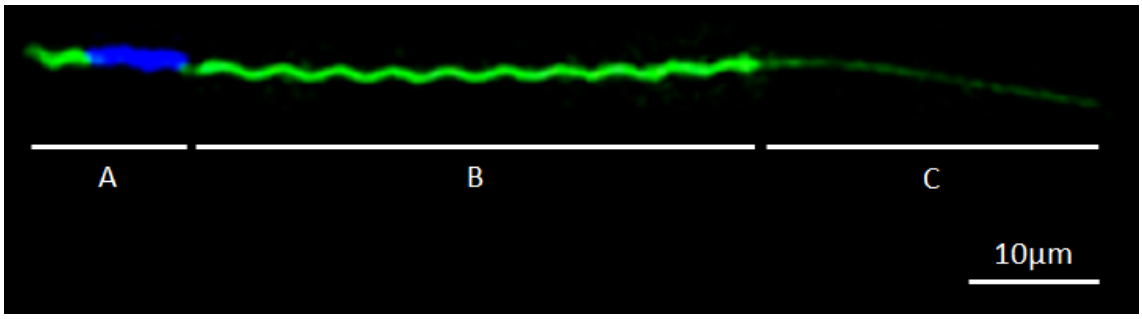


Figure 2. The structure of single zebra finch sperm. The sperm is counterstained with Hoescht 33342 and Mitotracker Green dyes for ease of viewing each component part. The head (A) is comprised of the nucleus (blue) and the acrosome (green). The midpiece helix (B) and the tail (C) together comprise the flagellum.

Statistical analyses

The founder cohort of each selection line was not included in analyses because sperm morphology data were available only for a subset of the founder males (S-line: n = 20, I-line: n = 13 and L-line: n = 14). Data used from cohort 1 to 3 comprise the sperm morphology of every male from each cohort (cohort 1: n = 139, cohort 2: n = 189 and cohort 3: n = 233).

The response of sperm morphology to artificial selection was analysed using linear mixed effects models (LMMs). Selection line and cohort were included as fixed effects with an interaction term between line and cohort fitted to detect divergence of sperm length occurring across the cohorts. Bird ID was included as a random effect as there were five measures of individual sperm per male. P values and highest posterior density (HPD) intervals were obtained using Markov Chain Monte Carlo (MCMC) sampling. The significance of the interaction between line and cohort was tested by comparing models with and without the interaction term, using log-likelihood tests against the Chi squared distribution with four degrees of freedom.

Pearson's correlations were used to examine the associations between the sperm components. Only data from cohort 3 were used so that the relationships observed were not obscured by between-cohort changes in sperm morphology. The mean sperm component values per male (n = 233) were used instead of the raw data to avoid achieving inflated significance values as a consequence of a large dataset.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package, lme4 (Bates et al. 2012) and languageR (Baayen 2011).

3.3: Results

There was a rapid response to artificial selection on sperm total length (Figure 3). Clear divergence was observed between the three selection lines in the predicted directions. Initial inspection of the data revealed that midpiece and straight midpiece length (SML), and flagellum and total length were positively correlated ($r = 0.92$ and $r = 0.99$ respectively, both $d.f = 874$ and both $p < 0.0001$). Therefore, only the effect of selection on midpiece length and total length is presented to avoid repetition.

The mean dimensions (± S.D) of the head, midpiece, tail and total length for each selection line across the three cohorts are shown in Table 2 and Figures 3 and 4. Because the founder cohorts are not included in the analyses, the points between the founder cohort and cohort 1 are not connected on the plots. Interestingly, sperm in the S-line (cohort 3) had approximately 50% less variation (coefficient of variation (CV) = 9.34) in midpiece length when compared to sperm from the L-line (CV = 22.40).

The effect of artificial selection on sperm morphology

The response of total sperm length to artificial selection is presented before the responses of the individual sperm components because total length was the target of the selective breeding regime.

Total length

There was a 5.86µm difference in mean total length between the L- and S-lines of the founder cohort (Figure 3A & B), because the founder cohort was divided into three lines by selecting pairs with the highest and lowest estimated breeding values (EBVs) for the L- and S-line respectively (Chapter 2 Section 2.2). Total length diverged significantly between the three lines in the predicted direction (Figure 3A and Table 3), such that in cohort 3, the L-line produced the longest sperm, I-line sperm were intermediate length, and S-line sperm were the shortest. Sperm in the S-line lines responded strongly to selection, with a 6.7% decrease in sperm total length between cohort 1 and 3 (compared to a 3.8% increase in the L-line). In addition, a significant interaction between line and cohort was detected due to the increasing overall divergence in sperm total length (Table 2 and Figure 3B).

Table 2. The mean (± S.D) of sperm components. Each selection line (S-line, I-line and L-line) produced three cohorts (one cohort produced per year of selective breeding). Sperm from the L-line had the shortest midpiece compared to sperm from the S- and I-line.

Sperm component (μm)	**Line**	**Cohort mean ± S.D**		
		1	**2**	**3**
Head	S	10.75 ± 0.43	10.59 ± 0.40	10.32 ± 0.52
	I	11.25 ± 0.54	10.70 ± 0.51	10.89 ± 0.53
	L	11.16 ± 0.60	10.87 ± 0.49	10.87 ± 0.59
Midpiece	S	30.68 ± 3.51	30.96 ± 2.89	30.87 ± 2.89
	I	27.21 ± 6.12	30.50 ± 4.65	30.81 ± 5.43
	L	30.69 ± 6.12	30.89 ± 6.42	28.08 ± 6.29
Tail	S	20.57 ± 5.52	18.89 ± 4.28	16.69 ± 4.04
	I	29.99 ± 9.07	24.09 ± 6.92	24.38 ± 7.28
	L	26.92 ± 8.55	28.47 ± 7.90	32.45 ± 8.33
Total length	S	62.00 ± 4.98	60.44 ± 4.55	57.88 ± 4.64
	I	68.45 ± 4.98	65.29 ± 4.77	66.08 ± 4.13
	L	68.77 ± 4.72	70.23 ± 4.48	71.40 ± 4.95

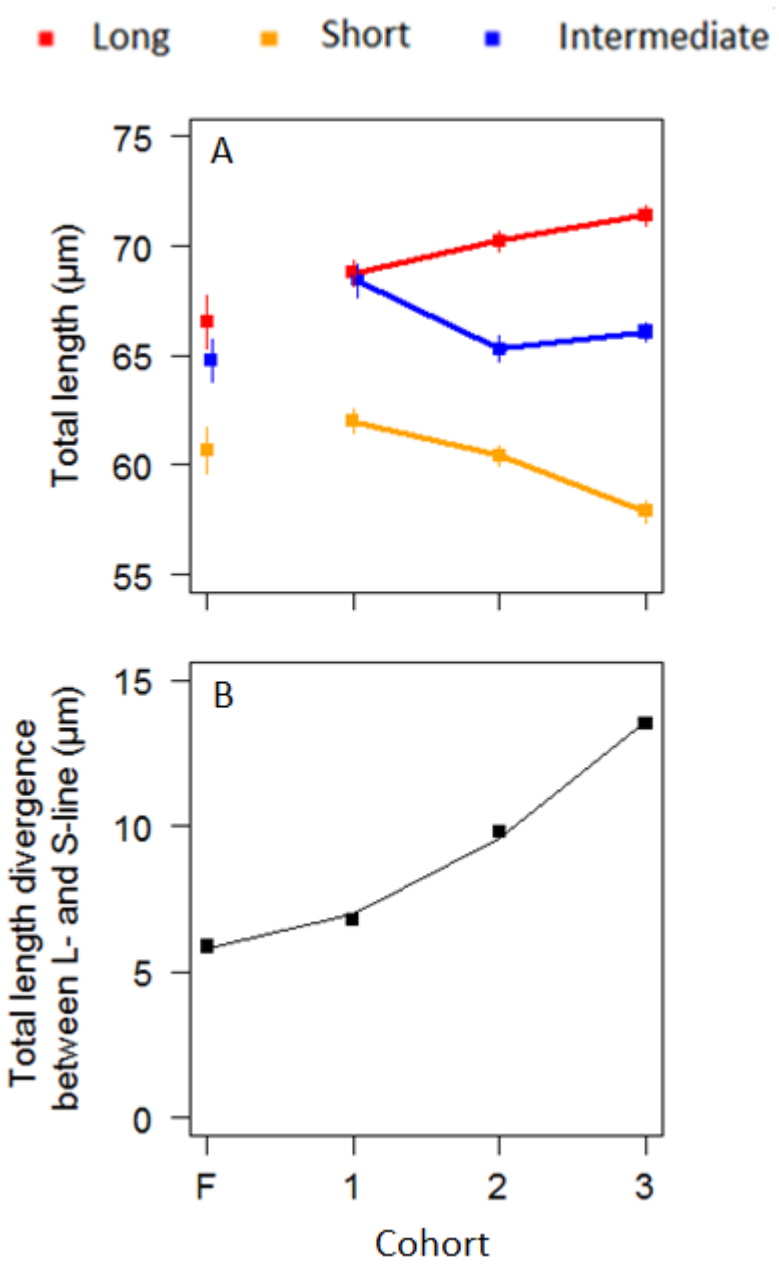


Figure 3. (A) The change in sperm total length in response to selection. Sperm measurements were available only for a subset of males in the founder cohort (S-line: n = 20, I-line: n = 13 and L-line: n = 14). The data for cohorts 1 to 3 are mean sperm measurements (5 sperm per male) from all males produced by the selective breeding regime (cohort 1 = 139; cohort 2 = 189; cohort 3 = 233). Bars represent 95% confidence intervals around the mean. (B) The divergence in mean sperm total length between the long and short selection line from the founder cohort to cohort 3. There was a 99% increase in divergence between cohort 1 and cohort 3. The equation of the regression line is $y = 0.48x + 0.705x^2 + 5.79$, where x is the cohort number and y is the difference between the S- and L-line in mean total length.

Head length

Mean sperm head length decreased across cohorts 1 to 3 in all three lines with selection on sperm total length. Head length was shortest in the S-line (Table 3; $p < 0.0001$) compared to the L- and I-line (Figure 4A). There was a significant interaction between line and cohort (Table 3) because the head length of short sperm decreased rapidly between cohort 2 and 3.

Midpiece length

Mean midpiece length remained more or less constant in the S-line across the cohorts (Figure 4B & Table 3). In cohort 3, sperm from the S- and I-line had similar midpiece lengths, whereas long sperm had significantly shorter midpieces. Overall, midpiece length did not respond to selection in a clear pattern.

Tail length

Mean tail length diverged significantly between the lines over the three cohorts (Table 3 and Figure 4C), with a similar pattern that was observed in total length (Figure 3A), suggesting that tail length may drive the observed change in total length. S-line sperm had significantly shorter tails compared to sperm from the L- and I-lines, where tails were of long and intermediate length respectively. Significant interactions were detected between line and cohort due divergence of tail length across the three cohorts.

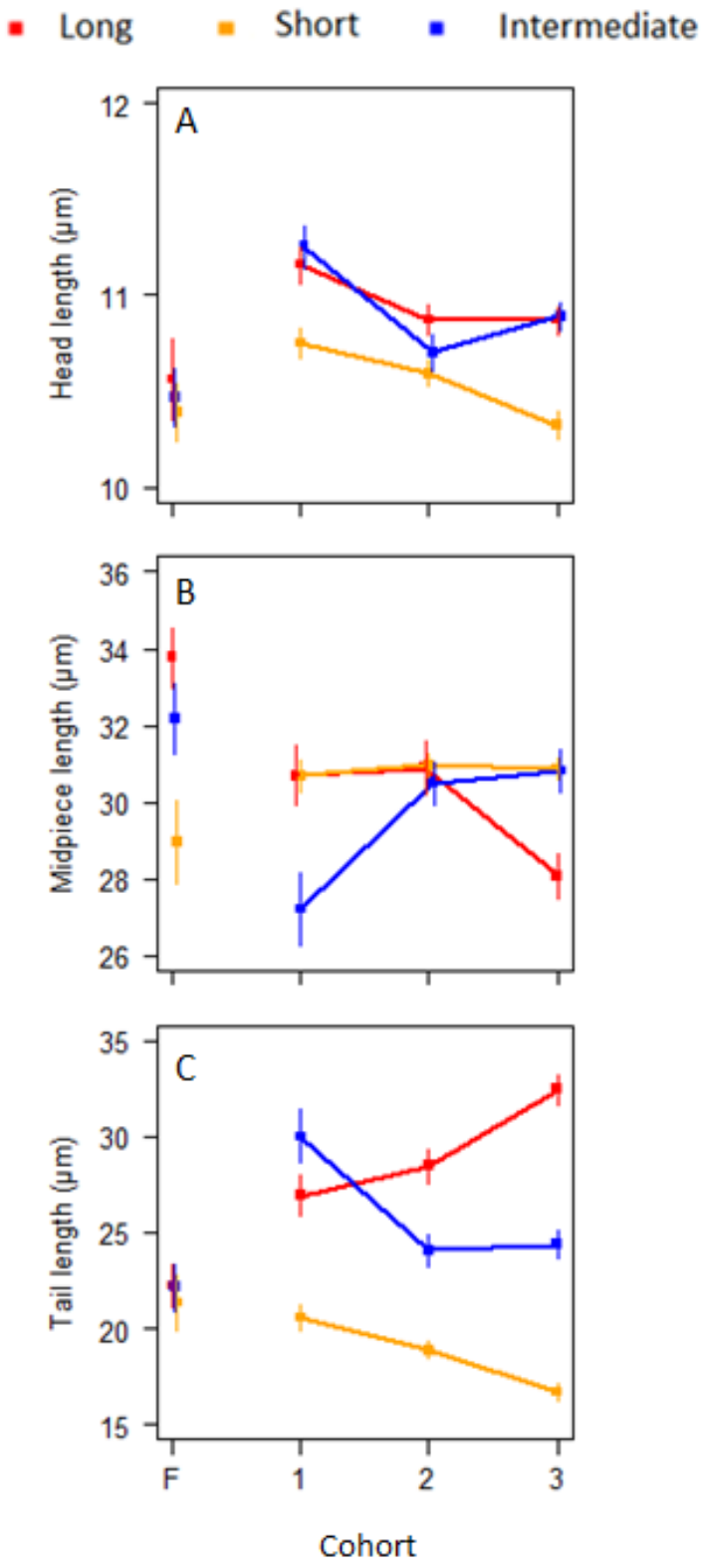


Figure 4. The change in mean sperm morphology in response to selection on sperm total length for (A) head length, (B) midpiece length and (C) tail length in the three selection lines over the three cohorts. Sperm measurements were only available for a subset of the founder (F) cohort (S-line: 20, I-line: 13; L-line: 14). The data for cohorts 1 to 3 are from males from the selective breeding regime (cohort 1: n = 139; cohort 2: n = 189; cohort 3: n = 233). Bars represent 95% confidence intervals around the mean.

Table 3. Results of LMMs analysing the effect of selection line and cohort on four components of sperm length[1]. There was a significant interaction ($p < 0.05$) between line and cohort on each sperm component (shown in bold)[b].

Sperm component (μm)	Fixed effect	T[a] $\chi 2$[b]	HPD (lower, upper)[2]	pMCMC
	Intercept[3]	---	11.11, 11.38	---
	Line L	-0.71[a]	-0.27, 0.10	0.3430
	Line S	-4.21[a]	-0.68, -0.33	**0.0001**
	Cohort 1	-4.50[a]	-0.72, -0.37	**0.0001**
Head	Cohort 2	-3.11[a]	-0.52, -0.19	**0.0001**
	L * Cohort 2	1.65[a]	0.02, 0.50	**0.0336**
	S * Cohort 2	2.52[a]	0.17, 0.63	**0.0012**
	L * Cohort 3	0.45[a]	-0.16, 0.28	0.5488
	S * Cohort 3	-0.43[a]	-0.29, 0.16	0.5700
	Overall effect of interaction	12.90[b]	---	**<0.0001**
	Intercept[3]	---	26.26, 28.21	---
	Line L	3.54[a]	2.16, 4.71	**0.0001**
	Line S	3.66[a]	2.28, 4.72	**0.0001**
	Cohort 1	3.36[a]	2.01, 4.52	**0.0001**
Midpiece	Cohort 2	3.93[a]	2.36, 4.72	**0.0001**
	L * Cohort 2	-2.40[a]	-4.74, -1.44	**0.0001**
	S * Cohort 2	-2.41[a]	-4.62, -1.418	**0.0004**
	L * Cohort 3	5.16[a]	-7.80, -4.72	**0.0001**
	S * Cohort 3	-2.86[a]	-4.98, -1.87	**0.0001**
	Overall effect of interaction	29.43[b]	---	**<0.0001**
	Intercept[3]	---	28.70, 31.28	---
	Line L	-2.12[a]	-4.76, -1.43	**0.0001**
	Line S	-6.76[a]	-11.05, -7.84	**0.0001**
	Cohort 1	-4.10[a]	-7.50, -4.22	**0.0001**
Tail	Cohort 2	-4.18[a]	-7.22, -4.09	**0.0001**
	L * Cohort 2	3.94[a]	5.29, 9.65	**0.0001**
	S * Cohort 2	2.29[a]	2.22, 6.34	**0.0001**
	L * Cohort 3	6.31[a]	9.11, 13.16	**0.0001**
	S * Cohort 3	0.98[a]	-0.29, 3.72	0.0932
	Overall effect of interaction	53.83[b]	---	**<0.0001**
	Intercept[3]	---	67.58, 69.27	---
	Line L	0.33[a]	-0.78, 1.43	0.5670
	Line S	-6.93[a]	-7.53, -5.42	**0.0001**
	Cohort 1	-3.30[a]	-4.26, -2.04	**0.0001**
Total length	Cohort 2	-2.64[a]	-3.36, -1.27	**0.0001**
	L * Cohort 2	3.67[a]	3.17, 6.09	**0.0001**
	S * Cohort 2	1.30[a]	0.20, 2.99	**0.0292**
	L * Cohort 3	4.24[a]	3.61, 6.32	**0.0001**
	S * Cohort 3	-1.50[a]	-3.15, -0.47	**0.0090**
	Overall effect of interaction	47.06[b]	---	**<0.0001**

[1]Data are based on 5 sperm measured per male for 561 males over the three cohorts.
[2]The highest posterior density intervals (HPD) were calculated by MCMC sampling.
[3]The intercept is the intermediate line; all other variables are compared against it in the model.
[a]The test statistic for each main effect in the LMM.
[b]The test statistic for the interaction between the main effects in each LMM, the significance of which was tested using log-likelihood tests against the Chi-squared distribution with 4 degrees of freedom.

Associations between all components of sperm morphology

Total length vs. head length

In all three selection lines there was a positive relationship between head length and the total length of the sperm, such that longer sperm had longer heads (Figure 5A). This relationship was strongest in the I-line (Table 4).

Total length vs. midpiece length

There was a highly significant positive relationship between total length and midpiece length in the S-line such that longer sperm had longer midpieces (Figure 5B and Table 4). However, this was in direct contrast to the L- and I-lines, where midpiece length decreased as sperm total length increased (Figure 5B and Table 4).

Total length vs. tail length

In all three selection lines there was a positive relationship between the total length and tail length of sperm, such that males with longer sperm had sperm with significantly longer tails (Figure 5C and Table 4).

Head length vs midpiece length

There was no relationship between head length and midpiece length of sperm across the three selection lines with (all $p > 0.05$; Figure 6A and Table 4).

Head length vs tail length

There was no relationship between head length and tail length of sperm in the L- and I-lines. Sperm with longer heads were more likely to have longer tails in the S-line, although this relationship was weak ($p = 0.03$) (Figure 6B and Table 4).

Midpiece length vs tail length

There were strong negative associations between midpiece length and tail length in the L- and I-lines. Sperm with longer tails had shorter midpieces (both $p < 0.001$) (Table 4). This contrasts with the S-line where there was no relationship between the midpiece and tail length ($p = 0.79$) (Figure 6C and Table 4).

Table 4. The phenotypic correlation coefficients between sperm components. Significant relationships are in bold. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. There was a signficant negative correlation between midpiece and total length of sperm in both the I- and L-line, in contrast to the positive relationship observed in the S-line.

Line	Component	Head	Midpiece	Tail
Short	Head	---	---	---
	Midpiece	-0.006	---	---
	Tail	**0.254***	0.032	---
	Total	**0.339****	**0.497*****	**0.873*****
Intermediate	Head	---	---	---
	Midpiece	-0.003	---	---
	Tail	0.135	**-0.861*****	---
	Total	**0.384*****	**-0.253***	**0.705*****
Long	Head	---	---	---
	Midpiece	0.083	---	---
	Tail	0.008	**-0.851*****	---
	Total	**0.263***	**-0.210***	**0.687*****

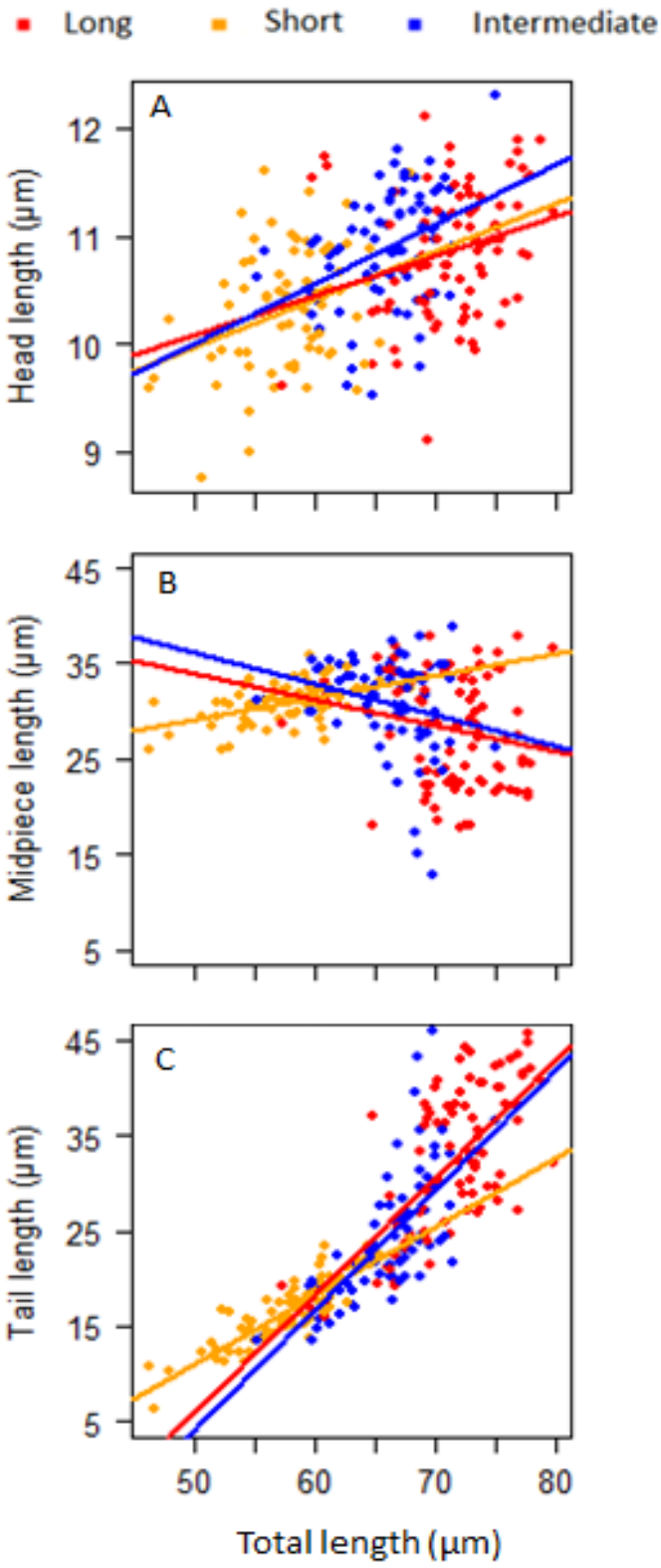


Figure 5. Pairwise phenotypic associations between the total length of sperm and three sperm components. Each data point is the mean value for each sperm component per male (n = 233). (A) mean head and mean total length, (B) mean midpiece length and mean total length and (C) mean tail length and mean total length. The three selection lines are represented by different colours: long (red), short (orange) and intermediate (blue). All associations between total length and other sperm components were significant ($p < 0.05$).

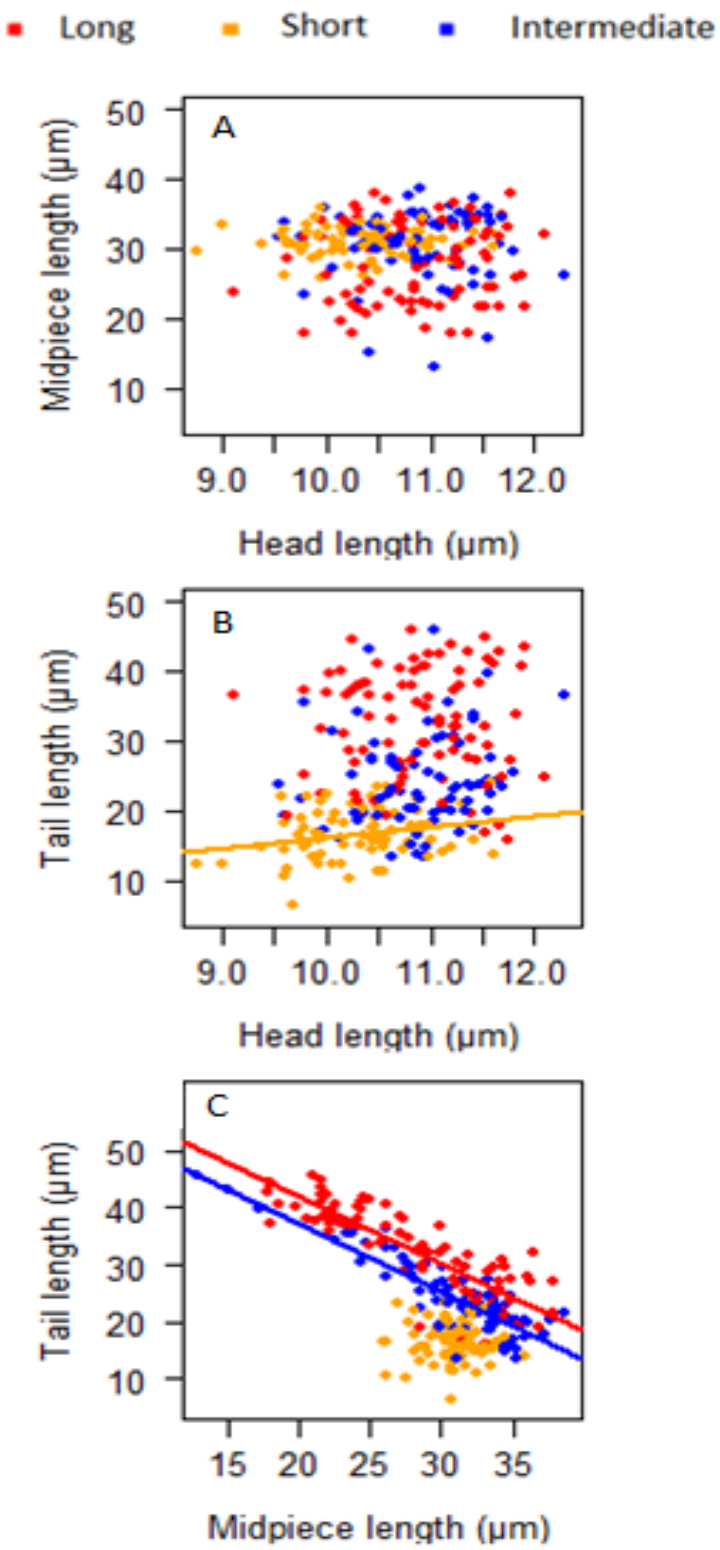


Figure 6. Pairwise phenotypic associations between the three sperm components. (A) mean head and mean midpiece length, (B) mean head length and mean tail length and (C) mean midpiece length and mean tail length. The three selection lines are represented by different colours: long (red), short (orange) and intermediate (blue). Significant associations ($p < 0.05$) are indicated by regression lines. Each data point is the mean value for each sperm component per male (n = 233).

3.4: Discussion

The present study demonstrated that artificial selection on sperm total length had a strong effect on multiple aspects of sperm morphology. Sperm total length diverged significantly between the L- and S-line, with a similar pattern of response occurring in tail length. In addition, there were major differences in the relationships between the sperm components across the selection lines, specifically between midpiece and tail length, and midpiece and total length. Here, the discussion centres on the phenotypic patterns observed; the underlying genetic factors of these relationships are addressed in Chapter 4.

The effect of selection on sperm morphology

There was a rapid and clear divergence in sperm total length in the expected directions (i.e. the L-line produced longer sperm than the S-line). In general, the mean values of the target phenotypic traits often fluctuate across generations during selection experiments, and although total length continued to diverge in the present study, it is possible that this trend may not be observed in the future. Morrow & Gage (2001a) also observed a large divergence between long- and short-sperm lines of crickets (from approximately 10 to 40µm) in only four generations of artificial selection. Both the present study and Morrow & Gage (2001a) used species that exhibited natural inter-male variation in sperm total length; this may have facilitated rapid between-line divergence. Of course, rapid selection responses early on may occur if the trait under selection is determined by few loci of large effects; if this is not the case then significant phenotypic divergence must usually be achieved through many generations of selective breeding, by small changes in the contributing allele frequencies (Barton & Keightley 2002).

Sperm tail length diverged between the lines in a similar manner to total length, suggesting that changes in tail length are driving the observed differences in total length. Only a reduction in tail length in the S-line can result in shorter sperm, because midpiece length remains relatively constant across the cohorts. Conversely, although both midpiece and tail length are variable in the L- and I-lines (tail length more so), it is more difficult to say which trait may actually drive the overall length of sperm. Previously, Birkhead et al. (2005) suggested that midpiece and flagellum length in the zebra finch were controlled in an antagonistic nature, such that sperm receiving genes determining long flagella also possessed genes for short midpieces. As the present study detected line-specific relationships between sperm

components (previously undetected in Birkhead et al. (2005) because a single analysis using all birds was preformed), it is also possible that different genetic relationships might exist between sperm components across the different selection lines. This possibility is explored in Chapter 4.

Neither head length nor midpiece length followed the same pattern of response as tail or total length. Due to the phenotypic correlation between head and total length (although it was weak), head length may have been expected to diverge similarly to total length. However, head length actually decreased in all three lines, suggesting that the phenotypic association may not be underpinned by a genetic association. A reduction in trait variance is a consequence of strong artificial selection, and it may be that random genetic drift (an important effect in small, closed populations) could have caused the reduction in head length. Without replicate selection lines; however, it is not possible to verify if this pattern of response is reproducible or a chance event.

In contrast, the change in midpiece length was variable between the selection lines. By cohort 3, the L- and S-line had diverged, such that males from the L-line had shorter midpieces compared to those from the S-line. This pattern of response was consistent with previous work, where a negative association between midpiece and flagellum length was detected (Birkhead et al. 2005; Mossman et al. 2009). It is interesting that, overall, midpiece length of sperm from the S-line remained constant through the selection regime. Minimal phenotypic change may be attributed to a lack of genetic variation in that particular trait, as the raw material for selection (either natural or artificial) is missing.

Sperm designs: associations between sperm components

There were interesting differences in sperm design between the three selection lines. When there was no association between sperm components (e.g. between head and midpiece) sperm could - in theory - evolve to have combinations of components of any dimensions, producing a range of possible designs. However, strong associations between components, for example between tail and total length, may only produce certain sperm designs because longer sperm are thus constrained to have longer tails. Of course, these constraints will be realised only if the phenotypic relationships are underpinned by identical genetic associations.

Across species of passerine birds (Immler & Birkhead 2007; Lüpold et al. 2009a), and within single bird species (Helfenstein et al. 2010), longer sperm have longer midpieces. This

relationship may indicate that strong energetic requirements govern sperm morphology in birds, i.e. a longer flagellum (and therefore longer total length) requires a longer midpiece to provide sufficient energy for sperm functions, such as motility. As mentioned earlier, previous research in the zebra finch observed significant negative relationships between midpiece and flagellum length (Birkhead et al. 2005; Immler & Birkhead 2012), in contrast to the examples in other birds (see above). Although negative relationships describe the overall association in the zebra finch populations, the association is not actually meaningful. This is because the present study indicated a division of sperm into at least two morphotypes (Figure 7B): (1) sperm with a midpiece that comprises the majority of the flagellum (sperm originating from all three selection lines), and (2) sperm where the midpiece is relatively short compared to the flagellum (sperm from the L- and I-lines: see Figure 7A & B comparing the phenotypic distribution of the present study with previous research). Therefore, to understand the relationships between sperm components, at least in the zebra finch, it may be appropriate to consider these morphotypes separately. Similar divisions by sperm morphotype were also evident from the associations between midpiece and tail where (1) sperm with long midpieces had relatively short tails, and (2) sperm with short midpieces had relatively long tails (Figure 6C). Relationships between midpiece and tail length were not reported in previous studies of birds (e.g. Helfenstein et al. 2010; Laskemoen et al. 2010) so it is unknown whether the pattern observed in the zebra finch can be generalised across species. What caused the division of the two morphotypes across the selection lines? As the distribution of sperm designs is similar to that in wild populations (Immler et al. 2012), it is likely that different morphotypes are naturally occurring. However, the separate morphotypes may be more distinct in the present study because the selective breeding regime has 'pulled apart' the distribution by producing males with the most extreme sperm designs.

The different sperm morphotypes observed across zebra finch sperm results in extensive morphological variation in sperm design, which is attributed to the low risk of sperm competition in this species (Birkhead et al. 2005). Producing the best quality sperm is then less important and suggests that there is an energetic cost to maintaining high quality sperm (Birkhead & Immler 2007). By contrast, in species where sperm competition risk is high, sperm morphology between males is remarkably similar and conforms on a species-specific optimal design (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008) that may result in the competing males producing comparably high quality sperm.

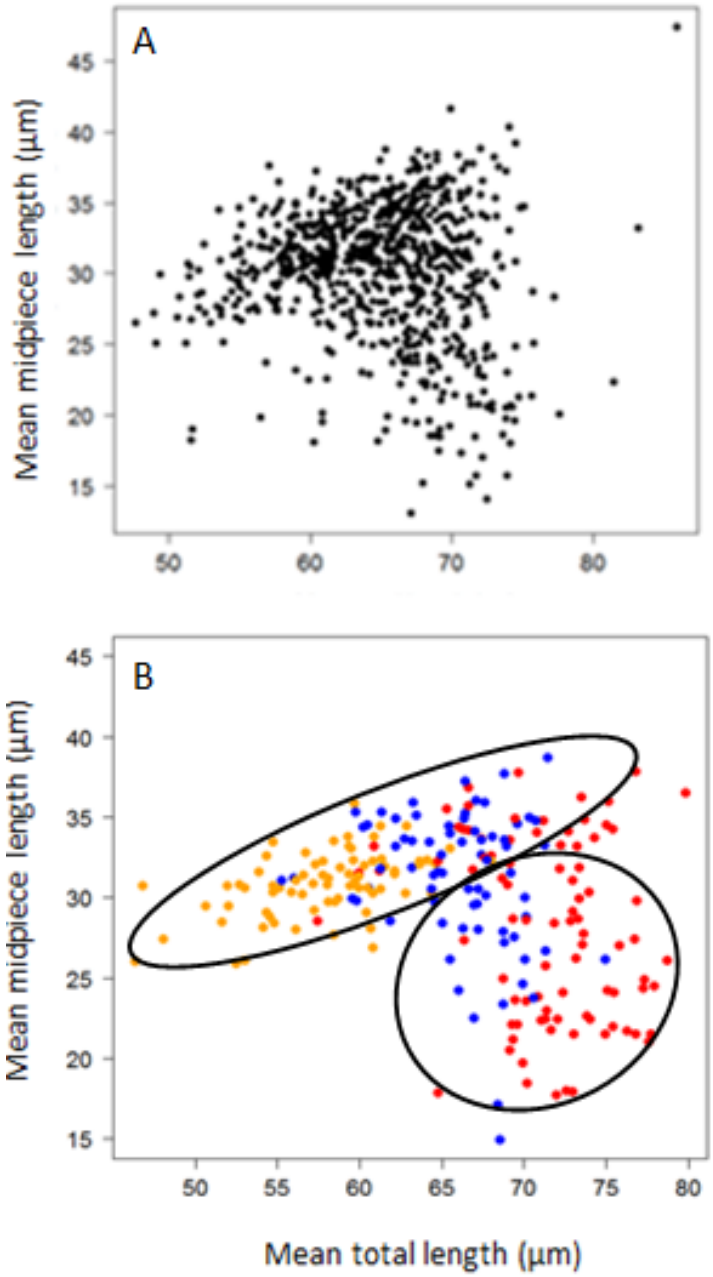


Figure 7. Relationships between midpiece length and total length in two datasets of zebra finch sperm morphology. This figure was created from Figure 1A and Figure 5B of this chapter for visual comparison. Each point is the mean value of sperm components per male. (A) A subset of birds analysed in Birkhead et al. (2005). There was a weak but significant negative correlation ($r = -0.085$, $d.f = 772$, $p < 0.018$) between the sperm components. (B) The birds from the present study ($n = 233$). There was a positive correlation in the S-line (orange), and negative correlation in the L- and I-line (red and blue respectively). The ellipse and circle suggest the distribution of the two sperm morphotypes (long midpiece relative to flagellum, and short midpiece relative to flagellum respectively: see p53 for details).

Maintaining phenotypic variation

Strong selection on phenotypic traits may be expected to erode variation on the target trait, especially in a small, closed population (Falconer & Mackay 1996). However, this did not occur in the long sperm (L- and I-lines) of the present study, in fact, the CV of midpiece length in the L-line increased from 11.44 to 22.40 between cohort 1 and 3, compared to a reduction in CV in the S-line from 11.44 to 9.36. There is greater variance in the L- and I-line because these males produce sperm of two morphotypes: (1) sperm where the midpiece comprises the majority of the flagellum, and (2) sperm with short midpieces relative to the flagellum. Because midpiece and flagellum length are negatively correlated in both the L- and I-lines (a strong design constraint: Birkhead et al. (2005), morphological variation and extreme sperm designs. This may be because strong constraints cannot be broken by the selection process. Where there are no such constraints, as in the S-line sperm, sperm designs could be subject to directional selection and variation reduced.

Other explanations for the maintenance of sperm phenotypic variation across taxa include: (i) new mutations (Falconer & Mackay 1996), (ii) male age (Green 2003), and (iii) environmental conditions (Blackenhorn & Hellriegel 2002), but they are not applicable to the present study. Although mutations are a source of variation, they must occur in the loci that determine the phenotype. In addition, the low mutation rates (0.1 – 1.0% per generation; Barton & Keightley 2002) would require many generations to have an appreciable effect on variation (Falconer & Mackay 1996). All birds were raised in identical environmental conditions and sperm were obtained from each male at the same age (when sexually mature at 100 d). There is no convincing evidence that male condition affects sperm morphology: in both zebra finches (Birkhead et al. 1998; Birkhead et al. 1999a) and moths *Plodia interpunctella* (Gage & Cook 1994), sperm morphology was not affected by rearing on standard or supplemented diets. Only sperm number decreased in the moths (Gage & Cook 1994).

An additional source of variation has been reported in the Gouldian finch *Erythrura gouldiae* (Immler et al. 2010). Plasticity of sperm morphology was determined by social status among colour-morphs. The change in midpiece length in red morphs was associated to hormone levels, although this did not explain changes observed in the black morph. In the present study, all birds were housed in groups of multiple males, although it was not possible to deduce the complex social hierarchy that could occur. However, given the strong genetic control of sperm morphology in the zebra finch (Birkhead et al. 2005; Mossman et al. 2009), and absence of

condition-dependence (Birkhead et al. 1998; Birkhead et al. 1999a), it seems unlikely that sperm morphology is a plastic trait.

The future of selection

Considering the strong selection response of sperm total length observed in this study and others (e.g. Morrow & Gage, 2001a; Miller & Pitnick 2002), the most interesting questions are: (i) how long can a response persist, (ii) what extreme phenotypes could be produced, and (iii) what are the consequences of these extreme phenotypes on sperm motility and male fertility? In theory, a selection response may be maintained for as long as there is sufficient additive genetic variation in that trait, and sufficient loci contributing to the variation. Large populations will also reduce the risk of genetic variation being eroded by genetic drift or inbreeding (a potential pitfall of strong artificial selection: Falconer & Mackay 1996). When there is ample genetic variation, the selection response might be assumed to continue indefinitely, thus producing sperm of the most extreme dimensions, such as the giant sperm produced by the fly *Drosophila hydei* (Pitnick & Markow 1994). However, these giant sperm appear to be exceptions to the norm. This may be because the sperm of species carrying out internal fertilisation have to function within the constraining environment of the female reproductive tract, and therefore cannot exceed certain dimensions that allow sperm to function normally. Although strong bidirectional selection on zebra finch sperm in the present study resulted in more males producing extreme sperm designs, with increased and decreased mean total length in the L- and S-lines respectively, the range of extreme sperm lengths were very similar (Figure 8).

The fact that overall variation in sperm length variation is unchanged, despite strong selection, suggests that total sperm length in the zebra finch may have an upper and lower limit beyond which selection cannot expand. These limits may be due to functional constraints exerted on spermatogenesis via the spermatogenic tissue. Across bird species, longer sperm are produced by wider seminiferous tubules (Lüpold et al. 2009c), although this relationship is yet to be established within a single species. It is reasonable to assume from evidence in other species (Pitnick 1996) that longer sperm in birds may also take longer to produce, and the testes architecture may have to undergo specialist changes to produce sperm of varying morphologies. The testes architecture may not be infinitely flexible to manufacture the most extreme sperm designs. The upper and lower limit on sperm design may also be enforced by the female, via the dimensions of the sperm storage tubules (SSTs). Again, there is evidence of coevolution between sperm and SST dimensions across bird species (Briskie & Montgomerie

1992; Briskie et al. 1997). Also, within single species, males producing sperm of comparable lengths to the female storage organs gained greater fertilisation success (Miller & Pitnick 2002). Therefore, the range of sperm dimensions able to be produced and carry out the intended functions may have already been reached in the zebra finch.

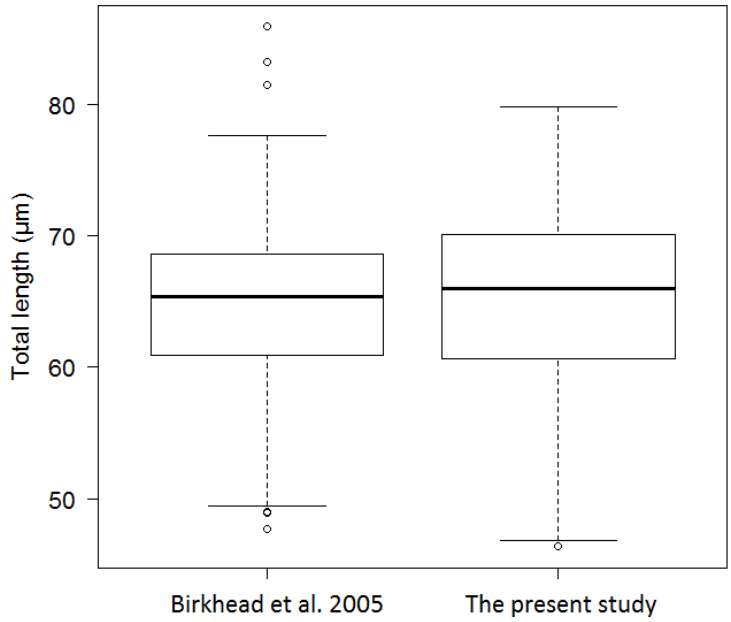


Figure 8. The range of sperm total lengths included in Birkhead et al. (2005) and the present study. The variation in total length is similar between the two studies, as demonstrated by the similar sizes of the box and whisker plot. The horizontal black lines within each box are the median values of sperm total lengths. Strong artificial selection did not extend the range of extreme sperm lengths, despite having a clear divergent effect on the mean values for each selection line (Figure 3).

In conclusion, this study demonstrated that zebra finch sperm morphology responds rapidly to artificial selection on sperm total length, such that differences in total sperm length were driven largely by changes in tail dimensions. Clear differences exist between the lines in the phenotypic relationships between the length of the midpiece and the tail because of naturally occurring, but contrasting, associations between these components. As this study described only the phenotypic patterns observed in zebra finch sperm, the next chapter will explore how the underlying genetic relationships determined both the response to selection of overall sperm morphology, and the relationships between the sperm components, which are likely to have important consequences regarding the evolutionary change of sperm design.

Chapter 4

The genetic architecture of zebra finch selection lines

4.1: Introduction

Postcopulatory sexual selection, in particular sperm competition (Parker 1970), is an important driving force behind the phenotypic change of traits determining reproductive success. For example, comparative studies across species have revealed that sperm morphology is one trait that varies according to intensity of sperm competition (e.g. Kleven et al. 2008; Calhim et al. 2007; Lüpold et al. 2009b). Sperm total length dimensions, and the lengths of individual sperm components in a single species can also be altered via artificial selective breeding regimes (e.g. Woolley 1971; Morrow & Gage 2001a; Chapter 3), which provides useful insights into the magnitude and direction of phenotypic change possible.

Regardless of how phenotypic change occurs, additive genetic variation (V_A) is required. Quantifying the contribution of V_A to the overall phenotypic variation (narrow sense heritability, h^2: Falconer 1989) is useful to understand the magnitude of evolutionary potential of a single trait such as sperm length; however, this single estimate does not take into account how other phenotypic traits may affect the overall change in sperm dimensions. Sperm total length is dependent on the dimensions of each individual component parts of the sperm, the head, midpiece and tail (Chapter 2 Section 2.4.2). As sperm total length and the lengths of the individual sperm components are likely to be quantitative traits, and affected by many loci with relatively small effects, this non-independence among sperm components may affect how the phenotype responds to selection.

Selection on one phenotypic trait may cause a correlated response in another trait if the traits genetically covary, for example if traits are controlled by pleiotropic loci, which are loci determining multiple phenotypic traits (Falconer & Mackay 1996). Phenotypic traits may also covary in the absence of pleiotropy if the governing loci are in linkage disequilibrium with each other, or via indirect processes such as epistasis (Falconer & Mackay 1996). Therefore, understanding how sperm total length responds to selection requires understanding of the genetic relationships between the head, midpiece and tail, and how these relationships may govern the change in each trait (Lande 1979). The matrix of additive genetic variances and covariances (the G matrix; Lande 1979) is a useful tool to reveal these genetic relationships (for an accessible overview regarding G matrix evolution, see Steppan et al. 2002).

The G matrix

Consider two traits - trait 1 and trait 2. The G matrix of the relationship between these traits (Figure 1) describes the amount of additive genetic variation for each trait, and the additive genetic covariance between the traits. The G matrix also describes the extent and the direction that combinations of traits can evolve and change together. In order to visualize how the genetic relationships between two traits can dictate evolutionary change, consider the different relationships between trait 1 and trait 2 in Figure 2. When there is no relationship between the genetic values of trait 1 and trait 2 (Figure 2A), each trait could increase or decrease in value independently of the change in the other trait. However, if trait 1 and trait 2 are genetically correlated (Figure 2B), a change in trait 1 will cause a correlated change in trait 2. Because there is a positive association between trait 1 and trait 2, the G matrix constrains the trajectory of evolutionary change for both traits, and the traits cannot evolve independently.

Therefore, the G matrix is a representation of the genetic relationships among traits for a given population or group of individuals. To compare the G matrices across populations or treatment groups, some complex statistical analyses are required. Eigenanalyses (related to Principle components analysis) are commonly used to compare G matrices (e.g. Arnold 1992; Jones et al. 2003), and involve the generation of new variables called eigenvectors from the original data. Eigenvectors represent linear combinations of the original data, with each eigenvector describing a single axis of independent variation (Hill & Thompson 1978). The eigenvalue describes the amount of additive genetic variance represented by the eigenvector.

Previously, in Chapter 3 a significant divergence in sperm total length was observed across three artificially selected lines of zebra finches. Because sperm total length is a phenotypic trait entirely controlled by the individual component parts (Chapter 2 Section 2.4.2; Chapter 3), the genetic relationships controlling the dimensions of the sperm component parts must be considered to fully understand the response of total sperm length to selection. For example, if there are positive genetic correlations between components such as midpiece and tail, selective breeding to increase sperm total length is likely to produce sperm with larger midpiece and tail dimensions. By examining the genetic variance and covariance matrix for each selection line, it is possible to understand the genetic relationships across the suite of phenotypic traits for each selection line, and how these differences control the phenotypic response to artificial selection.

	Trait 1	Trait 2	
Trait 1	G_1 (h^2)	G_3	**= G**
Trait 2	G_3	G_2 (h^2)	

Figure 1. The G matrix of the relationship between two traits (trait 1 and trait 2). The diagonals give the additive genetic variances for trait 1 (G_1) and trait 2 (G_2). The off-diagonals (G_3) give the additive genetic covariance between trait 1 and trait 2.

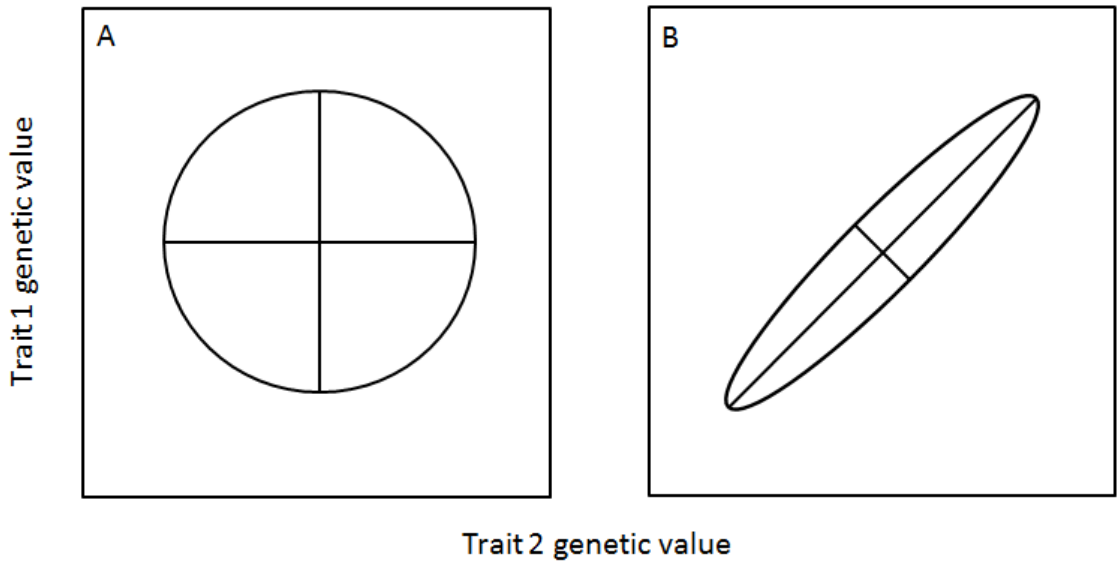


Figure 2. Schematic illustrating how the evolutionary trajectory of two traits (trait 1 and trait 2) is dependent on the genetic relationship between the traits. (A) A lack of association between the genetic values of trait 1 and trait 2 means that traits can evolve independently by increasing or decreasing in value. (B) A strong positive genetic correlation between trait 1 and trait 2 constrains evolutionary change of both traits to be in the same direction.

Aim

This study intended to describe differences in the genetic relationships of three components of sperm morphology (head, midpiece and tail) between the G matrices of three selection lines (long (L-line), intermediate (I-line) and short (S-line)), and relate any differences to the phenotypic associations observed in Chapter 3. Specifically, the expectation was that the G matrix of the S-line would differ significantly from the G matrices of the L- and I-line, in terms of the genetic correlation between the three sperm components. This expectation was due to the marked differences in phenotypic correlation of midpiece and tail length between the S-line and the L- and I-lines (Chapter 3). The genetic relationships across the suite of sperm components (head, midpiece and tail) for the three selection lines were described using two methods: (i) a matrix comparison approach (Robinson & Beckerman 2013), which draws on methods by Krzanowski (1979), Ovaskainen et al. (2008) and Kirkpatrick (2009), and (ii) a tensor approach (Robinson & Beckerman 2013), which uses methods described in Hine et al. (2009). These approaches are explained in detail below in the Methods (Section 4.2). All statistical analyses in this chapter were conducted in collaboration with M. Robinson who provided the necessary R scripts and advised on the suitable methodology.

4.2: Methods

Study population

The zebra finches used in this study were part of the selective breeding regime described in Chapter 2 Section 2.2. Sperm morphology data of males (n = 422) from cohort 2 and 3 of all three selection lines were used in the analyses because the sperm total lengths were completely diverged between the three lines (Chapter 3).

Sperm collection and morphology assessment

Samples of dead sperm were collected (Chapter 2 Section 2.3.3) and sperm morphology was assessed following the method in Chapter 2 Section 2.4.2. Briefly, dead sperm were collected from the faeces of each male and five morphologically normal sperm per male were measured to the nearest 0.01µm using ImageJ (Abramoff et al. 2004) for the following sperm

components (see Chapter 2 Section 2.4.2): (i) head, (ii) midpiece, and (iii) tail. Total length was calculated by adding the three values together.

Data preparation

Separate pedigrees were prepared for each of the three selection lines with all males (n = 422) from cohort 2 and 3 (see Table 1 for sample sizes). The parent birds were added to the pedigree using the R package MasterBayes (Hadfield et al. 2006). Sperm morphology data for these males were collated into three separate phenotype files, one file per selection line.

Table 1. Sample sizes of males used in the analyses for each selection line from cohort 2 and 3, and the number of different families that comprise each selection line.

Line ($N_{FAMILIES}$)	**Number of males**	
	Cohort 2	**Cohort 3**
Long (54)	64	88
Intermediate (45)	52	72
Short (51)	73	73

Statistical analyses

Separate analyses were carried out for each selection line using linear mixed models (animal models: Kruuk 2004) in the R package MCMCglmm (Hadfield 2010). The animal model (equation 1) partitions the phenotypic variance of each individual (y_i) into three parts: (i) a_i: the additive genetic effect (proportion of variance due to genetics), (ii) p_i :permanent environmental effect (proportion of the variance due to shared environment), and (iii) e_i :residual variance (all remaining variance). The population phenotypic mean is represented by μ. Each variance component was estimated using a Bayesian framework (see Beaumont & Rannala 2004; O'Hara et al. 2008 for overviews of the Bayesian approach).

$$y_i = \mu + a_i + p_i + e_i \qquad \text{Equation 1}$$

Initially, univariate (single trait) models were used to quantify the additive genetic variance of each sperm component. Models were constructed using each single sperm component as the response variable, e.g. head, midpiece or tail. The additive genetic and permanent environment effects were modelled as random effects. A variance-expanded non-informative

prior was specified for each model, such that the estimates of the posterior distribution were obtained using the data only.

Estimates of the posterior distribution were made via MCMC runs with a chain length of $2.5x10^5$ iterations, a burn-in period of $5x10^4$ and a sampling interval of 200, giving 1000 joint posterior estimates of the random effects. Heritability was calculated as the amount of additive genetic variance divided by the total phenotypic variance (V_A/V_P). Significant estimates of heritability and model parameters were indicated when the 95% credibility intervals (95% CI – the region with the 95% highest posterior density) did not overlap zero.

Multivariate models were then constructed to describe the genetic relationships between the three sperm components (one model per selection line). Each sperm component value was mean standardised by dividing it by the mean value and multiplying by 10, which ensures all sperm components values were the same scale, aiding model convergence. The head, midpiece and tail were modelled as a combined response variable to enable the genetic covariance between components to be estimated. The additive genetic and permanent environment effects were modelled as random effects. A variance expanded non-informative prior was specified for each model. The posterior distributions of the model parameters were estimated via MCMC runs with a chain length of $2.6x10^6$ iterations, with a burn-in period of $1x10^5$ and a sampling interval of 2500, giving 1000 joint posterior estimates of the model. Model parameters were regarded as significant if their 95% CIs did not overlap zero.

Pairwise matrix comparison

The G matrices were estimated from the joint posterior distributions of the multivariate model from each selection line and used to examine the difference in the underlying genetic variance-covariance distributions between the three selection lines, as described in Robinson & Beckerman (2013). The following equation was used:

$$y_i = u + X\beta + Z\alpha + e \qquad \text{Equation 2}$$

where y is a vector of all trait values of each selection line. Z is the design matrix which relates individuals to additive genetic effects *a*. This equation estimates an unstructured n x n variance-covariance matrix of the genetic effects where n is the number of phenotypic traits (i.e. 3). The matrix has dimensions of n (n + 1)/2 for each selection line, referred to hereon as G_L, G_I and G_S (the G matrices of the long, intermediate and short selection line respectively).

The distribution of the unconstrained additive genetic variation for each trait combination in a given G matrix can be visualized as an ellipsoid (Figure 3), which can then be decomposed into a set of eigenvectors. Each eigenvector represents the linear combination of traits with independent variances which respond to selection independently. The principle eigenvector (G_{MAX}) is the eigenvector measuring the largest dimension of the ellipsoid. The ratio of the genetic variance of the G_{MAX} and the second eigenvector describe the shape of the distribution of variance, for example a ratio of 1 indicates the distribution is spherical with a similar amount of variation in all dimensions. The associated eigenvalues (λ) describe the genetic variation associated with each eigenvector. These measures allow estimation of the amount of genetic variation and the potential for evolutionary change of specific trait combinations, i.e. the combination of traits where there is maximum genetic variation for proportional change, referred to as the trait evolvability (Kirkpatrick 2009).

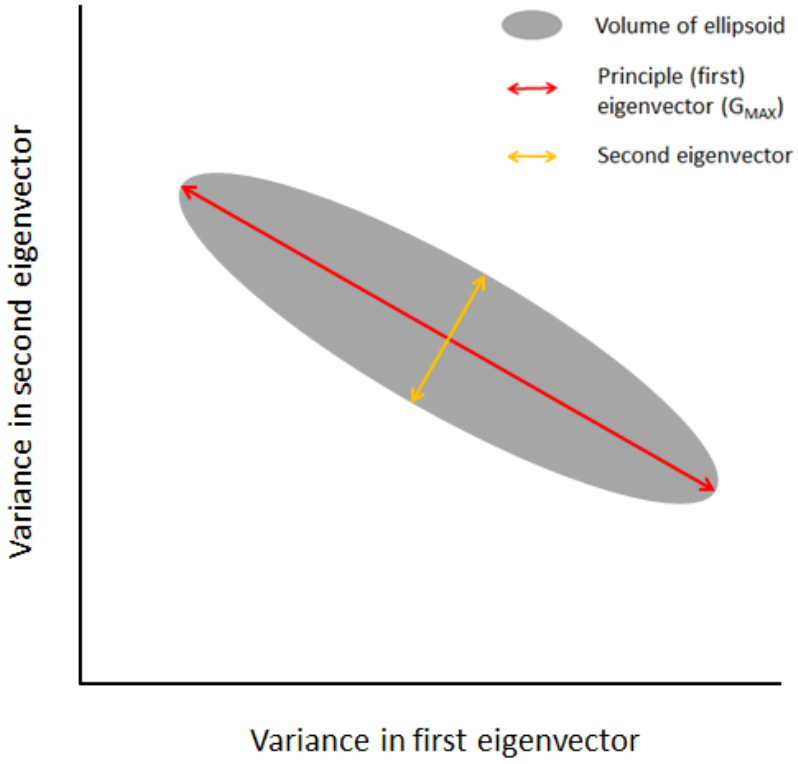


Figure 3. Schematic illustrating how the statistics are obtained using the probability distribution of a single G matrix. Comparisons between two G matrices (G_L and G_S) are made by calculating the difference between the two matrices (e.g. G_L minus G_S) for each test statistic in Table 2. Note that the G matrix in the present study is three dimensional because three traits were analysed.

Statistics describing each G matrix were generated using the following process. One thousand sets of random samples (A and B) were drawn from each G matrix, providing estimates of the respective G matrix from each selection line. The following statistics were then calculated for each G matrix using the equations in Table 2: (i) the volume of the ellipsoid, (ii) the number of significant eigenvectors, (iii) the total genetic variance, (iv) the genetic variance of G_{MAX}, (v) the genetic variance of the second eigenvector, (vi) the ratios of genetic variance of G_{MAX} and second eigenvector, and (vii) the evolvability. Because each statistic was calculated 1000 times per selection line, a measure of confidence in the estimate was obtained using 95% CIs.

Then a matrix comparison approach (Ovaskainen et al. 2008; Robinson & Beckerman 2013) was applied to test for differences in genetic variation and covariance between the G matrices of the three selection lines. Comparisons were made in a pairwise manner as follows: L-line against S-line, L-line against I-line and finally S-line against I-line. The differences between the G matrices of each selection line pair were calculated by making the following comparisons (here the method is described using comparisons between G_L and G_S): (i) the difference in ellipsoid volume between G_L and G_S, (ii) the angle between G_{MAX} of both G_L and G_S, (iii) the difference in the variance of G_{MAX} of G_L and G_S, and (iv) the difference in the ratios of G_{MAX} and the second eigenvector between G_L and G_S (see Table 3 for explanations of each statistical test). Because each test was carried out 1000 times (due to comparisons between 1000 sample G matrices of each line, see above), 95% CIs were calculated for each estimate. A significant difference between the G matrices for each pair occurred when the 95% CI did not overlap zero. The genetic variance covariance matrices of each line were visualized by plotting the first three axes (there are three axes because there are three phenotypic traits) of the eigensystem of the estimated G matrix. Plotting the two matrices on the same axes allows easy visual comparison.

Table 2. Descriptive statistics used to summarize the genetic variation of the G matrix associated with each of the three selection lines. All equations used were taken from Robinson & Beckerman (2013). In some cases the notation may have been adapted to fit the conditions of the present study.

Descriptive statistic	**Equation**
Volume of ellipsoid	$V_d = (1/2)\,\pi^2 \prod (r_n)$ (r_n are the semi-axes described as $\sqrt{\lambda}$ and λ is the eigenvalue of the eigenvector *n*)
Number of significant eigenvectors	$\psi_\omega\ (G_L) = (G_L^A \lambda_1 - G_L^B \lambda_1) - (G_L \lambda_1 - \Sigma\lambda_n)$ (the difference in eigenvalue of the first eigenvector of $G_L^A \lambda_1$, is compared to the difference in eigenvalue between the first eigenvector and total eigenvariance of G_L
Total genetic variance	$V_T = \Sigma_n \lambda$ (summing the eigenvalues gives the total genetic variance)
The genetic variance of G_{MAX}	$V_{GMAX} = \lambda_1 / \Sigma_n \lambda$ (variance of G_{MAX} relative to the total variance)
The genetic variance of the second eigenvector	$V_{SECOND} = \lambda_2 / \Sigma_n \lambda$ (variance of V_{SECOND} relative to the total variance)
The ratio of G_{MAX} and the second eigenvector	$R = \lambda_1 / \lambda_2$ (describes the shape of the distribution of total variance)
Maximum evolvability	$e_{MAX} = \sqrt{\lambda_1}$ (the square root of G_{MAX}. Describes the ability of a population to respond to natural or artificial selection)

Table 3. Example statistics used to formally compare the G matrices using pair-wise comparisons. Here the examples are explained using the G matrices for the long (G_L) and short lines (G_S). See Tables 4, 5 and 6 for the results of these comparisons. All equations used were taken from Robinson & Beckerman (2013). In some cases the notation may have been adapted to fit the conditions of the present study.

Statistic	**Equation and definition**
Difference in volume (G_L - G_S)	V_d (G_L - G_S) = $G_L V_d$ - $G_S V_d$ (describes the difference in genetic variation between G_L and G_S)
Angle between G_{MAX} of G_L and G_S	Ψ_θ (G_L, G_S) = $[\theta(G_L^A, G_L^B) + \theta(G_S^A, G_S^B)] - [\theta(G_L^A, G_L^B) + \theta(G_S^A, G_S^B)]$ where $\theta = \text{acos}(\sqrt{\lambda_1} / (\pi/180))$. (describes whether the genetic covariance of the three traits is similar in both G_L and G_S by describing the difference in orientation of the ellipsoids. The angle in radians is calculated from the arc cosine of the eigenvalue of the first eigenvector, divided by pi over 180)
Difference in variance of G_{MAX} (G_L - G_S)	$V_{d\,GMAX} = (\lambda_{1L} / \Sigma_n \lambda_L) - (\lambda_{1S} / \Sigma_n \lambda_S)$ (describes the difference in genetic variation in the principle eigenvector of G_L and G_S)
Difference in the ratios of G_{MAX} and the second eigenvector between G_L and G_S)	$R_{LS} = (\lambda_{1L} / \lambda_{2L}) - (\lambda_{1S} / \lambda_{2S})$ (describes the difference in the shape of the genetic variation between G_L and G_S)

Matrix comparison: a tensor approach

Another approach used to characterize differences in the G matrices uses tensors, which allows comparison of multiple G matrices across groups or environments (Robinson & Beckerman 2013) in one test, and removes the need for multiple comparisons using individual tests. Here, tensors are geometric objects that describe linear relationships among the three traits (head, midpiece and tail), and estimates of the G matrices describing these relationships at points among groups are obtained using multi linear algebra

$y_{ij} = u + X\beta + Z\alpha + \varepsilon$ Equation 3

where separate multi-trait models are run for each point i for each selection line j. Z estimates an unstructured n x n variance–covariance matrix of the genetic effects G_i, where n is the number of traits, with a dimension n(n + 1)/2 for each point i.

Multiple G matrices (G_i) obtained by equation 3 were used as second order tensor variables. The variation among each of these tensor variables were then described using a fourth order genetic covariance tensor Σ_G (Basser & Pajevic 2007; see also Figure 4) within a Bayesian MCMC framework. The number of independent axes of genetic variance (given by the number of significant eigentensors) across the three selection lines was estimated (Hine et al. 2009; Robinson & Beckerman 2013). The fourth-order covariance tensor, Σ_G, can be represented as a covariance matrix (S) of dimension n (n + 1)/2. S represents the genetic variance covariance structure in tensor form. S is decomposed into eigenvectors ε^k_G where each eigenvector has an eigenvalue σ^k_G. 95% CIs were obtained for each eigenvalue of each eigenvector of S.

The variation contributed to each significant eigentensor by each phenotypic trait was estimated (Robinson & Beckerman 2013). Each eigentensor of Σ_G was decomposed into eigenvectors $v^{k\ i}_G$, which has eigenvalues $e^{k\ i}_G$. The following projection was used: $V^n_A = (n)^T C^{i,j} E^j_G n$ where the additive genetic variation V_A from each phenotypic trait *n* from each significant eigentensor was calculated. Eigenvalues of each vector estimate the directional change of variance with each eigentensor. Squaring this eigenvalue, then dividing by the sum of all eigenvalues of the tensor gave the proportion of genetic variance explained by each trait.

All analyses were performed in R version 2.15.1 (R Development Core Team, 2012) using the package MCMCglmm (Hadfield 2010) and the tensor function (Robinson & Beckerman 2013).

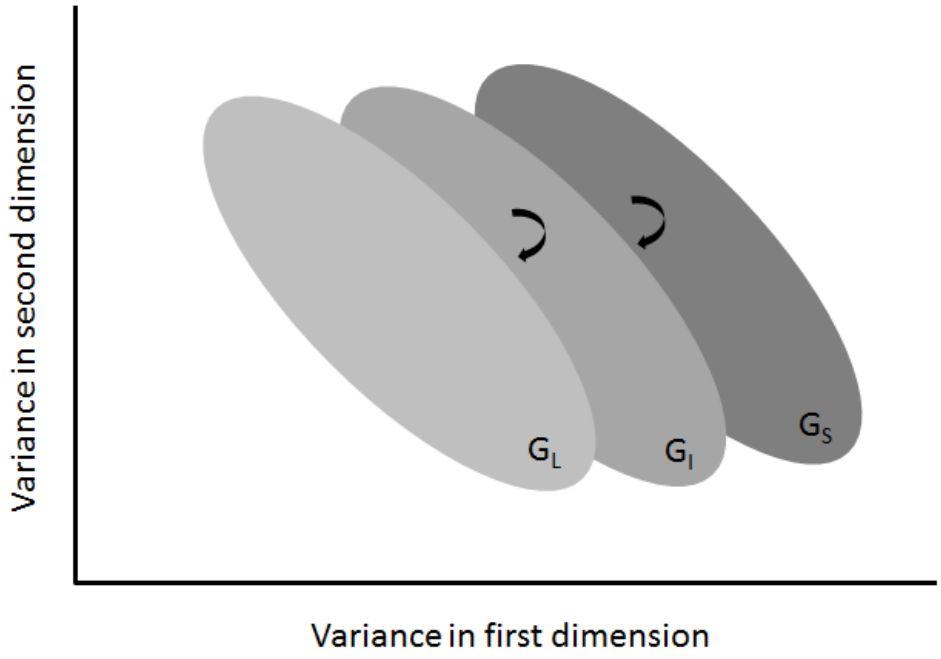


Figure 4. Schematic illustrating how the G matrices are compared in during the tensor analysis. Comparisons between the three G matrices (G_L, G_I and G_S) are made simultaneously. Each G matrix (represented by three filled grey ellipsoids) becomes a second order tensor and the variation among them described by a fourth order tensor covariance tensor Σ_G represented by the two curved arrows.

4.3: Results

Partitioning variance components

The majority of the sperm components had a significant proportion of variance due to additive genetic effects, with the exception of head length in the I-line and midpiece length in the S-line (Table 4). Consequently, these two sperm components in these lines were not significantly heritable. All sperm components across the lines had significant amounts of residual unexplained variance (Table 4). All data for head, midpiece and tail were used in the next set of analyses because each sperm component had some additive genetic variation, even if the estimate did not reach significance (Table 4).

Genetic correlation between sperm components

The genetic correlations between sperm components in the S-line were generally low (Table 5). Head and tail length were positively correlated; however, the relationship was weak, and there was no correlation between midpiece and tail length. In contrast, there was a significant negative correlation between midpiece and tail length in both the L- and I-line, suggesting that loci determining a long midpiece also results in a shorter tail, and vice versa.

Table 4. The additive genetic variance (V_A), permanent environment effect (P_E), residual variance (V_R) and heritability (h^2) derived from the univariate analyses. The values in parenthesis are the 95% credibility intervals. Values in bold and indicated by the asterisk are significant, where the 95% credibility intervals do not overlap zero. Refer to Table 1 for samples sizes.

Line	Component	V_A	P_E	V_R	h^2
	Head	**0.19***	0.11	**0.28***	**0.32***
		(0.04, 0.34)	(3.0e-06, 0.21)	(0.25, 0.31)	(0.09, 055)
L	**Midpiece**	**28.19***	1.91	**9.99***	**0.69***
		(18.94, 37.33)	(5.2e-06, 6.49)	(8.97, 11.14)	(0.55, 0.79)
	Tail	**50.30***	3.15	**12.15***	**0.76***
		(33.34, 65.98)	(2.2e-07, 10.49)	(10.76, 13.36)	(0.63, 0.86)
	Total	**14.28***	4.58	**4.11***	**0.61***
		(5.60, 23.15)	(1.9e-06, 10.03)	(3.66, 4.56)	(0.33, 0.87)
	Head	0.14	0.15	**0.28 ***	0.24
		(2.0e-06, 0.30)	(2.7e-05, 0.26)	(0.24, 0.31)	(3.5e-06, 0.49)
I	**Midpiece**	**16.76 ***	1.17	**8.44***	**0.63***
		(9.87, 22.72)	(3.7e-08, 4.08)	(7.37, 9.43)	(0.48, 0.58)
	Tail	**38.81***	2.2	**10.29***	**0.75***
		(27.17, 51.1)	(1.5e-05, 8.63)	(9.03, 11.58)	(0.62, 0.84)
	Total	**12.48***	4.787	**4.613***	**0.56***
		(3.257, 22.0)	(2.6e-04, 10.79)	(4.20, 5.15)	(0.24, 0.84)
	Head	**0.19***	0.08	**0.24***	**0.36***
		(0.06, 0.32)	(2.5e-08, 0.17)	(0.21, 0.29)	(0.15, 0.58)
S	**Midpiece**	0.92	**2.67***	**4.49***	0.11
		(7.1e-08, 2.84)	(0.72, 4.04)	(4.02, 4.99)	(9.1e-09, 0.33)
	Tail	**10.22***	3.54	**6.01***	**0.51***
		(4.06, 16.73)	(3.6e-06, 7.60)	(5.40, 6.74)	(0.25, 0.74)
	Total	**16.04***	**4.47***	**4.51***	**0.63***
		(7.35, 26.38)	(0.0001, 10.23)	(4.01, 5.12)	(0.37, 0.86)

Table 5. Genetic correlations between the three sperm components for each selection line. There was a significant negative correlation between midpiece and tail length in the long and intermediate selection lines, indicated in bold[1].

Line	Component	Head	Midpiece
Short	**Head**	---	---
	Midpiece	-0.004	---
	Tail	0.181	-0.016
Intermediate	**Head**	---	---
	Midpiece	-0.089	---
	Tail	-0.052	**-0.835**
Long	**Head**	---	---
	Midpiece	0.102	---
	Tail	0.054	**-0.860**

[1]Significant correlations indicated when the 95% CIs of the model's posterior mode did not span zero (data not shown).

Matrix comparisons: pairwise approach

Short vs. Intermediate

The genetic variation illustrated in Figure 4 for each line was observed in one dimension, demonstrated by a single significant vector in both the S- and I-line (Table 6). There were similar ratios between the first and second vectors for each line, indicating that the shape of the distribution of genetic variance between G_S and G_I was similar. However, the orientation of the principle eigenvector of G_S and G_I were significantly different, such that the genetic covariance of the three traits has changed between the lines. There was less genetic variation (illustrated by the volume of the ellipsoid) in G_S compared to G_I (Figure 5), although this difference was not significant because of the wide 95% CIs around the estimate of the variance for G_I (Table 6). G_S had significantly less variation in G_{MAX} compared to G_I. Overall, these differences between the lines resulted in the G_S having a reduced potential for adaptive evolutionary change.

Short vs. Long

As above, the genetic variation for both the S- and L-line was observed in one dimension, described by a single significant vector and the similar ratios between the first and second vectors for each matrix (Figure 6 & Table 7). The orientation of the principle eigenvector of G_S and G_L were significantly different, indicating that the genetic covariance of the three traits has changed between the lines. There was significantly less genetic variation overall in G_S compared to G_L, with less variation in G_{MAX} in G_S compared to G_L. These differences result in reduced potential for adaptive evolutionary change in G_S compared to G_L.

Intermediate vs. Long

The genetic variation in both G matrices was observed in one dimension, described by a single eigenvector and the similar ratios between the first and second vector for each line (Figure 7 & Table 8). The genetic covariance between the traits was the same in G_L and G_I because there was no difference in the angles of the principle eigenvectors of each G matrix. The total variation in both G matrices was similar. The only significant difference between the G matrices was the variance in G_{MAX} where G_I had slightly less variation compared to G_L. Therefore, the L-line had slightly higher evolvability than the I-line.

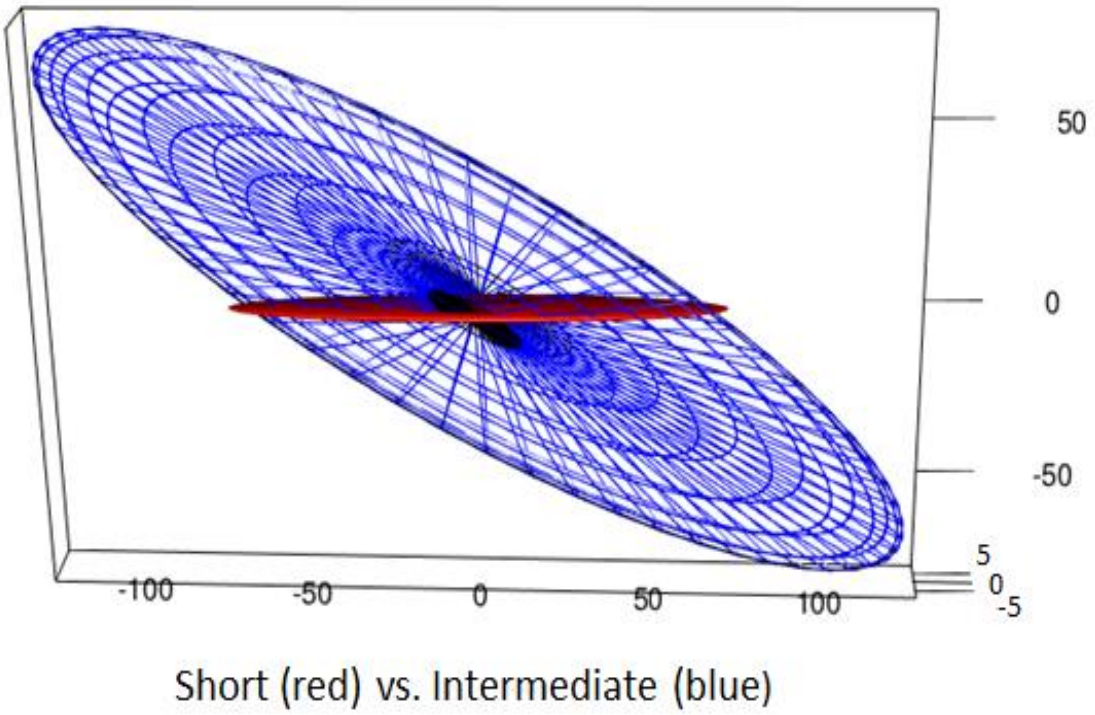


Figure 5. Comparison of the G matrices of the short (red) and intermediate (blue) selection lines. The amount of genetic variance in G_{MAX} varied between the lines, illustrated by the different lengths of G_{MAX}. The majority of the genetic variation existed in the 'x' dimension (left to right). The genetic covariance across the traits also differed, demonstrated by the shift in orientation of the ellipsoid of G_I (blue) compared to G_S (red).

Table 6. Matrix comparison statistics comparing the short (G_S) and intermediate G_I matrices. There were significant differences in the variances of G_{MAX}, and in the genetic associations between the suite of traits (head, midpiece and tail) between the two lines (indicated in bold).

	Short vs. Intermediate
Test statistic[1]	**Mode (95% CI lower, upper)[2]**
Angle between G_{MAX}	**30.99 (25.03, 41.18)**
Variance of G_{MAX} (G_S)	1119.82 (443.69, 1755.54)
Variance of G_{MAX} (G_I)	5557.57 (3935.22, 7041.41)
Difference in variance of G_{MAX}	**-4520.81 (-6011.74, -2619.25)**
Volume G_S	902.12 (1.83, 8835.87)
Volume G_I	13254.69 (2178.93, 32056.76)
Difference in volume	-10555.73 (-29577.01, 1570.03)
Ratio of 1st to 2nd vector G_S	7.02 (1.39, 109.12)
Ratio of 1st to 2nd vector G_I	15.75 (6.77, 31.01)
Difference in ratio	-3.82 (-27.22, 97.28)
Evolvability of G_S	36.24 (22.55, 42.51)
Evolvability of G_I	72.56 (60.82, 81.26)
Number of significant vectors (G_S, G_I)	1,1

[1]Refer to main text for explanation of the test statistics.
[2]Significant results are indicated when the 95% credibility intervals did not overlap zero and are in bold.

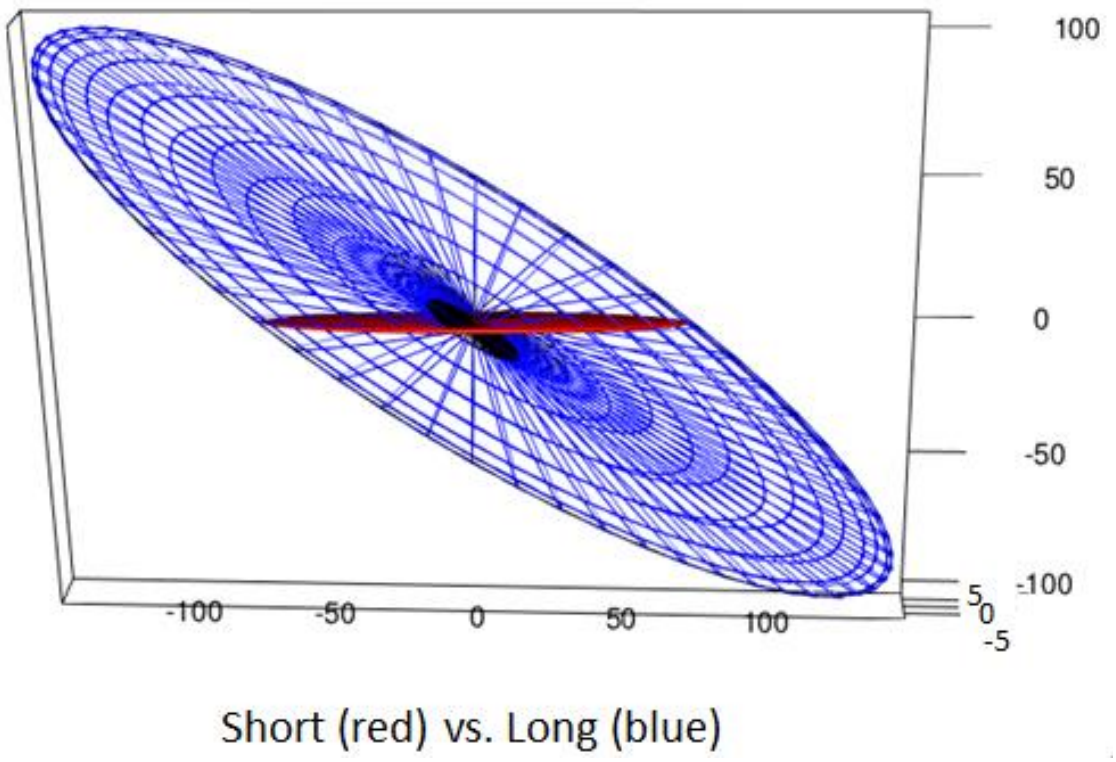


Figure 6. Comparison of the G matrices of the short (red) and long (blue) selection line. The amount of variance in G_{MAX} was significantly different between the lines. The majority of the genetic variation existed in the 'x' dimension (left to right). The genetic covariance across the traits also differed, demonstrated by the shift in orientation of the ellipsoid of G_L (blue) compared to G_S (red).

Table 7. Matrix comparison statistics comparing the short (G_S) and long (G_L) matrices. There were significant differences in the variances of G_{MAX}, and in the genetic associations between the suite of traits (head, midpiece and tail) between the two selection lines (indicated in bold).

	Short vs. Long
Test statistic[1]	**Mode (95% CI lower, upper)[2]**
Angle between G_{MAX}	**38.77 (31.20, 47.83)**
Variance of G_{MAX} (G_S)	1119.82 (443.69, 1755.54)
Variance of G_{MAX} (G_L)	7720.04 (5841.83, 9779.02)
Difference in variance of G_{MAX}	**-6576.76 (-8825.64, -4642.74)**
Volume G_S	902.12 (1.83, 8835.87)
Volume G_L	37602.69 (11522.60, 60551.16)
Difference in volume	**-30116.60 (-58125.64, -7688.58)**
Ratio of 1st to 2nd vector (G_S)	7.02 (1.39, 109.12)
Ratio of 1st to 2nd vector L matrix (G_L)	13.24 (7.53, 30.89)
Difference in ratio	-4.96 (-29.39, 94.50)
Evolvability of G_S	36.24 (22.55, 42.51)
Evolvability of G_L	84.36 (73.46, 96.26)
Number of significant vectors (G_S, G_L)	1,1

[1]Refer to main text for explanation of the test statistics.
[2]Significant results are indicated when the 95% credibility intervals did not overlap zero and are in bold.

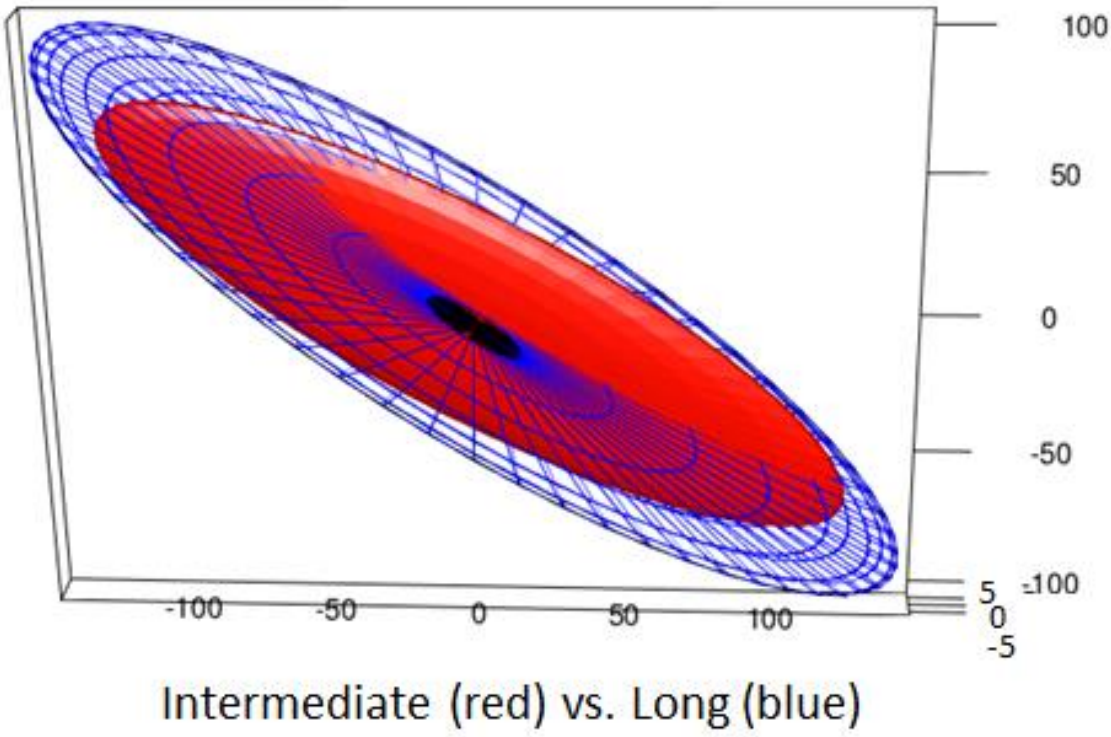


Figure 7. Comparison of the G matrices of the intermediate (red) and long (blue) selection line. There was a small but significant difference in the amount of variance in G_{MAX} between the lines such that G_L was the most variable. The genetic covariance across the traits was similar between G_L and G_I because of the similar orientation of both ellipsoids.

Table 8. Matrix comparison statistics comparing the intermediate (G_I) and long (G_L) matrices. There were significant differences in the variances of G_{MAX}, and in the genetic associations of the suite of traits (head, midpiece and tail) between the two selection lines (indicated in bold).

	Intermediate vs. Long
Test statistic[1]	**Mode (95% CI lower, upper)[2]**
Angle between G_{MAX}	4.73 (1.68, 10.12)
Variance of G_{MAX} (G_I)	5557.57 (3935.22, 7041.41)
Variance of G_{MAX} matrix (G_L)	7720.04 (5841.83, 9779.02)
Difference in variance of G_{MAX}	**-2351.86 (-5222.28, -96.11)**
Volume G_I	13254.69 (2178.93, 32056.76)
Volume G_L	37602.69 (11522.60, 60551.16)
Difference in volume	-11976.20 (-46841.10, 10923.97)
Ratio of 1st to 2nd vector (G_I)	15.75 (6.77, 31.01)
Ratio of 1st to 2nd vector (G_L)	13.24 (7.53, 30.89)
Difference in ratio	-0.98 (-19.27, 20.30)
Evolvability of G_I	72.56 (60.08, 81.26)
Evolvability of G_L	84.36 (73.46, 96.26)
Number of significant vectors (G_I, G_L)	1,1

[1]Refer to main text for explanation of the test statistics.
[2]Significant results are indicated when the 95% credibility intervals did not overlap zero and are in bold.

Matrix comparisons: a tensor approach

There was one significant eigentensor across the three selection lines representing one dimension of multivariate genetic variation, with 0.9998% (lower 95% CI: 0.0960, upper 95%CI: 0.999) of the genetic variation explained by this single axis of variation.

Tail length was the phenotypic trait that contributed the majority of the genetic variation of the single significant eigentensor (Table 9). Although the 95% CIs for tail length overlapped zero, the modal estimate was several orders of magnitude greater than the estimates for both head and midpiece length, suggesting that tail length is the most important trait influencing phenotypic change across the three lines.

Table 9. The amount of genetic variation contributed to the single significant eigentensor by each phenotypic trait. Tail length contributed virtually all the genetic variation, although the 95% CIs overlapped zero.

Phenotypic trait	**Proportion of variance of tensor explained by each trait Mode (95% CI)**
Head	-4.23e-05 (1.054e-10, 0.005)
Midpiece	4.915e-06 (1.212e-11, 0.003)
Tail	1.000 (9.945e-01, 0.999)

4.4: Discussion

The main finding from this study was that the underlying additive genetic variation differed between sperm from the S-line compared with sperm from both the L- and I-lines. The pattern of these differences was similar to the phenotypic relationships presented in Chapter 3. Sperm from the S-line had significantly lower genetic variance; however, the genetic covariance across the three sperm components was different between the lines, which will ultimately dictate the differences in the evolutionary trajectory of sperm design across the selection lines. Here, these results are discussed in more detail.

Variance and covariance of sperm components

Initially, estimates of heritability were calculated to ensure that each component actually had additive genetic variation before proceeding with the more complex modelling. In general, the heritability estimates were similar to previously published estimates of zebra finch sperm morphology (Birkhead et al. 2005; Mossman et al. 2009). However, there were two important exceptions. First, in the present study the heritability of head length in all lines was lower than reported previously, and was actually non-significant in the I-line. Second, the heritability of midpiece length in the S-line was also non-significant. The power to detect significant heritability increases as a function of sample size because larger datasets will have greater number of relatives, which are used to characterize the genetic relationship between traits. The present study used a smaller dataset (n = 422 compared to n = 923 in Mossman et al. 2009) and then each line was analysed separately, resulting in a reduction of power to detect heritability. Head length is also the phenotype with the lowest range of absolute values, for example most head lengths are between 8 and 12µm, which may make detecting and estimating heritability more difficult, especially if the extent of variation is similar within and between families. The low heritability estimate of midpiece length in the S-line may be because the males with shorter sperm have less variable midpieces. The founder cohort of the S-line had more similar sperm designs, and comparably low levels of genetic variation within and between families, which is reflected by the inability to detect additive genetic effects. Midpiece length did have a significant permanent environment effect (P_E), which does suggest some degree of genetic control as the phenotype is consistent across sperm from the same families. Overall, the low power of the dataset is a likely cause of the non-significant heritability estimate of head and midpiece length discussed above; consequently, the

significant heritability estimates reported previously (Birkhead et al. 2005; Mossman et al. 2009) may be more robust.

The magnitude and direction of the strong negative genetic correlations between midpiece and tail length observed in the present study are similar to the phenotypic correlations reported in Chapter 3, indicating that these genetic relationships in zebra finch sperm are accurately represented by the phenotypes. Sperm with shorter midpieces are more likely to have longer tails, and this sperm design occurs commonly in the longest sperm. In contrast, midpiece and tail length was unrelated in the S-line. The genetic correlations between head, midpiece and flagellum length were calculated previously by Birkhead et al. (2005). In that study midpiece and flagellum length had considerable negative genetic correlations indicating that as sperm length increased then midpiece length decreased. Although Birkhead et al. (2005) did not include tail length in the analysis; given that longer sperm (with longer flagella) have longer tails in the L- and I-lines, the results of the present study corroborate that result. The difference in genetic correlation between midpiece and tail in the S-line, compared to that of the L- and I-line, is because groups of sperm with different designs formed the founder cohort for the three selection lines: (i) the short sperm where midpiece and tail lengths were independent, and (ii) the longer sperm where a short midpiece was associated with a longer tail, or vice versa.

Comparison of G matrices

The G matrix describes the additive genetic variance and covariance between sperm components (Lande 1979), and has been used to compare the genetic relationships within single species (Schluter 1996; Cano et al. 2004; Hine & Blows 2006; Robinson & Beckerman 2013). As discussed above, the patterns of distribution of genetic variance between the three selection lines was similar to the phenotypic variation in each line described in Chapter 3, such that the G matrices of the L- and I-line were comparable, yet both matrices were different from the G matrix of the S-line. The S-line had less additive genetic variance (represented by ellipsoid volume) than the L-line, although the comparisons between the S- and I-line were not actually statistically significant. This is likely to be a consequence of the extreme uncertainty around the volume estimate in the I-line, represented by the wide 95% CIs. Given that there was (i) no difference in volume between the L- and I-line, and (ii) a significant difference in volume between the L- and S-line, it is likely that the lack of difference between the S- and I-line volume estimates is a spurious result. Because additive genetic variation is required for

phenotypic change, less genetic variance in the S-line could mean a reduced potential for phenotypic change in S-line sperm. In a situation where sperm dimensions may need some degree of flexibility, for example if the internal dimensions of the female reproductive tract evolve and change in response to sperm competition, males with short sperm may be disadvantaged by an inability to adapt to the change in female internal physiology. In contrast, if the longer sperm from the L- and I-line have greater flexibility to respond to this change, males producing these longer sperm may benefit via enhanced fertilisation success.

The most interesting difference comparing the S-line G matrix with both the L- and I-line G matrices was the difference in covariance between the three sperm components across the matrices. This was represented by the difference in orientation of the ellipsoids, and a significant difference in G_{MAX} between the lines. The consequence of the differing genetic covariance between lines is that the evolutionary trajectory of phenotypic change is altered. As the orientation of the ellipsoid represents the direction of least resistance for phenotypic change (Schluter 1996), applying selection pressure to alter sperm dimensions in the L- and S-lines is likely to result in different phenotypic outcomes, as each line will respond to the selection according to the direction of least resistance. Sperm designs are then constrained to change in a certain manner. If there was strong selection for the L- and S-lines to reach an optimum sperm length phenotype midway between their existing dimensions (Figure 8), then it may not be possible for the S-line sperm to reach that optimum phenotype, because it is constrained to phenotypic change along the principle axis of variation, and also has minimal variation in other dimensions (y and z) in phenotype space. In contrast, the L-line sperm may reach the optimum phenotype by changing along the path of least resistance. Although there was also only one significant dimension to the variation, there still is potential for some phenotypic change in the ‘y’ dimension due to the greater amount of genetic variation compared to the S-line. Overall, these differences in the G matrices result in the S-line having a lower measure of evolvability (Kirkpatrick 2009) and less potential for evolutionary change than either the L- or I-lines.

Comparing the three G matrices using the tensor approach also indicated that there was a single dimension to the additive genetic variation, such that phenotypic change is constrained to evolve along that single axis of variation. This result agrees with the pairwise matrix comparisons discussed above. This strongly suggests that there is minimal variation in the other dimensions of phenotypic shape space, i.e. in the S-line there is virtually no genetic variation along the y dimension (along the y-axis of the plots; Figure 5 & 6). If there was an

additional significant dimension to the genetic variation in one selection line, this could suggest that there were line-specific allelic effects determining genetic variation in that line. This strong restriction of phenotypic change could mean some phenotype combinations are unlikely to occur, perhaps explaining why there were no short sperm with a tiny midpiece and longer tail, producing the odd distributions seen in plots of midpiece and total length in both captive and wild populations (Immler & Birkhead 2012; Chapter 3). The tensor analysis also highlights that the overwhelming majority of the genetic variation across the three G matrices is contributed by differences in tail length. This agrees with the observations discussed in Chapter 3, as tail length was completely diverged between the three lines over the selection regime, and also appeared to the primary constituent of the change in total length of sperm.

An important question leading from this result is what is the basis for the differences in the genetic relationships between the S-line, and the L- and I-line? The results presented here suggested that L- and I-lines have different genetic architecture. It is not known whether this difference in genetic architecture is a consequence of the selection regime or naturally occurring in this population of zebra finches, because replication of the selection lines was not possible. It was also not possible to analyse each cohort separately to visualize the changes in genetic architecture over the selection regime as the sample size would have been too small and with too little power to confidently draw conclusions.

Given that the genetic and phenotypic relationships are described in this thesis are similar, and the phenotypic patterns shown in Chapter 3 are similar in wild zebra finches (Immler & Birkhead 2012), it is possible that the difference in genetic architecture was naturally occurring. Therefore, the differences in the genetic relationships between the selection lines could be due to the division of available genetic variation during selection of the founder cohort (Chapter 2 Section 2.2): one group of males where the phenotypic and genetic variation of the sperm midpiece was low, and the second group with greater amounts of genetic variation due to the increased range of possible sperm designs. These differences in genetic architecture then dictated how the sperm phenotypes changed further in response to artificial selection; with a range of phenotypes in the L- and I-line maintained by the genetic variation and change in covariance across sperm components compared to the restricted sperm design of the S-line. The extensive genetic variation in tail length was a major driver in the phenotypic change across the lines, although the difference in genetic covariance between the lines meant that in the L- and I-line, changing tail length was combined with changing midpiece length, where in the S-line tail length change was independent of midpiece length.

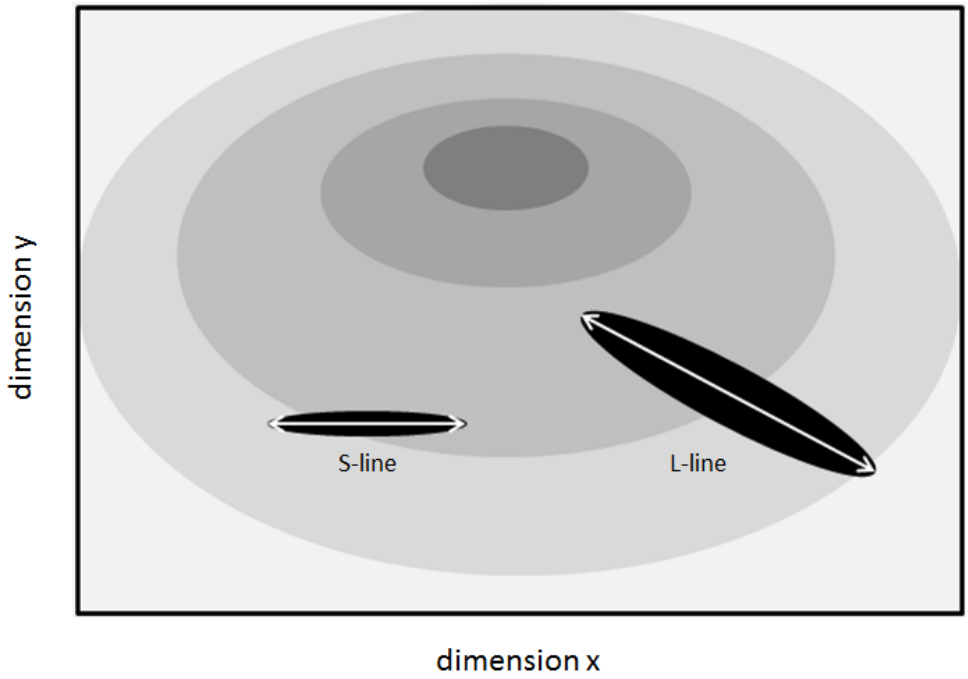


Figure 8. Schematic of an adaptive landscape illustrating how the genetic architecture of the S and L-lines control phenotypic change. The lengths of the ellipsoids representing additive genetic variation are drawn to an arbitrary scale. The S-line is constrained to phenotypic change in the x dimension, however the difference in genetic covariance of sperm components in the L-line mean that phenotypic change can occur along the direction of least resistance (white arrows) therefore obtaining the optimum, fittest phenotype (the darkest grey shading).

Despite the fact that sperm competition is low in the zebra finch, and extreme variation in both sperm morphology and genetic variation may be expected (Birkhead et al. 2005), very strong directional constraints on phenotypic traits were evident. Phenotypic change could really only occur in one dimension, for example by changing the length of each sperm component, even in the more variable L-line. This is likely to originate from a functional constraint where the sperm of birds must adhere to strict designs, to ensure the sperm can function effectively in the female reproductive tract. Constraining the evolutionary trajectories of the sperm may ensure phenotypic change does not deviate from the tried and tested designs.

In conclusion, the results from this study indicate that the phenotypic and genetic relationships observed across the three selection lines were qualitatively similar. Large differences in genetic variation were found between the G matrices of the three lines, especially between the S- and L-lines. These differences, and the differences in the genetic covariance of the individual sperm components in the S-line compared to the L- and I-line causes the variation in response to artificial selection between the lines, and control the evolutionary trajectory of sperm designs. This study has demonstrated that the similar phenotypic response of the L- and I-lines to selection occurred because of the similar underlying genetic architecture. Overall, despite observing the possibility for strong design constraints, there is still potential for further adaptive change in sperm dimensions in this population of zebra finches, which agrees with speculation by Simmons & Moore (2009). This potential for adaptive change may be beneficial in a sperm competition context. The focus of the following chapter is to investigate exactly how these changes in sperm morphology may affect one of the most important functions of the sperm; their motility and swimming velocity.

Chapter 5

The coevolution of swimming velocity following artificial selection on sperm morphology

5.1: Introduction

In birds, sperm are inseminated into the cloaca and rely on being motile to traverse the vagina (Allen & Grigg, 1957) and reach the sperm storage tubules (SSTs). The small proportion of sperm that reach the SSTs (around 2% of inseminated sperm: Brillard 1993; Bakst et al. 1994) are later released and are transported passively towards the infundibulum (Allen & Grigg, 1957), where fertilisation takes place (Olsen & Neher 1948; Bakst et al. 1994). In sperm competition, where multiple males inseminate a single female and compete to fertilise a set of ova (Parker 1970), sperm motility and swimming velocity may be important factors in determining the relative numbers of each male's sperm that enter the SSTs, and hence, the set of sperm with reasonable likelihood of fertilising ova (Birkhead et al. 1999b; Donoghue et al. 1999).

Across species, longer and faster swimming sperm are associated with increasing sperm competition risk (Gomendio & Rolden 1998; Lüpold et al. 2009b but see Stockley et al. 1997). Midpiece volume in mammals also correlates with sperm competition intensity (Anderson & Dixson 2002; Anderson et al. 2005); therefore, sperm morphology and swimming velocity are assumed to be inter-dependent. The theory behind this assumption relates to: (i) the sperm flagellum length, where longer flagella in mammals are predicted to produce greater forward propulsive forces via an increased beat frequency (Cardullo & Baltz 1991), (ii) the sperm midpiece length or volume, where a longer midpiece or larger midpiece volumes are expected to have more mitochondria, producing more energy in the form of adenosine triphosphate (ATP) (Rowe et al. 2013), and (iii) the relative lengths between sperm components, such that sperm with smaller heads relative to the length of the flagellum are less affected by drag from the external environment (Humphries et al. 2008), which could result in faster swimming velocity.

Empirical tests of the three predictions above using single species suggest that longer sperm do indeed swim faster (Mossman et al. 2009; Fitzpatrick et al. 2010 but see Gage et al. 1998; Gage et al. 2002; Fitzpatrick et al. 2007). Indirect evidence suggests that the midpiece should have an important influence on swimming speed; sperm with longer midpieces had a higher ATP concentrations in salmon *Salmo salar* (Vladic et al. 2002), and sperm with more ATP have greater mobility in domestic fowl *Gallus domesticus* (Froman & Feltmann 1998) and the bluegill *Lepomis macrochirus* (Burness et al. 2004). Yet the actual effect of midpiece length on sperm velocity is equivocal, as sperm with absolutely longer midpieces swam faster in *Mus*

domesticus (Firman & Simmons 2010), slower in deer *Cervus elaphus hispanicus* (Malo et al. 2006), and were unaffected by midpiece length in zebra finches *Taeniopygia guttata* (Mossman et al. 2009). Finally, sperm with a larger flagellum: head ratio (small head relative to flagellum length) (e.g. Mossman et al. 2009; Helfenstein et al. 2010), and a long midpiece relative to total sperm length (Laskemoen et al. 2010) achieve higher swimming velocities. This effect of the latter 'sperm design' on swimming velocity should be investigated, even if midpiece length *per se* is unrelated to velocity. If two sperm have very different flagella lengths yet a similar midpiece length, and if all else is equal, then theoretically less power may be available per unit length of the longer flagellum, affecting swimming velocity. Midpiece length relative to total length or tail length is, however, largely ignored in the literature.

Whereas in some taxa there is no relationship between morphology and velocity (Gage et al. 2002; Fitzpatrick et al. 2007) and sperm motility must be determined by other processes (Fitzpatrick et al. 2007), it is possible that in other species, some relationships may exist but remain undetected. In species with extensive intra-ejaculate morphological variation (e.g. the bullfinch *Pyrrhula pyrrhula;* Birkhead et al. 2006a), a lack of association between morphology and velocity may occur if mean values of sperm morphology per male are used (even if many sperm are measured per male). Similarly, as sperm velocity and motion can vary greatly within a single ejaculate (e.g. Abaigar et al. 2001), simply using a single mean value per male may also be misleading (Holt et al. 2007). Hence, separating the sperm into distinct subpopulations based on sperm velocity may be a good approach to disentangle the relationship between morphology and velocity (Mossman et al. 2009). Ideally, where possible, direct linkage between sperm morphology and velocity estimates for individual sperm should be made (Fitzpatrick et al. 2010). This approach may also be useful when predictable morphological variation between males is low, such as in domestic fowl (T. R. Birkhead, pers. com).

The relationship between sperm morphology and sperm velocity can also be investigated using species with minimal intra-ejaculate morphological variation, such as the zebra finch. In the zebra finch, all morphological traits are highly repeatable (Chapter 2 Section 2.4.3), yet there is extensive between-male variation across all sperm components (Birkhead et al. 2005). Although some of the relationships between morphology and velocity have already been described in this species (in general, longer sperm, and sperm with longer tails and larger flagellum: head ratios swim faster: Mossman et al. 2009), the indication from Mossman et al's (2009) data is that some of the longest sperm do not actually achieve the fastest swimming speeds. It is not possible to explain exactly why this may occur given the low numbers of

extremely long and short sperm included in Mossman et al's (2009) dataset (Appendix 4). However, long sperm are more likely to have shorter midpieces and longer tails (Chapter 3), perhaps resulting in less energy produced by the midpiece to power the longer tail. By exploiting the divergence in mean sperm total length resulting from the selective breeding regime (documented in Chapter 3), there is potential to disentangle the effect of sperm morphology on swimming velocity using a greater numbers of males with extreme sperm phenotypes.

Chapter 3 also yielded some additional interesting findings. Although there was divergence in mean sperm total length between the three selection lines, where the long line (L-line), intermediate line (I-line) and short line (S-line) produced the longest, medium and shortest sperm respectively, there were marked differences in the phenotypic relationships between the sperm components (Chapter 3). Specifically, midpiece and tail lengths were negatively associated in the L- and I-lines, yet unrelated in the S-line. Determining how sperm velocity differs between the three selection lines, and whether similar sperm designs result in comparable swimming speeds is of interest.

Aim

The general aim of this part of the study was to understand how sperm morphology influences swimming velocity in two ways: (i) by investigating the differences between the three selection lines across a suite of kinematic parameters (see Section 5.2), and (ii) characterising the effect of each component of sperm morphology on sperm velocity. Longer sperm from L-line are predicted to swim faster than shorter sperm from the S-line, due to greater propulsion from longer tails and flagella (Mossman et al. 2009). However, it is speculated that the combination of an extremely long tail and short midpiece, as described in Chapter 3, may not promote the fastest swimming speeds, as a short midpiece may be unable to provide sufficient energy to power a long tail.

5.2: Methods

Study population

The zebra finches used in this study were from the selection lines that were part of a breeding regime selecting for divergence in sperm total length (described in Chapter 2 Section 2.2).

Male zebra finches used (n = 144) were from the L-line (n = 51), I-line (n = 40) and S-line (n = 53). Live sperm samples were obtained by dissection from the left seminal glomerus (SG) from each male (Chapter 2 Section 2.3.4).

Sperm motility assay

Sperm motility (a general term for how sperm swim) was measured as described in Chapter 2 Section 2.4.1. Briefly, five kinematic parameters were obtained for each sperm from each male using Computer Assisted Sperm Analysis (CASA): (i) average path velocity (VAP), (ii) curvilinear velocity (VCL), (iii) straight line velocity (VSL), (iv) linearity (LIN: given by VSL/VCL), and (v) straightness (STR: given by VSL/VAP). VAP, VCL and VSL describe sperm swimming speed (velocity in μm/s) while STR and LIN describe sperm swimming motion. Because VAP, VCL and VSL covary (Mossman et al. 2009), a Principle Component Analysis (PCA) was used to reduce these three parameters into a single index of sperm velocity (PC1) for each individual sperm, referred to as sperm velocity from hereon (see Statistical Analyses for details of the PCA).

Following the PCA, the dataset was divided into three subpopulations of sperm (as in Mossman et al. 2009) because there was considerable variation in swimming velocity within the ejaculate of a single male (for example, a typical range of sperm velocity is 0 - 70μm/s), and so using average values per male in analyses may be misleading (Holt et al. 2007). The subpopulations were as follows: (i) the total population of sperm, (ii) the fastest 10% of sperm, and (iii) the fastest single sperm. The latter two subpopulations of sperm were selected using the highest 10%, and highest single PC1 scores respectively (PC1 was highly correlated to VAP, VCL and VSL; Pearson's correlation: $r = 0.99$, 0.93 and 0.97 respectively, all $d.f = 147$ and all $p < 0.0001$). PC1 was then used as an index of sperm velocity in all analyses (mean PC1 scores per male for the total population and the fastest 10% of sperm, and the single highest PC1 score per male for the fastest sperm subpopulation).

Sperm morphology

The sperm morphology of each male was assessed as described in Chapter 2 Section 2.4.2. Briefly, ten morphologically normal sperm per male were measured to the nearest 0.01μm using ImageJ (Abramoff et al. 2004) for the following sperm components: (i) head, (ii) midpiece, and (iii) tail. Flagellum and total length were calculated by adding together the values for the relevant component parts (midpiece plus tail, head plus midpiece plus tail respectively).

Statistical analysis

Three kinematic parameters describing sperm velocity (Chapter 2 Section 2.4.1) were obtained using Computer Assisted Sperm Analysis (CASA). Three Principle Components (PCs) were obtained from the motility dataset and the proportion of variance explained by each PC is presented in Table 1. PC1 was used as the index of swimming velocity in all subsequent analyses in this Chapter, and in Chapter 6, because it explained the majority of the variance (93%) in the data set (Table 1). The factor loadings from the PCs on the three kinematic parameters (VAP, VCL and VSL) are given in Table 2. Mean PC1 values were calculated for each male for each the three sperm subpopulations (see above), and used as the response variable in the models described below.

The effect of selection line on multiple kinematic parameters was analysed using linear models (LMs). VAP, VCL, VSL and PC1 were analysed in separate models with selection line included as an explanatory variable. The effect of selection line on sperm motion (STR and LIN) was analysed using generalised linear models (GLMs) with a binomial error distribution and a logit link function (STR and LIN are proportions). Line was included as an explanatory variable as above. The analyses were repeated with models specified as above for the fastest 10% of sperm, and the fastest single sperm from each male.

The effect of sperm morphology (head, midpiece, tail, total length, flagellum: head ratio and midpiece: tail ratio) on PC1 was investigated using LMs. A separate model was used for each sperm component because the sperm components covary. The mean value of each sperm component was calculated per male and included as an explanatory variable. Weighting the models by the number of sperm from each male did not change the conclusions of the analyses (data not shown).

All statistical analyses were conducted using R v 2.15.1 (R Core Team, 2012) with the base package.

Table 1. Descriptive statistics obtained from the PCA. PC1 was used as the index of sperm velocity as it explained 93% of the variance in the data set (highlighted in bold).

	PC1	**PC2**	**PC3**
Standard deviation	1.68	0.43	0.08
Proportion of variance	0.937	0.060	0.002
Cumulative proportion	**0.937**	0.998	1.000

Table 2. The factor loadings from each PC on the three kinematic parameters from the dataset (n = 144).

	PC1	**PC2**	**PC3**
VCL	-0.559	0.821	0.118
VSL	-0.582	-0.490	0.649
VAP	-0.590	-0.294	-0.752

5.3: Results

The effect of selection on sperm velocity

Sperm from the S-line swam more slowly compared to sperm from the L- and I-line (Table 3 & Figure 1), given by lower values of VAP, VCL, VSL and PC1 (Table 3). Sperm from the L- and I-lines swam at similar speeds, with no differences in any kinematic parameter between those lines. There were no differences in measures of sperm motion given by STR and LIN between the three selection lines.

The same patterns as above were observed in the fastest 10% and the fastest single sperm subpopulations of sperm (for results of all analyses see Table 3; refer to Appendix 5 for plots describing these relationships).

Table 3. Results of models analysing the differences in six measures of sperm motility between the three selections lines for each subpopulation of sperm[1]. For all kinematic parameters, the short selection line had significantly lower mean values compared to the long and intermediate line. The intermediate line achieved slightly higher kinematic values compared to the long line, although this difference was non-significant. P values in bold italics indicate significance ($p < 0.05$).

Sperm population	Kinematic parameter	Mean ± S.D Long	Intermediate	Short	$F_{2,141}$[a] / deviance[b]	p
Total population	VAP (µm/s)	43.70 ± 14.54	44.33 ± 15.19	***34.21 ± 12.02***	*8.34*[a]	***0.0004***
	VCL (µm/s)	51.42 ± 14.15	58.11 ± 15.49	***46.61 ± 18.44***	*5.74*[a]	***0.0037***
	VSL (µm/s)	40.09 ± 15.16	39.99 ± 15.45	***30.67 ± 11.33***	*7.55*[a]	***0.0008***
	PC1	1.10 ± 0.91	1.23 ± 0.93	***0.58 ± 0.82***	*7.27*[a]	***0.0009***
	LIN	0.63 ± 0.17	0.57 ± 0.16	0.59 ± 0.18	-0.34[b]	0.8432
	STR	0.74 ± 0.15	0.74 ± 0.13	0.75 ± 0.17	-0.03[b]	0.9862
Fastest 10%	VAP (µm/s)	76.88 ± 15.01	78.43 ± 17.03	***58.82 ± 14.90***	*24.43*[a]	***< 0.0001***
	VCL (µm/s)	83.45 ± 12.86	90.11 ± 11.13	***74.24 ± 17.82***	*14.12*[a]	***< 0.0001***
	VSL (µm/s)	73.90 ± 15.90	74.65 ± 18.01	***55.04 ± 14.69***	*23.84*[a]	***< 0.0001***
	PC1	3.18 ± 0.90	3.35 ± 0.94	***2.18 ± 0.91***	*23.42*[a]	***< 0.0001***
	LIN	0.89 ± 0.11	0.83 ± 0.14	0.75 ± 0.18	-3.26[b]	0.1955
	STR	0.96 ± 0.03	0.95 ± 0.03	0.92 ± 0.12	-0.68[b]	0.7112
Fastest sperm	VAP (µm/s)	89.01 ± 15.48	90.63 ± 16.57	***72.38 ± 19.02***	*17.27*[a]	***< 0.0001***
	VCL (µm/s)	94.81 ± 18.00	100.26 ± 18.33	***83.63 ± 18.07***	*10.41*[a]	***< 0.0001***
	VSL (µm/s)	85.45 ± 16.48	86.91 ± 16.79	***68.76 ± 19.37***	*31.09*[a]	***< 0.0001***
	PC1	3.92 ± 0.95	4.09 ± 0.96	***2.97 ± 1.11***	*17.51*[a]	***< 0.0001***
	LIN	0.91 ± 0.13	0.88 ± 0.14	0.82 ± 0.19	-1.81[b]	0.4046
	STR	0.96 ± 0.06	0.96 ± 0.04	0.94 ± 0.11	-0.32[b]	0.8507

[1]Mean values between the selection lines were calculated from the raw data (n = 144).
[a]Differences in means between the lines were compared using LMs
[b]Differences in means between the lines were compared using GLMs by comparing models with and without line as a fixed effect with log likelihood tests.

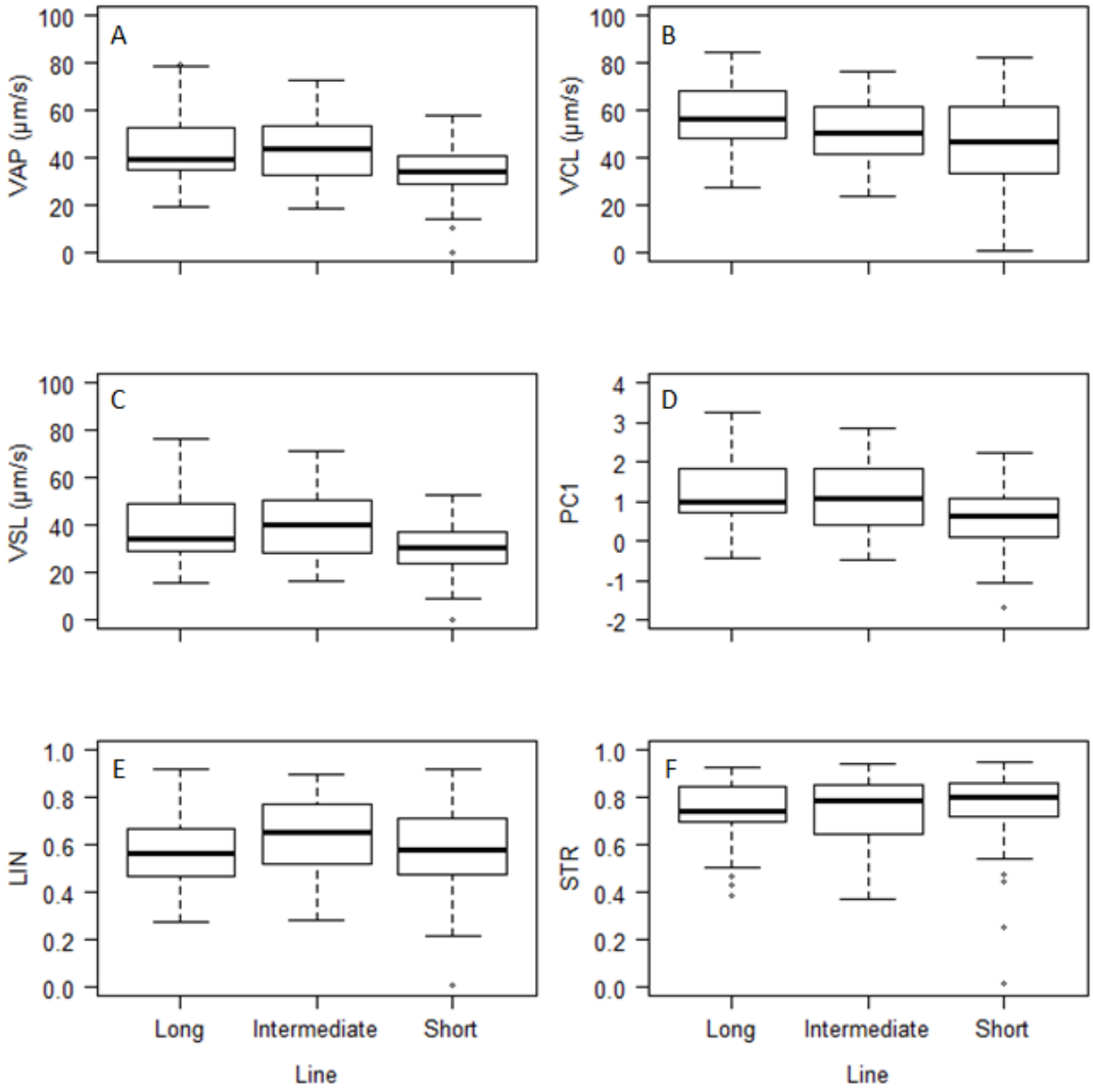


Figure 1. The effect of selection line on six kinematic parameters of zebra finch sperm (n = 144). VAP (A), VCL (B) and VSL (C) were analysed using PCA to give (D) an index of sperm velocity (PC1). LIN (E) and STR (F) are given by VSL/VCL and VSL/VAP respectively. Median values are represented by the thick black line across each individual boxplot, and the box and whiskers represent the variation in the data. (A-D): the S-line had significantly lower values for the kinematic parameter. (E-F): there are no differences between the lines in either STR or LIN.

The effect of sperm morphology on sperm velocity

Sperm morphology was a significant determinant of swimming velocity (given by PC1), with all sperm components associated with swimming velocity in the total sperm subpopulation (Table 4 & Figure 2), although the relationship between midpiece length and swimming velocity was weak. All components of sperm morphology also had a significant influence on swimming velocity in the fastest 10% and fastest single sperm subpopulation (Table 4 & Appendix A5) with the exception of midpiece length, where positive but non-significant trends were observed.

Across the three subpopulations, sperm head length was linearly associated with swimming velocity, such that sperm with longer heads swam faster, although the R^2 values were low compared to values associated with other sperm components (Table 4). However, tail length and total length determined swimming velocity via a quadratic relationship where swimming velocity increased as tail and total length increased until a certain value was reached (tail: approximately 30μm; total length: approximately 70μm), after which swimming velocity decreased (Table 4 for all data; Figure 2C & D for plots of the total sperm subpopulation; see also Appendix A5 for the plots of fastest 10% and fastest single sperm subpopulation).

Sperm velocity was also determined by relative lengths of sperm components; flagellum: head ratio and midpiece: tail ratio, via a quadratic relationship in all subpopulations of sperm (Table 4 for all data, Figure 2E & F for plots of the total sperm subpopulation; see Appendix A5 for the plots of fastest 10% and fastest single sperm subpopulation). Sperm swam faster as flagellum length increased relative to the head length (larger flagellum: head ratio), but swimming velocity declined when ratios increased beyond 5. Swimming velocity was fastest when the lengths of the midpiece and the tail were similar (ratios around 1), and then declined with increasing ratios, where sperm had relatively longer midpieces compared to the short tails.

Table 4. Results of models analysing the effect of six measures of sperm morphology on sperm velocity (PC1)[1]. Head and midpiece length were modelled as linear explanatory variables[a]. All other sperm components (tail, total, flagellum: head ratio and midpiece: tail ratio) were associated with PC1 via a quadratic relationship. Model estimates for both the linear[a] and quadratic[b] terms from these models are presented. Significant results are indicated in bold italics ($p < 0.05$). The table and footnotes continue overleaf.

Sperm subpopulation	Sperm component	Estimate ± S.D	t	$F_{1,142}$[a] $F_{2,141}$[b]	Adjusted R^2	p
Total	Head[a]	0.386 ± 0.120	3.21	10.30[a]	0.061	***0.0016***
	Midpiece[a]	0.034 ± 0.017	2.00	4.02[a]	0.021	***0.0470***
	Tail[a]	0.195 ± 0.047	4.14	---	---	---
	Tail[b]	-0.003 ± 0.001	-3.97	8.79[b]	0.098	***0.0002***
	Total length[a]	0.614 ± 0.188	3.27	---	---	
	Total length[b]	-0.004 ± 0.001	-3.10	10.28[b]	0.115	***<0.0001***
	Flagellum: head[a]	6.682 ± 1.769	3.78	---	---	---
	Flagellum: head[b]	-0.642 ± 0.176	-3.65	8.45[d]	0.094	***0.0004***
	Midpiece: tail[a]	0.937 ± 0.447	2.10	---	---	---
	Midpiece: tail[b]	-0.339 ± 0.130	-2.62	5.16[b]	0.055	***0.0069***
Fastest 10%	Head[a]	0.525 ± 0.135	3.89	15.14[a]	0.090	***0.0002***
	Midpiece[a]	0.032 ± 0.019	1.66	2.76[a]	0.012	0.0989
	Tail[a]	0.340 ± 0.048	7.09	---	---	---
	Tail[b]	-0.006 ± 0.001	-6.54	28.76[b]	0.280	***<0.0001***
	Total length[a]	0.974 ± 0.189	5.15	---	---	---
	Total length[b]	-0.007 ± 0.001	-4.81	33.22[b]	0.311	***<0.0001***
	Flagellum: head[a]	10.543 ± 1.819	5.80	---	---	---
	Flagellum: head[b]	-0.989 ± 0.181	-5.79	26.70[b]	0.264	***<0.0001***
	Midpiece: tail[a]	1.018 ± 0.482	2.11	---	---	---
	Midpiece: tail[b]	-0.451 ± 0.140	-3.23	14.34[b]	0.157	***<0.0001***

Table 4 continued.

Sperm subpopulation	Sperm component	Estimate ± S.D	t	$F_{1,142}$ $F_{2,141}$[b]	Adjusted R^2	p
Fastest	Head[a]	0.490 ± 0.146	3.35	11.24[a]	0.067	***0.0010***
	Midpiece[a]	0.035 ± 0.021	1.67	2.78[a]	0.012	0.0974
	Tail[a]	0.346 ± 0.053	6.56	---	---	---
	Tail[b]	-0.006 ± 0.001	-6.11	23.78[b]	0.242	***<0.0001***
	Total length[a]	1.051 ± 0.209	5.03	---	---	---
	Total length[b]	-0.008 ± 0.002	-4.74	27.07[b]	0.267	***<0.0001***
	Flagellum: head[a]	10.145 ± 2.021	5.02	---	---	---
	Flagellum: head[b]	-0.952 ± 0.201	-4.74	19.92[b]	0.209	***<0.0001***
	Midpiece: tail[a]	1.215 ± 0.522	2.33	---	---	---
	Midpiece: tail[b]	-0.504 ± 0.151	-3.34	12.72[b]	0.141	***<0.0001***

[1]All analyses were carried out using LMs (n = 144).
[a]The linear term from the model and the associated model estimates.
[b]The quadratic term from the model and the associated model estimates. The significance of the quadratic term was tested by comparing models with the without the quadratic term using log likelihood tests (data not shown).

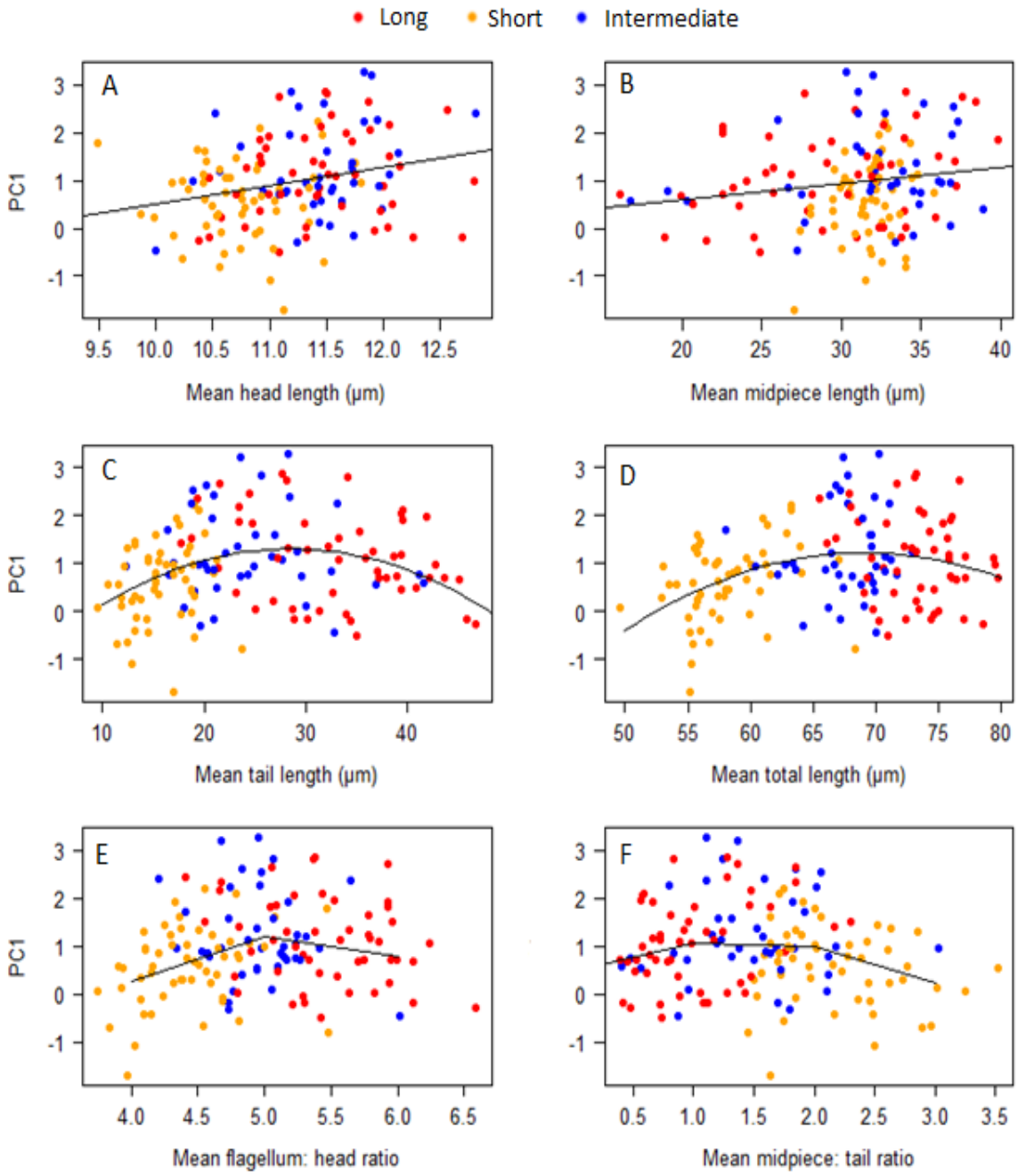


Figure 2. Relationships between sperm velocity (PC1) and six measures of sperm morphology for the total population of sperm: head (A), midpiece (B), tail (C), total length (D), flagellum: head (E) and midpiece: tail (E). The coloured points represent the three selection lines: long (red), intermediate (blue) and short (orange). Each point represents data from a single male zebra finch (n = 144). Each sperm component (A-F) had a significant effect on sperm velocity (Table 4). Head and midpiece length were linearly associated to sperm velocity, whereas increasing the length of the other sperm components only resulted in faster velocity up to a certain value, before velocity declined.

5.4: Discussion

This study demonstrated that sperm morphology is an important determinant of swimming velocity; in particular longer sperm and sperm with longer tails swam faster than shorter sperm. This was evident from (i) comparisons of kinematic parameters between the selection lines, and (ii) associations between the sperm components and the index of sperm swimming velocity (PC1). This means that the artificial selection that increased sperm morphology has also caused a correlated response in sperm swimming velocity. In addition, this study also provides the first evidence that the fastest swimming sperm are not achieved by simply increasing tail length to the extreme; rather, an extremely long tail may actually cause a decline in swimming velocity. Here, the specific relationships between sperm morphology and swimming velocity are discussed in more detail.

Kinematic parameters between the selection lines

Sperm from each selection line swam with similar patterns of motion (LIN and STR). This indicates that the relationships between VAP, VCL and VSL (which are used to calculate STR and LIN) were not significantly different. However, the absolute values of VAP, VCL, VSL and PC1 varied significantly between the selection lines. As expected, shorter sperm from the S-line swam significantly slower than longer sperm from the L- and I-lines. Slower swimming velocity is most likely a consequence of reduced forward propulsion generated by shorter tails and shorter flagella, and the clear effect observed here may be due to the stronger selection response of the S-line (Chapter 3). The most interesting result, however, was the lack of difference in mean values of VAP, VCL, VSL and PC1 between sperm from the L- and I-line, despite the L-line sperm being longer than the I-line (Chapter 3). In fact, the majority of the above values were actually higher (although not significantly) in sperm from the I-line. This suggests that simply increasing absolute sperm length does not necessarily result in faster swimming sperm in the zebra finch. Chapter 3 and Chapter 4 demonstrated that, despite the difference in absolute values of sperm total length between the L- and I-lines, the phenotypic and genetic relationships between sperm components (e.g. midpiece and tail length) were similar. This may explain why L- and I-line sperm swim at similar speeds, and supports the idea that sperm design and the relative measures of sperm components have an important influence on how sperm swim (Humphries et al. 2008; Mossman et al. 2009; Helfenstein et al. 2010).

Sperm morphology and sperm velocity

Sperm head length had a small but significant effect on sperm velocity (PC1), such that sperm with longer heads swam faster than sperm with shorter heads. This was in direct contrast to Mossman et al. (2009), who found that head length was unrelated to swimming speed. However, in this present study, it is unclear whether this relationship is due to head length *per se*, as longer sperm also have longer heads (Chapter 3), and, as discussed below, sperm total length has a more influential effect on sperm swimming speed. However, it was not possible to disentangle the effects of head and total length to PC1 in the same model due to covariance between them.

There was a weak, but marginally significant association between midpiece length and PC1 in the total sperm population ($p = 0.047$), where sperm with longer midpieces swam faster. However, this relationship was not evident in either the fastest 10% of sperm, or the fastest single sperm subpopulations (both $p = 0.09$). A non-significant association may be a consequence of using values of mean midpiece length in the analysis. If midpiece length does (albeit weakly) influence motility, and if midpiece length is more variable (although still significantly repeatable) within a single male's ejaculate, then it is feasible that the fastest swimming sperm have slightly longer midpieces. This relationship between midpiece length and velocity may then be masked by using the mean midpiece value. A similar issue is dealt with by Fitzpatrick et al. (2010) and may explain why some relationships between sperm components and sperm velocity remain undetected or inconclusive. Unfortunately, in the present study it was not possible to match sperm component measurements with individual sperm velocity data to further investigate this issue.

Mossman et al. (2009) also found no association between midpiece length and swimming velocity, but this result may be affected by the same issue discussed above, as similar analyses were performed. Midpiece length is often expected to covary with swimming velocity because of the assumption that a longer midpiece, with presumed greater mitochondrial loads, can produce more ATP via the oxidative phosphorylation (OXPHOS) pathway, although in reality, support for this idea across taxa remain equivocal (e.g. Malo et al. 2006; Mossman et al. 2009). In mammals, less emphasis is placed on the dimensions of the midpiece influencing motility because energy can also be produced via glycolysis, which can occur along the fibrous sheath of the flagellum and may provide energy directly to the flagellum (Storey & Kayne 1975, cited in Ford 2006; Eddy et al. 2003), bypassing the need for ATP to be diffused down the flagellum

from the midpiece. If glycolysis occurred in the zebra finch, this may mean that even sperm with tiny midpieces could obtain ample ATP direct from the flagellum, rendering absolute midpiece length unimportant. However, in the domestic fowl, it appears that glycolysis is not a major factor in sperm motility (instead energy is produced by respiration: Froman & Feltmann 1998; Froman et al. 1999), but it is not known whether the same is true in passerine birds.

Tail length and the total length of sperm both significantly influenced sperm velocity across all three subpopulations of sperm. However, the relationship for both sperm components was quadratic and there was a trend for declining motility above certain dimensions. The same pattern was found for both sperm components because the longest sperm also had the longest tails (Chapter 3). This result was in contrast to Mossman et al.'s (2009) study on the same populations of zebra finches. However, re-analysing a subset of Mossman et al's (2009) data for the total sperm population revealed that a quadratic relationship was actually a better fit to their data (Appendix A6). The greater numbers of extreme sperm phenotypes included in the present study may have facilitated the detection of the quadratic relationship (Appendix A6), because the ranges of sperm component dimensions are similar between the two studies.

In general, studies investigating associations between morphology and swimming velocity report results relating to head, midpiece and flagellum length, without referring to tail length (e.g. Helfenstein et al. 2010). Distinguishing between tail and flagellum length in bird species is important because of the structure of the sperm. Briefly, in the majority of species investigated (see Birkhead et al. 2006a for an exception), the midpiece consists of a single, fused mitochondrion wound around the flagellum, sometimes making up a large proportion of the flagellum length and leaving only a very small tail (e.g. Lüpold et al. 2009a). While two sperm from different males may have flagella of similar lengths, the lengths of the midpiece and the tail can vary, as in the zebra finch (Birkhead et al. 2005; Chapter 3; see also Figure 3B & 3C). Following observations of the swimming patterns of sperm with varying midpiece: tail ratios, the relationship between these variables and swimming velocity was particularly interesting. Sperm with a very long midpiece swam rigidly as if the midpiece was unyielding, and the whole sperm appeared to vibrate and spin down its total length, similar to the description by Vernon & Woolley (1999). In contrast, sperm with a very short midpiece and a long tail appeared more flexible and the tail moved with an undulating wave-like movement. Of course, these observations are made in two dimensions, not three dimensions, in which the sperm actually swims. Differences in flagella motion could not be captured by CASA because the CASA software tracks sperm movement using only the sperm head. Perhaps one reason for slow swimming speed association with extremely long and short tails is that both sperm

designs preclude the tail from providing propulsion for fast swimming; the long midpiece restricting tail movement and the very short midpiece providing too little power. This may explain why the fastest swimming sperm from the fastest single sperm subpopulation (see Appendix 5 Figure A5.4) had midpiece: tail ratios between 1 and 1.5, a sperm design where the difference between midpiece and tail lengths are less extreme (Figure 3).

In common with several other studies of passerine bird sperm (Mossman et al. 2009; Helfenstein et al. 2010; Laskemoen 2010), the present study detected a significant effect of flagellum: head ratio on velocity. Sperm with relatively smaller heads compared to the flagellum (larger flagellum: head ratio) achieved higher swimming velocities, but this relationship was evident only in short sperm from the S-line. As the flagellum: head ratio increased in longer sperm, swimming velocity decreased slightly. An explanation for this may be that an increase in flagellum length in the L-line is insufficient to offset the additional drag experienced by the longer sperm head (Humphries et al. 2008; Chapter 3), combined with the potential reduction in energy output from the shorter midpieces.

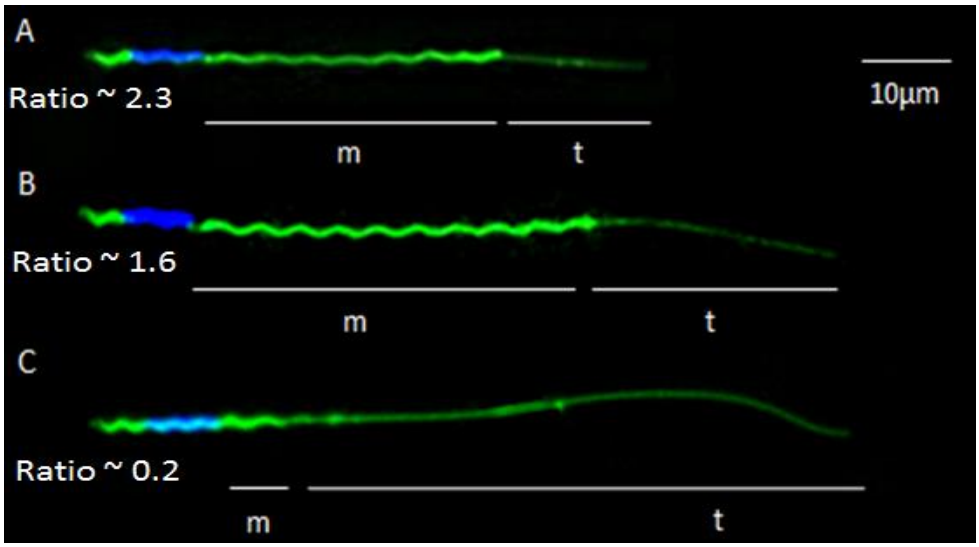


Figure 3. Zebra finch sperm from three different males with varying midpiece (m): tail (t) ratios. (A) A short sperm with a large midpiece: tail ratio (approximately 2.3). (B) A long sperm where the midpiece: tail ratio is approximately 1.6. (C) A long sperm with a small midpiece: tail ratio (approximately 0.2). The sperm with the fastest swimming velocity would likely be sperm B, where the difference between the midpiece and tail less are less extreme.

Stabilising selection on sperm morphology

With the exception of the present study, the evidence to date suggests either a linear association between sperm length and swimming velocity (in species including mammals, birds and invertebrates, e.g. Malo et al. 2005; Mossman et al. 2009; Fitzpatrick et al. 2010), or that these two traits are independent (in fish, e.g. Gage et al. 2002; Fitzpatrick et al. 2007). Therefore, the present study is the first to demonstrate that increasing absolute sperm length does not result in a linear increase in swimming velocity. Increasing sperm length is beneficial only up to certain trait values, after which sperm velocity declines. This suggests that sperm morphology in the zebra finch, and potentially other species, is maintained - in part - by stabilising selection, which may reduce the number of sperm with extreme phenotypes, and possibly prevent the range of sperm lengths from further increase. This could occur by two methods. (1) If males producing sperm with the extreme phenotypes (very small or very long) are less successful during sperm competition because their sperm swim more slowly than average length sperm, males with average length sperm will then sired more offspring. The number of males producing extremely long or short sperm in the population will decrease. (2) If the female reproductive tract exerts stabilising selection on the distribution of sperm phenotypes, occurring if there is coevolution between sperm length and storage organ dimensions, meaning that extremely long or short sperm are not storage as efficiently. Evidence from comparative studies suggests that there may be an optimal length for successful sperm storage (Dybas & Dybas 1981; Briskie & Montgomerie 1992). Invertebrate studies using single species have also demonstrated that the female reproductive tract anatomy does influence fertilisation success (e.g. Miller & Pitnick 2002; Otronen et al. 1997), and consequently may represent a functional constraint on sperm morphology, with the magnitude of the effect dependent on the interaction between male sperm and female tract dimensions (Miller & Pitnick 2002).

The morphological variation across zebra finch males was still extremely high (Birkhead et al. 2005; Chapter 3), despite stabilising selection on zebra finch sperm morphology, presumably because erosion of variation occurs gradually at either extreme of the phenotypic distribution, thus maintaining variation for longer. Another explanation for the variation is the low intensity of sperm competition (Birkhead et al. 2005), demonstrated by low levels of extra-pair paternity (Birkhead et al. 1990; Griffith et al. 2010). With low levels of sperm competition, there may be a reduced chance of extreme sperm being outcompeted by average length (and faster swimming) sperm from other males. The significant additive genetic component of sperm

morphology (e.g. total length: 0.63 ± 0.11 (mean ± S.E); Mossman et al. 2009) ensures that sperm designs, even those of extremely long and short sperm, are inherited from father to son.

In conclusion, this chapter provides strong evidence that sperm morphology has a pronounced effect on sperm swimming velocity. In other words, the artificial selection regime has caused a change in sperm morphology and produced a correlated response in sperm swimming velocity. In addition, it is shown for the first time that sperm velocity may be constrained by extremes of sperm design, such that longer sperm swim faster up to a point, but beyond a certain sperm length swimming velocity declines. Potentially, this could result in stabilising selection of sperm morphology, providing that sperm at the extremes of the sperm length distribution are competitively inferior to the sperm with an average morphology. Determining whether or not evolutionary changes in sperm morphology have also influenced fertilisation success in a competitive context is the focus of Chapter 6.

Chapter 6

The coevolution of competitive fertilisation success following artificial selection on sperm morphology

6.1: Introduction

The primary role of sperm is to fertilise ova. When sperm of multiple males compete to fertilise a given ovum (sperm competition; Parker 1970), the male producing competitively superior sperm will be more successful. Given that the females of most species are promiscuous (Birkhead & Møller 1998), the competition between the sperm of rival males results in intense selection on male specific phenotypic traits that enhance sperm competitive ability.

Adaptations to sperm competition include: (i) increased sperm production through larger testes (Møller 1991; Pitnick et al. 2001; Byrne et al. 2002), (ii) increasing the proportion of viable and morphologically normal sperm (Hunter & Birkhead 2002; Rowe & Pruett-Jones 2011), and (iii) changes to sperm morphology (LaMunyon & Ward 1999; Byrne et al. 2003; Calhim et al. 2007; Gomendio & Rolden 2008). These adaptations also result in competitively superior ejaculates (Firman et al. 2011) within individual species in the following ways. First, theoretical predictions (Parker 1990), and empirical work (Martin et al. 1974; Gage & Morrow 2003) together provide strong evidence that the fertilisation success of a given male is proportional to the number of sperm that he inseminates – the 'fair raffle' model of sperm competition (Parker 1990). However, differences in sperm quality (see General Introduction) mean that not all sperm have an equal chance of fertilising the egg (Dziuk 1996; Birkhead et al. 1999b). Second, males producing better quality ejaculates, with more viable and morphologically normal sperm have greater fertilisation success (Garcia-Gonzalez & Simmons 2005; Malo et al. 2005). Third, sperm morphology often determines fertilisation success (for exceptions see Morrow & Gage 2001b; Gage et al. 2004; Dziminski et al. 2010), with larger or longer sperm being advantageous in some species (Radwan 1996; LaMuynon & Ward 1998), and shorter sperm determining competitive success in others (Gage & Morrow 2003; Garcia-Gonzalez & Simmons 2007).

Therefore, the association between sperm morphology and fertilisation success can be unclear across species. Differences in sperm morphology are assumed to influence fertilisation success through variation in sperm motility, specifically, the swimming speed of the sperm. Evidence both across and within species suggest that longer sperm swim faster (across species – Gomendio & Rolden 2008; Lüpold et al. 2009a but see Gage et al. 2002: within species – Mossman et al. 2009; Fitzpatrick et al. 2010) and is based on the following reasoning. First, the longer flagella of longer sperm are suggested to create greater forward propulsive forces

(Cardullo & Baltz 1991). Second, when sperm length and midpiece length positively covary (e.g. Immler & Birkhead 2007), longer sperm could have greater energy output, due to increased production of adenosine triphosphate (ATP) from the midpiece mitochondria (e.g. Rowe et al. 2013), which, in some species, determines sperm motility (e.g. Froman & Kirby 2005). Third, the relative lengths of individual sperm components also influence swimming speed by affecting the hydrodynamics of the sperm. For example, sperm with small heads relative to flagella length are less likely to experience negative effects of drag (Humphries et al. 2008), hence achieving faster swimming speeds (Mossman et al. 2009; Helfenstein et al. 2010). Sperm with a relatively longer midpiece for a given total length may also have a greater energy budget for swimming compared to the same length sperm with a smaller midpiece, which can benefit fertilisation success (Laskemoen et al. 2010).

A body of evidence also demonstrates that males producing faster swimming sperm gain higher fertilisation success (Birkhead et al. 1999b; Donoghue et al. 1999; Gage et al. 2004; Denk et al. 2005; Casselman et al. 2006; Liljedal et al. 2008; Dziminski et al. 2009); however, the link between sperm morphology, sperm swimming speed and fertilisation success is not clear. To my knowledge, the amoeboid sperm of nematodes *Caenorhabditis elegans* (LaMunyon & Ward 1998) is the only species in which this relationship has been demonstrated, where larger, faster crawling sperm out-compete smaller hermaphrodite self-sperm. An indirect link between morphology, velocity and fertilisation success also exists in sparrows *Passer domesticus* (Laskemoen et al. 2010), such that males producing sperm with longer relative midpieces had both faster swimming sperm and greater fertilisation success. However, this relationship was not conclusive as the association between midpiece length and fertilisation success arose only in multivariate regressions. In addition, the fertilisation success data used by Laskemoen et al. (2010) originated from natural incidences of sperm competition (the sum of within- and extra-pair offspring), and therefore is subject to additional uncontrolled variation (see also Cramer et al. 2013). Hence, the relationship between morphology, swimming velocity and fertilisation success is yet to be rigorously tested in a species producing flagellate, swimming sperm.

The zebra finch provides an appropriate model to determine the link between sperm morphology, sperm velocity and male fertilisation success. The population of zebra finches produced by artificial selection (Chapter 3) are ideal to investigate this relationship. Bidirectional artificial selection produced males with extreme sperm designs, building on the natural inter-male variation of sperm total length (the Sheffield inter-male population range is approximately 40-80µm, see Birkhead et al. 2005; Chapter 3). Males from the long selection

line (L-line) produced sperm that were longer, had longer flagella relative to head length and shorter midpiece relative to tail length, and importantly, swam faster compared to males from the short selection line (S-line). Sperm morphology is also highly repeatable in the ejaculate of a single male and over time (Birkhead & Fletcher 1995b; Birkhead et al. 2005; Chapter 2 Section 2.4.2). The relationship between sperm morphology and swimming velocity is well understood; in general, longer sperm swim faster than shorter sperm, and the relative lengths of sperm components have significant effects on swimming speed (Mossman et al. 2009; Chapter 5). Male zebra finches can be used to test the effect of sperm morphology (and consequently sperm swimming velocity) on fertilisation success by conducting competitive fertilisation trials between pairs of males that producing long and short sperm.

Due to the reproductive biology of birds, there are additional factors to consider that could affect male fertilisation success: (i) sperm storage by females, (ii) mating order, and (iii) male-female interactions. First, prolonged sperm storage in birds (in excess of a month in some species: Birkhead & Møller 1993) temporally separates insemination and fertilisation, and affords the female some control over paternity (via cryptic female choice: Thornhill 1983; Eberhard 1996). Limited access to the sperm storage tubules (SSTs) is one reason why males producing faster swimming sperm have greater fertilisation success (Birkhead et al. 1999b; Donoghue et al 1999; Froman et al. 1999). As only 1-2% of inseminated sperm enter the SSTs (Brillard 1993; Bakst et al. 1994), faster swimming sperm are more likely to reach the SSTs before slower sperm and hence make up the fertilising set of sperm. Additionally, faster swimming sperm are thought to be retained in the SSTs for longer periods of time (Froman 2003), so males producing highly mobile sperm can fertilise more eggs over longer periods of time (Pizzari et al. 2008). Second, under certain circumstances, when females mate multiply, the last male to copulate has greater fertilisation success (last male sperm precedence; demonstrated in Birkhead et al. (1988b). This is because passive sperm loss from the SSTs occurs, which means more of the first male's sperm have been lost from the SSTs, and relatively more of the last male's sperm are available to fertilise eggs (Colegrave et al. 1995). These effects may be difficult to detect with natural copulations where variables such as sperm number are not rigorously controlled. Third, individual male fertilisation success may vary from female to female (Birkhead et al. 2004) depending on the specific female and rival male combination, making each sperm competition event unique. This means that an unsuccessful male in one competitive combination may be superior in another combination due to an interaction between the sperm and the female physiology. For example, this could occur between the sperm length and dimensions of the sperm storage organs (Otronen et al. 1997; Miller & Pitnick 2002), or by influencing the storage or utilisation of related males sperm

(Bretman et al. 2009). Ideally, these three important variables must be controlled during sperm competition experiments.

Aim

The primary aim of this study was to investigate the effect of sperm length on fertilisation success, using two indices of fertilisation success: (i) the proportion of sperm from each male that reached the ovum, and (ii) the paternity of the resulting embryo (see Methods). Males producing long sperm were expected have more sperm reaching the ovum and a greater proportion of the paternity of the embryos. This is because long sperm swim faster (Mossman et al. 2009; Chapter 5), reach the SSTs first and may be retained for longer (Froman 2003) compared to the shorter sperm, and therefore become the fertilising set of sperm.

6.2: Methods

Study population

All zebra finches used in this study were raised as part of the selective breeding regime that produced selection lines of birds divergent in sperm total length (Chapter 2 Section 2.2; Chapter 3). All female zebra finches used in the experiment were from the L-line (n = 8) and S-lines (n = 10) from cohort 3 (see Terminology in Chapter 1 Section 1.4). Male zebra finches were from the L- (n = 18) and S-line (n = 18) were from cohort 2 (n = 8) and cohort 3 (n = 28).

Each male zebra finch had a sample of dead sperm collected (Chapter 2 Section 2.3.3) and measured (Chapter 2 Section 2.4.2) prior to the start of the experiment. Based on this, males were divided into two categories, short sperm (S) or long sperm (L), defined as follows: short sperm males produced sperm with a mean total length of less than 60μm and long sperm males produced sperm greater than 70μm. Male pairs (1 L male and 1 S male) were chosen by matching individuals by nearest hatching date to minimise possible male age effects on sperm competitiveness (Jones et al. 2007; Gasparini et al. 2010a). Pairs of males were housed together (Chapter 2 Section 2.3.1) so living conditions were as similar as possible.

Female zebra finches were housed individually in double cages (each cage with dimensions: 0.6 x 0.5 x 0.4m: Figure 1), with a 'primer' male that was not being used in the main experiment behind a wire divider. The divider prevented physical contact between the male and female, while allowing the pair to bond. Each cage had a nest box with nest material and an internal wire divider separating the two nest boxes. This allowed visual and auditory interactions that encouraged the females to begin laying eggs while preventing copulation. The experiment began once each female had laid a clutch of eggs and incubated these eggs for 5 d. This is because each female was then in breeding condition and would lay a new clutch within approximately one week after removal of the incubated clutch of eggs.

Figure 1. The double cage arrangement used in the sperm competition experiment housed a single male (left hand cage) and female zebra finch (right hand cage) separated with an internal wire divider.

Experimental methods

Sperm competition experiment: sequential mating

Artificial insemination (AI) of a heterospermic mixture from the long and short sperm males is a procedure that allows rigorous control of sperm numbers and mating order effects while testing sperm competitive ability (Birkhead et al. 1999b). While this may have been the preferable experimental approach, preliminary trials determined that this method was not feasible in the zebra finch (Appendix A7).

Therefore, a mating switching experiment was conducted, using similar methods to Birkhead et al. (1988b). Females used in the experiment were in breeding condition (described earlier) and were from either the long or short selection line. Each female was allocated a single pair of males (one long- and one short-sperm male) for the duration of the experiment. At the start of the experiment, the 'primer' male was removed from the double cage (Figure 1) to another room to prevent auditory contact with that female. The order that the pair of experimental males were given access to the female (e.g. long-sperm male first, short-sperm male second) was randomised. The entire experiment was then repeated with the same male pair given access to the same female in the reverse order, referred to as mating round 1 and 2 (described fully below) in a fully factorial experimental design.

Mating round 1

Three hours following the removal of the 'primer' male (around midday), all the eggs were removed from the nest and the first experimental male (either long or short sperm male) was added to the female's cage (right hand cage: Figure 1) and the pair were left to copulate freely for 3 d. After 3 d the male was removed (around midday) to another room, and after an interval of 3 h, the second male was introduced to the female's cage and left for a further 3 d. After 3 d, he was moved to the left hand cage (Figure 1), separated from the female by a wire divider to prevent any further copulations.

Nest boxes were checked daily and any eggs laid were removed, marked with the pair ID and egg number, and incubated for 48 h at 36^{o}C in an incubator (Brinsea Octagon 20 Advance; relative humidity: 60%). The first egg laid was replaced with a dummy egg; all other eggs were removed without replacement to encourage the female to lay larger than average clutches to

maximise the data from each trio (Haywood 1993; N. Hemmings, pers. comm). Eggs were collected until 14 d after separation of the second male and the female, as this is the maximum time that sperm are stored by female zebra finches (Birkhead et al. 1989; N. Hemming, pers. comm).

Mating round 2

Once the male and female had been separated for 14 d, any additional eggs were not collected to encourage the female to stop laying. Once the clutch was completed, the female was allowed to incubate the eggs for 5 d, after which the eggs were removed and the same pair of males given access to the female following the protocol as above, except the order of mating was reversed. Eggs were collected and incubated as above.

Quantifying male competitive success: proportions of sperm on egg perivitelline layer

Once a single sperm has fused with the female pronucleus and fertilisation has occurred, a second outer perivitelline layer (OPVL) forms around the original PVL (now called the inner PVL (IPVL)) and traps all remaining sperm around the ovum (Bobr et al. 1964). Measuring the length of these trapped sperm, and assigning the sperm to one of the competing males provides a measure relative numbers of each male's sperm that reached the ovum.

The IPVL and the OPVL were separated as described by Birkhead et al. (2008). Briefly, the egg was gently opened into a petri dish of phosphate buffered saline (PBS) and the yolk was cut in half with a pair of sharp dissecting scissors. The half of the OPVL with the embryo attached was laid out flat on a microscope slide, stained with 10µl Hoescht 33342 fluorescent dye (0.5mg/ml) and covered with a coverslip before incubating in the dark for 2 minutes. Only this half of the OPVL was examined for the presence of sperm as the majority of sperm are found there (Birkhead & Fletcher 1994). The slide was then examined using fluorescence combined with darkfield microscopy at 400x magnification using a Leica DMBL microscope. The sperm nuclei exhibit blue fluorescence while the flagella appear white against a grey/black background. Each sperm was photographed using an Infinity 3 camera (Luminera Corporation), and the sperm total length was measured to the nearest 0.01µm (6.21µm per pixel) using ImageJ (Abramoff et al. 2004). This measurement was used to assign each sperm to one of the two males based on previous sperm length data. There was no overlap in total length between the sperm of the long and short sperm males pairs (see Section 6.3 Figure 2).

Quantifying male fertilisation success: paternity of embryo

Paternity analyses were conducted on all eggs where one or more sperm were embedded in the OPVL to ensure all fertile eggs were included in the dataset. Paternity would not be assigned to infertile eggs because only DNA from the mother would be detected. Embryos were removed from all eggs using a clean hair loop (Birkhead et al. 2008), and preserved in 100% ethanol for molecular paternity analyses (see below). If the fertility status of an egg was unclear, then the germinal disc (GD) was removed and preserved in the same manner. DNA was extracted from the embryos and GDs at a later date using the ammonium acetate extraction protocol (based on Bruford et al. 1998, see Appendix A8). Blood samples were obtained (under license) from all experimental females and the two potential sires. DNA was extracted from blood using the same extraction protocol (Appendix A8) with minor modifications.

PCR reactions were run with 8 microsatellite markers in a pre-optimised multiplex (Dawson et al. 2010; see Table 1) with markers of similar size distinguished by labelling with different colour fluorolabels: either 6-FAM or HEX (Geneworks). Each individual was also sex-typed using the marker set Z-002E (Dawson 2007) (see Appendix A9 for the results). The PCR reactions were set up as follows: 2µl of extracted DNA per sample (20ng/µl) was dried in a PCR plate. Two microliters of primer mix was added to each well (the primer mix consisted of 10µl of forward and reverse primers of each 0.2µM marker, 250µl of Quigen master mix (Qiagen Inc.) (Kenta et al. 2008), 90µl of lowTE and a drop of mineral oil. Thermocycling was performed on a DNA Engine Tetrad PCR machine. The thermocycling profile was as follows: an initial denaturing incubation (95^{o}C for 15 minutes) followed by 44 cycles at the following temperatures: 94^{o}C for 30 s, 56^{o}C for 1 minute 30 s and 72^{o}C for 1 minute 30 s. This was followed by a final extension step at 72^{o}C for 10 minutes. The PCR products were then diluted to 1 in 800 and 1µl of this dilution added to 9.5µl mixture of formamide and ROX 500 size standards (Applied Biosystems, Warrington, UK). The samples were denatured at 95^{o}C for 3 minutes then immediately placed in an iced water bath to prevent re-annealing, before being sequenced using an ABI 3730 48-well capillary sequencer (Applied Biosystems, California, USA). The sequencing products were visualised and scored for each of the marker loci using GeneMapper v 3.7 ® (Applied Biosystems, California, USA). Two markers (TG01-124 and TG07-022) failed to amplify in the majority of the samples; therefore, the PCR and sequencing were repeated for all samples using those two markers only. The chromatograms of these rerun loci were analysed separately using GeneMapper® and the genotypes added to the main data set.

The paternity analysis was conducted using Cervus v 3.0 (Kalinowski et al. 2007). An allele frequency analysis was carried out to determine to suitability of each locus for use in the paternity assignment, then a simulation of the paternity assignment was conducted to identify the threshold values for paternity assignment at specific confidence levels in the real data set. Two of the loci (TG05-053 and TG13-009) were excluded from the analysis due to high levels of null alleles (Table 1). The allele frequency analysis and simulation were repeated without these markers and used to assign parentage in the real data set, using the female bird as the known mother and the specific male pair as the two candidate fathers. Paternity assignments were accepted when paternity was assigned with 80% confidence (or above) to one of the candidate sires.

Table 1. The multiplex of 8 markers use the PCR reactions and the results of the allele frequency analysis. All 8 markers required an annealing temperature of 56°C. The number of alleles (n), number of individuals genotyped (N_A), the observed and expected heterozygosity (H_O, H_E) and the frequency of null allele (F_{NULL}) are given for each loci. Note that marker Z-002E is the sex marker and was not used in the allele frequency or parentage analysis (see Appendix A9 for the results). Significant departures from Hardy Weinberg equilibrium are in bold.

Marker name	Fluorescent dye	Allele size range (bp)	n	N_A	H_O	H_E	F_{NULL}
TG01-124	FAM	400-402	277	3	0.278	0.276	-0.0059
TG03-002	FAM	124-128	306	5	0.444	0.453	0.0055
TG05-053	FAM	194-198	292	3	0.264	0.410	**0.2229**
TG13-017	FAM	293-297	271	4	0.587	0.667	0.0652
TG01-147	HEX	277-285	296	5	0.659	0.693	0.0301
TG07-022	HEX	414-420	262	4	0.294	0.292	0.0129
TG13-009	HEX	195-195	278	3	0.126	0.158	**0.1051**
Z-002E	HEX	114-117	-	-	-	-	-

Sperm quality assays

At the end of the sperm competition experiment, all male zebra finches used were humanly killed by cervical dislocation. The left and right testes were removed, blotted dry and their mass recorded (to the nearest 0.001g). Live sperm samples were obtained from the left seminal glomerus (SG) of each experimental male zebra finch by dissection (Chapter 2 Section 2.3.4) The following sperm quality assays were performed: (i) concentration (an estimate of the concentration of sperm in the left SG), (ii) longevity (the length of time the sperm were motile), (iii) morphology (the dimensions of sperm components), (iv) motility (the swimming speed of sperm), (v) normality (the proportion of sperm with a normal morphology), and (vi) viability (the proportion of sperm with an intact head membrane and then able to fertilise ova). Further details of these assays are in Chapter 2 Section 2.4.1 to 2.4.6. These assays were carried out to determine if there were any systematic differences in sperm quality between the long- and short-sperm males (besides the expected difference in the morphology and swimming velocity of sperm) that could bias the fertilisation success towards either male (see Section 6.3 for a comparison between the long- and short-sperm experimental males; see also Appendix A10 for comparisons using males from Chapter 5).

Statistical analyses

The experimental design required two males (one long- and one short-sperm male) to be mated sequentially (in a randomised order) to a female prior to clutch initiation. To control for last male sperm precedence, each pair of males was then re-paired to the same female in the alternate order. Therefore, the female produced one clutch of eggs per mating round. Hence, the response variables in the models described below used (i) the sperm proportions of the second male to mate, and (ii) the proportion of paternity by the second male to mate (P2). If neither male had a competitive advantage, then the second male to mate (regardless of whether a long- or short-sperm male) would have significantly higher proportions of sperm on the OPVL, and a higher P2 in both mating rounds.

Sperm proportion data from the OPVLs were analysed using a generalised linear mixed model (GLMM) with a binomial error distribution and a logit link function, and modelled as the number of ‘successes’ or ‘failures’ (number of sperm from the second male to mate/number of sperm from the first male to mate) incorporated in to the response variable ‘y’. This retained sample size information (i.e. total number of sperm). Male mating order (i.e. short first = yes/no), female line and the number of days between the male swap and the laying of the

focal egg (referred to as 'number of days' from hereon) were included as fixed effects. The variable 'male mating order' controlled for the reciprocal experimental design and simultaneously coded for male selection line. Trio ID (i.e. 1 female and 1 pair of males) was fitted as a random effect. P2 was analysed using a GLMM, and modelled with a binomial error distribution with a logit link function, due to the binary nature of the response variable (i.e. sired by second male/not sired by second male). All other fixed and random effects were identical to the model described above. Interactions between the fixed effects were included in the maximal models. Minimal adequate models were obtained via model reduction, removing the least significant term, and comparing models with and without the term using log-likelihood tests and Akaike information criterion (AIC) values. Degrees of freedom are not presented from the GLMMs as opinions are divided regarding suitable methods to obtain them; instead, the numbers of observations are presented, with details of their grouping within the data.

Data from the sperm motility assay comprised multiple kinematic parameters obtained via Computer Assisted Sperm Analysis (CASA). Three of these parameters (Chapter 2 Section 2.4.1) were analysed using Principle Components Analysis (PCA) to produce a single index of sperm swimming velocity (PC1). This was carried out for all birds during the analyses for Chapter 5 (including the experimental birds in this chapter); therefore, the PC1 scores for the experimental birds were extracted from this larger dataset (for further details see Chapter 5 Section 5.2). These PC1 values were used in models (described below) to verify that sperm velocity was significantly different between the L- and S-lines.

Paired t tests were used to establish whether there were differences between the long- and short-sperm male pairs in the following sperm quality measures: (i) the proportion of viable sperm, (ii) the proportion of morphologically normal sperm, (iii) swimming velocity (PC1) for three sperm subpopulations (total population, fastest 10% and fastest single sperm: see Chapter 5 Section 5.2.2), (iv) sperm morphology, (v) sperm concentration, and (vi) sperm longevity (proportion of motile sperm at the start of the assay and the length of time for all sperm to be motile). All proportion data were arcsine transformed to meet requirements of the paired t tests. The relationships between sperm quality parameters were also tested using Pearson's correlations (using the transformed proportion data). Testes mass between the long- and short-sperm males was also compared. The combined mass of the left and right testis per male was divided by body mass (this is a crude method of controlling for differences in testes mass due to body mass: Appendix A10 uses a linear model (LM) with a larger dataset, although the overall conclusion is unchanged).

All statistical analyses were conducted using R v 2.15.1 (R Core Team, 2012) with the base package and lme4 (Bates et al. 2012).

6.3: Results

Sperm quality assays

There were no significant differences in the proportion of viable sperm, the proportion of morphologically normal sperm or the concentration of sperm dissected from the left SG between the pairs of males used in the sperm competition experiments (Table 2). Sperm longevity was similar overall; with no differences between the proportion of motile sperm at the start of the assay, or in the length of time taken until all sperm were immotile (Table 2). However, as expected, sperm total length was significantly different between the long- and short-sperm males (the distribution was non-overlapping: Figure 2), and all other components of sperm morphology (except midpiece length) were different between the males (Table 2). This resulted in clear differences in mean sperm motility index PC1 (Table 2). Longer sperm swam faster than the short sperm in all subpopulations of sperm (total, fastest 10% of sperm and the fastest single sperm). There was a small but significant difference in combined testes mass (corrected for body mass), such that the short-sperm males had slightly lighter testes than the long-sperm males (see also Appendix A10 for results from a larger dataset). The difference in testes mass was unlikely to affect sperm quality because the concentrations of sperm collected from the left SG of the long- and short-sperm males were similar. Because overall sperm quality was comparable between the long- and short-sperm male pairs, the sperm competition experiment therefore simultaneously tested the effect of sperm morphology and sperm velocity on competitive success between the competing males.

In general, there were no correlations between the sperm quality parameters, with the exception of sperm concentration and longevity (Table 3). Sperm longevity increased, i.e. sperm swam for longer, with increasing sperm concentration (Pearson's correlation; $p < 0.0001$; Table 3) (here, the concentration relates to that in the original aliquots; see Chapter 2 Section 2.4.5). The same relationship was also detected using a LM (LM; d.f = 39; t = 3.64; p = 0.0008), but the sperm longevity of the long- and short-sperm males was comparable (LM; d.f = 39; t = 0.38; p = 0.70).

Table 2. Comparison of sperm quality parameters and combined testes weight between the long- and short-sperm males used in the sperm competition experiments. The mean values were calculated from the raw data. Comparisons between the male pairs were made using pair t tests to establish if there were systematic bias in sperm quality that could affect the outcome of sperm competition. Proportion data were arcsine transformed before analyses. Significant values are in bold ($p < 0.05$).

Sperm quality assay		Mean ± S.D		Test statistic	
		Long	Short	$t_{d.f = a,b,c}$	p
Proportion of motile sperm[1]		0.89 ± 0.11	0.88 ± 0.14	0.23[a]	0.82
Proportion of normal sperm		0.74 ± 0.08	0.72 ± 0.14	0.21[b]	0.84
Proportion of viable sperm		0.74 ± 0.11	0.75 ± 0.14	-0.71[b]	0.48
Sperm concentration ($x10^6$ per ml)		107.70 ± 95.27	142.02 ± 117.60	-1.77[b]	0.45
Sperm longevity	*Proportion motile at t_0*[2]	0.67 ± 0.18	0.64 ± 0.25	0.60[a]	0.55
	All sperm immotile (min)	113.33 ± 50.47	127.5 ± 80.08	-0.61[c]	0.55
Sperm motility (PC1)	*Total population*	1.51 ± 0.79	0.50 ± 0.71	6.36[b]	**<0.0001**
	Fastest 10%	3.60 ± 0.77	2.11 ± 0.72	10.24[b]	**<0.0001**
	Fastest single sperm	4.27 ± 0.75	2.95 ± 0.97	5.04[b]	**0.0001**
Sperm morphology (µm)	*Head*	11.42 ± 0.58	10.72 ± 0.43	4.34[b]	**0.0005**
	Midpiece	29.75 ± 5.37	31.06 ± 1.66	-1.20[b]	0.25
	Tail	33.56 ± 6.42	14.77 ± 2.43	10.97[b]	**<0.0001**
	Total length	74.73 ± 2.19	56.55 ± 1.58	25.98[b]	**<0.0001**
	Flagellum: head	5.56 ± 0.35	4.28 ± 0.20	13.44[b]	**<0.0001**
	Midpiece: tail	0.95 ± 0.35	2.17 ± 0.43	-8.99[b]	**<0.0001**
Combined testes mass (g)[3]		0.0029 ± 0.0007	0.0024 ± 0.0007	2.64[c]	**0.02**

[1]Calculated from data obtained during the motility assay

[2]t_0 denotes the proportion of motile sperm at the start of the longevity assay.

[3]Combined testes mass comprises the sum of the left and right testes mass divided by the body mass.

[a,b,c] Refer to the degrees of freedom for each test, which are 15, 16 and 17 respectively.

Further details of the sperm quality assays can be found in Chapter 2 Section 2.4.1 to 2.4.6.

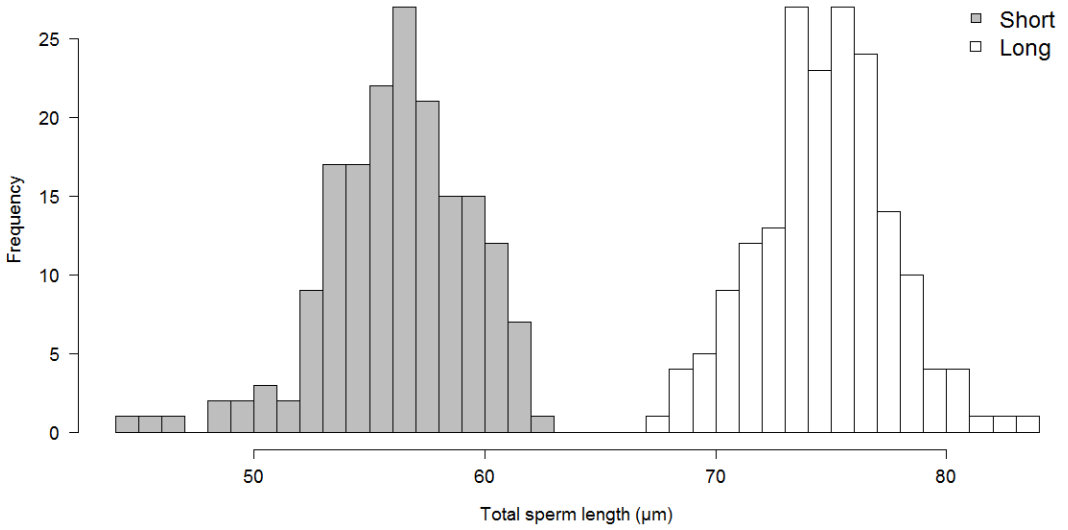


Figure 2. The range of sperm total lengths from the male zebra finches used in the sperm competition experiment (n = 36). Ten sperm were measured per male.

Table 3. The correlations between sperm quality parameters. Sperm concentration and sperm longevity were significantly correlated (indicated in bold italics, $p < 0.001$).

	Viability[1]	**Normality[2]**	**Concentration**	**Longevity[3]**
Viability[1]	---	---	---	---
Normality[2]	0.09	---	---	---
Concentration	-0.006	0.35[†]	---	---
Longevity[3]	-0.04	0.27	***0.55***	---
Swimming velocity (PC1)[4]	0.11	0.11	0.02	-0.11
Total length[5]	-0.07	0.09	-0.14	-0.12

[1,2]Proportion data were arcsine transformed before analyses.
[3]Longevity was quantified as the number of minutes until all sperm were immotile
[4]Data were obtained from analyses carried out in Chapter 5. PC1 relates to the total sperm population.
[5]Data were obtained from measurements of ten sperm per male.
[†]Indicates correlations that were marginally non-significant.
All d.f = 33.

Sperm competition experiment: sequential mating

Proportion of sperm from the second male on the egg PVL

Data from 18 trios were analysed, of which 12 trios produced a clutch of eggs from both mating rounds of the experiment. Clutch data (n = 30) were included in analyses when at least one egg from the clutch had sperm from both the long- and short-sperm male observed on the PVL (192 eggs in total; 74.7% of eggs laid), to ensure that both males had copulated with the female. Sperm numbers observed on PVLs varied widely within and between clutches (mean sperm per PVL = 28.2; range 1-132). Damaged or obscured sperm were recorded as 'male unknown', which accounted for 960 out of 5380 (18%) of sperm (range per clutch 0-32.3%). Refer to Appendix A11 for further details regarding the unassigned sperm. Sperm quality was not included in these analyses because there were no differences between the long- and short-sperm males in measures of sperm quality (Table 2).

Overall, more long sperm were found on the OPVL than short sperm (Exact binomial test: $p < 0.0001$; Figure 3). Higher proportions of long sperm reached the ova whether the long-sperm male mated first (0.62 ± 0.32 (mean ± S.D)) or second (0.55 ± 0.34 (mean ± S.D), although when mated second, this proportion did not differ significantly from 0.5 (Exact binomial test: $p = 0.38$). In other words, long sperm were more successful reaching the ovum when the long-sperm male was first to mate.

There were three significant two–way interactions (Table 4) but these are not discussed further because only the highest order interactions are biologically meaningful. Lower order interactions and main effects are, by definition, part of the higher order interaction. Intriguingly, sperm proportions from the second male to mate were determined by a significant three-way interaction between male mating order, female line and number of days between the male swap and egg lay ($p < 0.0001$; Figure 4). When the female and second male selection line matched, i.e. the female and second male were both from the long line, the sperm proportions from the second male increased over time (Figure 4A & 4D). This pattern was expected according to the passive loss model of last male sperm precedence (Colegrave et al. 1995). Conversely, and contrary to expectations, when the female and second male selection line mismatched, i.e. the female and second male were from different lines, the sperm proportions from the second male decreased with each successive egg (Figure 4B & 4C).

There is an important point to note about the relationship illustrated in Figure 4C. Initially the regression line indicated that sperm proportions on the OPVL increased across successive eggs (dashed line). The three points (highlighted in red; Figure 4C) that strongly influenced this relationship were based on only one or two sperm. Removing these data points from the analysis resulted in a negative relationship (solid line). Although the direction of the relationship changed, the overall model output and conclusions were unchanged, i.e. the presence of a significant three-way interaction. All other model outcomes remained the same when every data point comprising either one or two sperm were removed from the analysis (data not included).

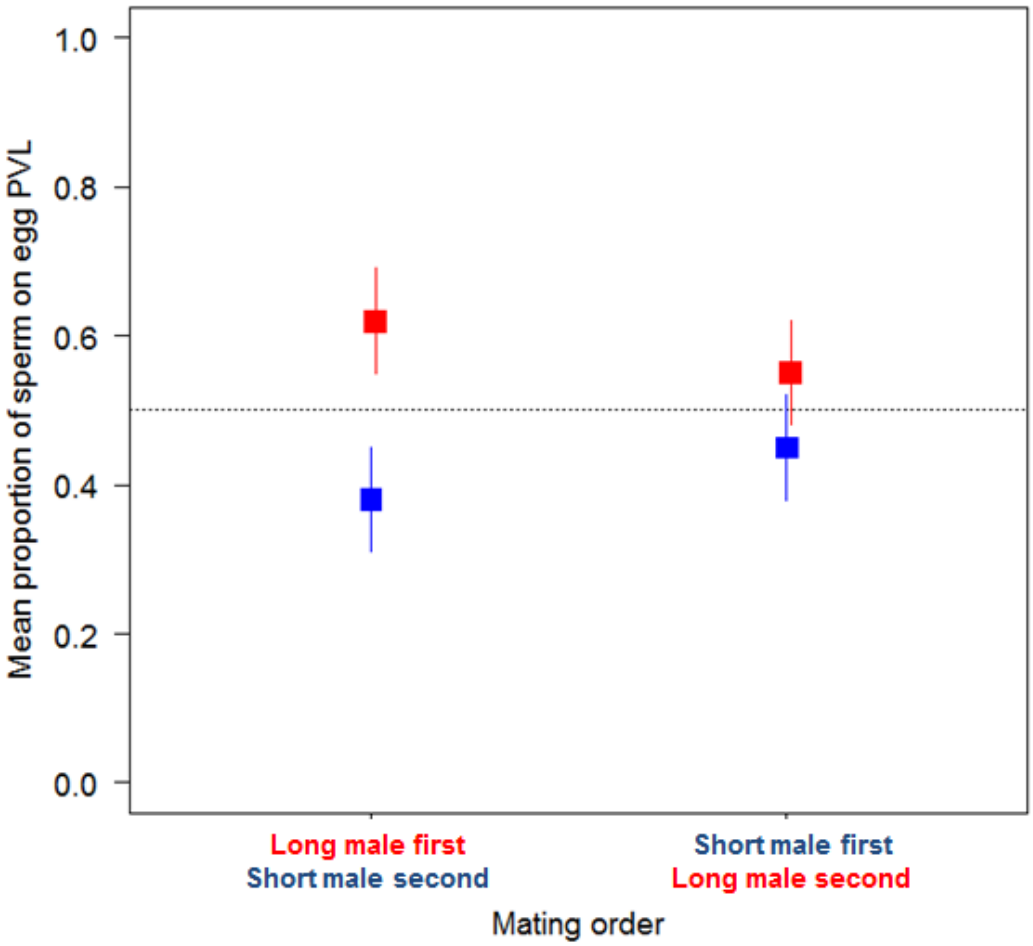


Figure 3. The proportion of sperm on the OPVL from the long- (red) and short- (blue) sperm males depending on whether they mated first or second with a female. The two points (red and blue) arranged vertically sum to 1.0, but both points are shown for ease of comparison. The long-sperm male has a paternity advantage regardless of the order of mating, although this is greater when the long-sperm male mated first. The dotted grey line at 0.5 indicates the expected P2 if neither male had a competitive advantage. Bars are 95% confidence intervals around the mean.

Table 4. Results of the GLMM analysing the effect of male mating order, days between male swap and onset of laying and female line on the proportions of sperm on the OPVL from the second male to mate[1]. Sperm proportions were determined by a three-way interaction; therefore, the lower order interactions and effects are not important for overall conclusions. Significant values ($p < 0.05$) are in bold.

Explanatory variable	Model estimate ± S.E	z	P
Male mating order	-1.42 ± 0.28	-5.04	**<0.0001**
Days between male swap and lay	-0.08 ± 0.04	-1.96	**0.049**
Female line	-0.66 ± 0.60	-1.11	0.269
Male mating order * Days between male swap and lay	0.32 ± 0.05	6.28	**<0.0001**
Male mating order * Female line	4.02 ± 0.39	10.27	**<0.0001**
Days between male swap and lay * Female line	0.15 ± 0.05	3.09	**0.002**
Days between male swap and lay * Female line * Male mating order	-0.40 ± 0.07	-5.85	**<0.0001**

[1]Data comprise 192 eggs from 18 trios. 12 trios produced clutches in both mating rounds.
Z is the test statistic and p is the significance level.
Further details of the statistical models can be found in the main text.

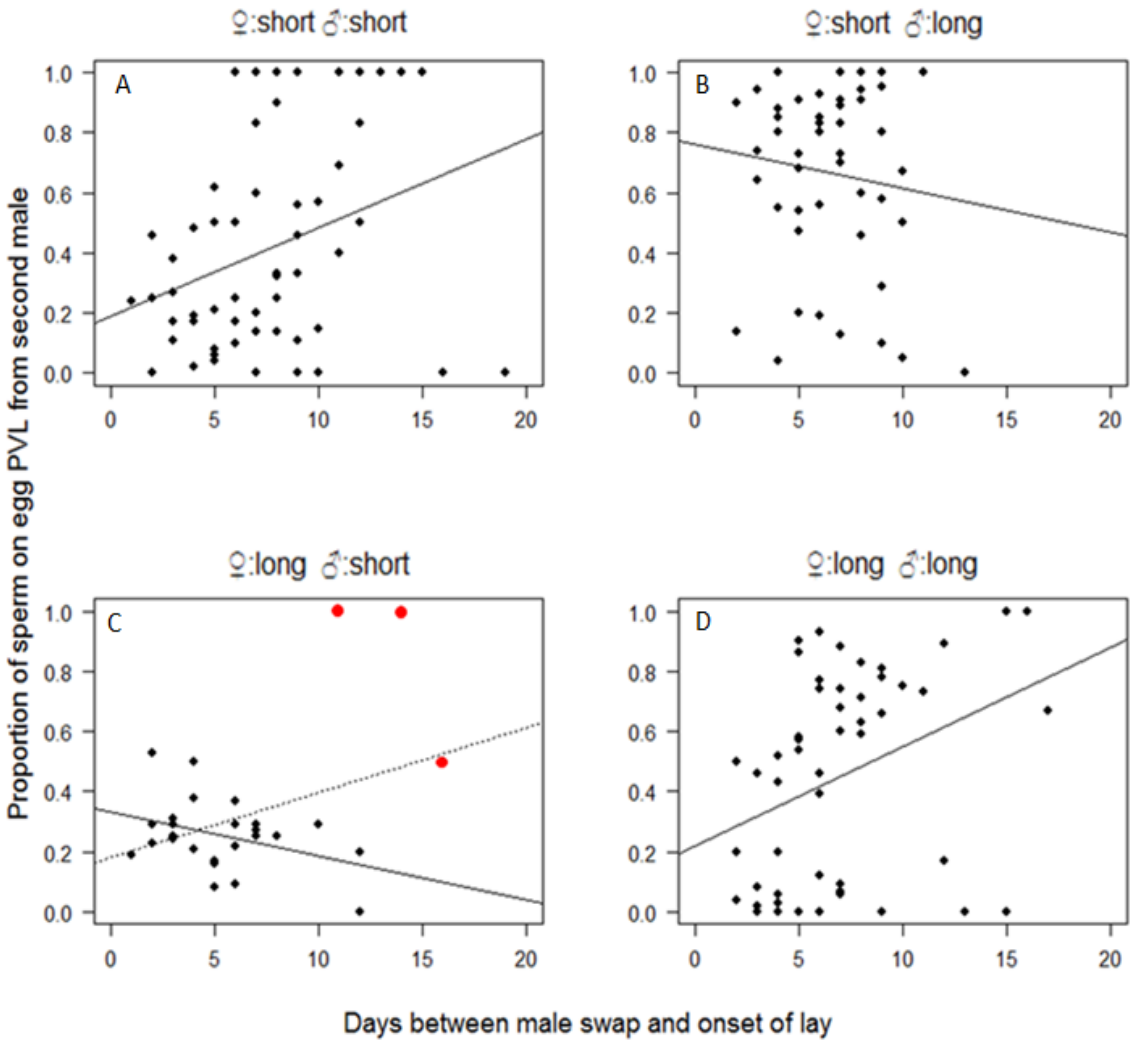


Figure 4. The significant three-way interaction determining sperm proportions from the second male to mate. The interacting variables are the female line, male mating order and the number of days between the male swap and lay. The title of each plot refers to the specific combination of female line and the line of the second male to mate. (A & D) Sperm proportions increase over time when the male and female selection line match. (B & C) Sperm proportions decrease over time when the male and female selection line do not match. (C) The red points are highly influential and proportions are based on one or two sperm. The dashed line is the relationship when these points are included, although the model conclusions are the same with and without these points. The model conclusions were also unchanged when all data points representing one or two sperm were removed.

Paternity of embryos

Paternity was assigned to 166 out of 196 embryos (84.7%), from 18 trios across 30 clutches, with at least 80% confidence. Embryos for whom paternity could not be assigned orginated from different clutches so systematic bias was unlikely (refer to Appendix A11 for details regarding the numbers of embryos with unassigned paternity). Sperm quality was not included in these analyses because there were no differences between the long- and short-sperm males in measures of sperm quality.

Overall, long-sperm males sired more embryos than short-sperm males (Exact binomial test; $p < 0.0001$). This test remained significant even when half of the embryos with unassigned paternity were assumed to be sired by the short-sperm male (Exact binomial test; $p < 0.0001$). The long-sperm male obtained a greater proportion of paternity when mated first (0.69 ± 0.46 (mean ± S.D), and when mated second 0.60 ± 0.40 (mean ± S.D) to the female (Figure 5).

P2 was determined by the interaction between male mating order and female line (Figure 6; Table 5), such that long-sperm males sired considerably more embryos when mated to a short-line females (Table 6). The proprortion of embryos sired by short-sperm males remained similar regardless of the selection line of the female.

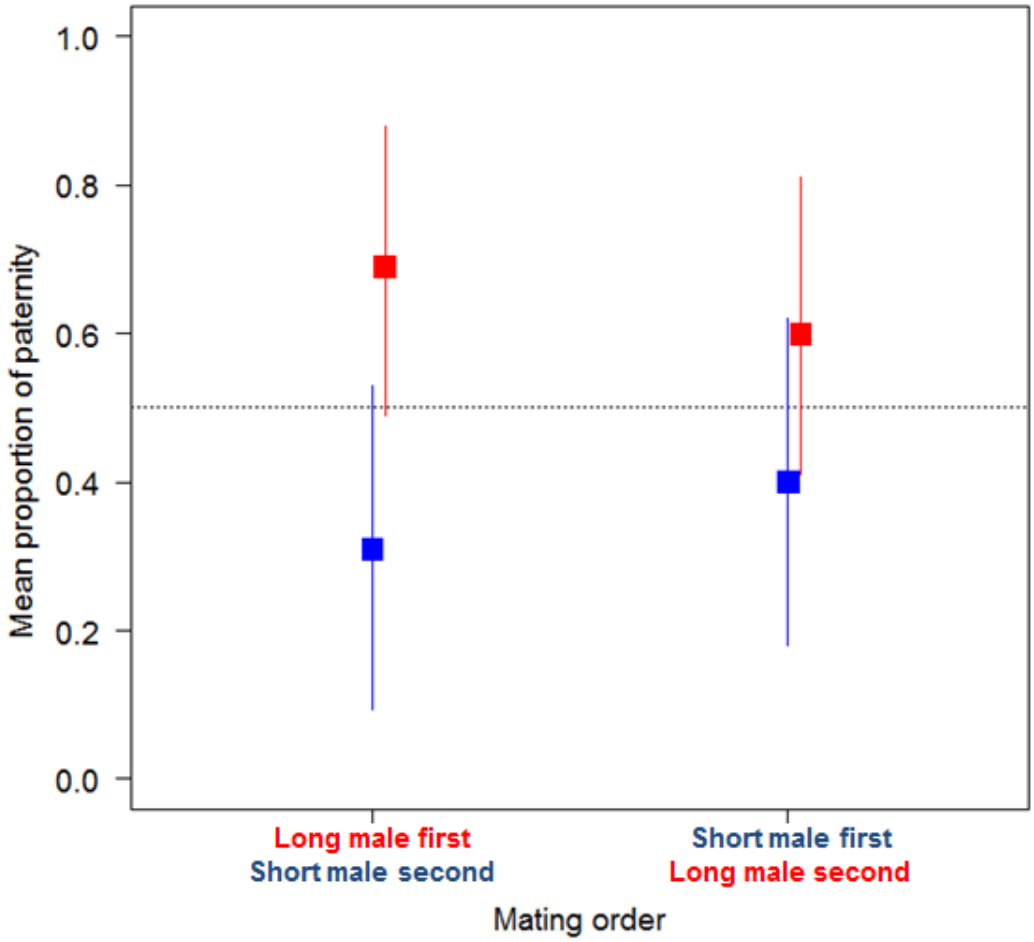


Figure 5. The proportion of paternity (P2) from the long- (red) and short-sperm (blue) males depending on whether they mated first or second with a female. The two points (red and blue) arranged vertically sum to 1.0, but both points are shown for ease of comparison. The long-sperm male has a paternity advantage regardless of the order of mating, although this is greater when the long-sperm male mated first. The dotted grey line at 0.5 indicates the expected P2 if neither male had a competitive advantage. Bars are 95% confidence intervals around the mean.

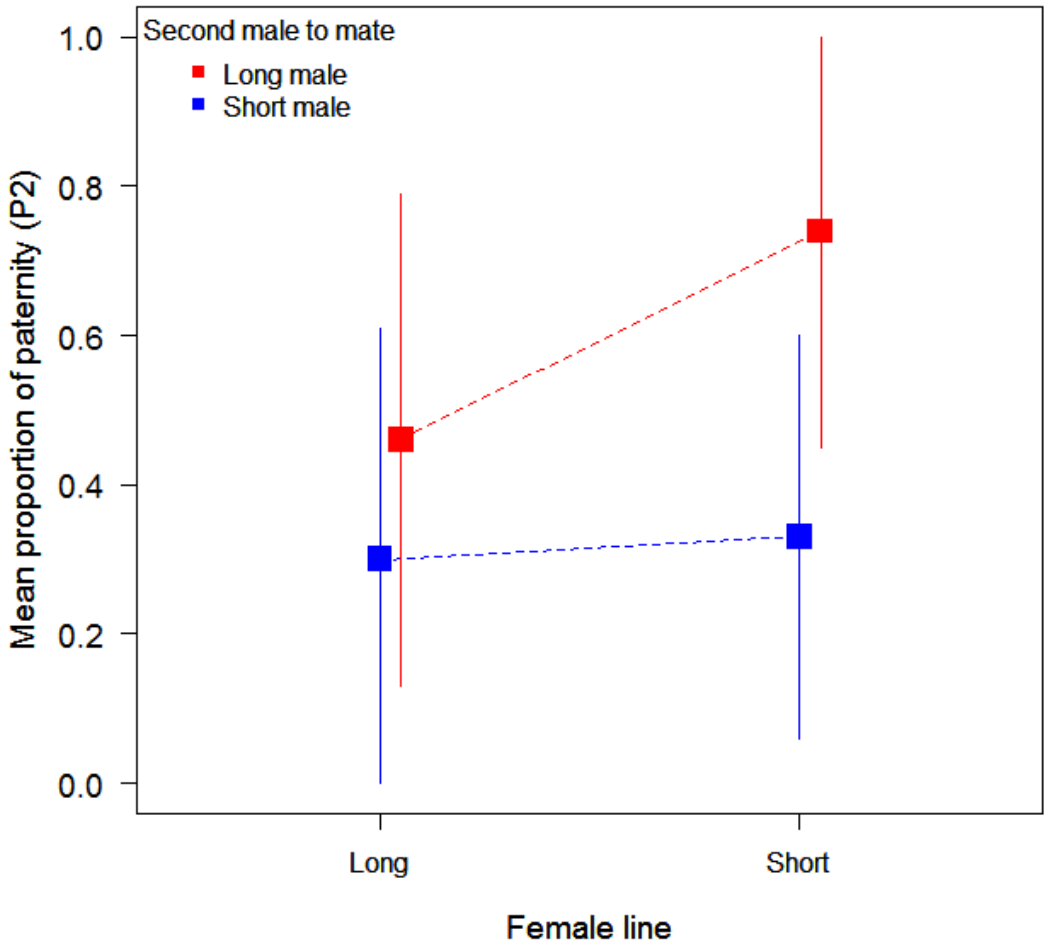


Figure 6. The interaction between male mating order and female line determines the proportion of paternity from the second male to mate. Each point is an independent mating combination, i.e. the short-sperm male mated second to the long line female (blue square, bottom left). The long-sperm male (red) gains higher P2 compared to the short-sperm male (blue) regardless of female line; however, there is a pronounced paternity advantage when mated to the short-line female. Bars are 95% confidence intervals around the mean.

Table 5. Result of the GLMM analysing the effect of male mating order and female line on the proportions of paternity from the second male to mate[1]. Paternity proportions of the second male are determined by an interaction between mating order and female line (in bold).

	Model estimate ± S.E	z	p
Male mating order	0.57 ± 0.59	0.96	0.33
Female line	-0.40 ± 1.44	-0.28	0.78
Male mating order * female line	3.60 ± 1.12	3.20	**0.001**

[1]Data comprise 166 embryos from 18 trios. 12 trios produced clutches in both mating rounds.
Z is the test statistic and p is the significance level. Further details of the statistical models can be found in the main text.

Table 6. The proportions of paternity to the long-sperm male in each mating combination of males by female line. The long-sperm male gained a greater share of paternity in three out of four mating combinations.

Female line	Mating order (1^{st} male – 2^{nd} male)	N (total = 30)	Proportion of paternity to long male ± S.E
Long	Short – Long	8	0.46 ± 0.17
	Long – Short	5	0.72 ± 0.17
Short	Short – Long	8	0.74 ± 0.15
	Long – Short	9	0.67 ± 0.14

6.4: Discussion

Overall, long-sperm males were competitively superior to short-sperm males in two ways: (i) more long sperm reached the ovum, and (ii) long sperm males sired a greater proportion of embryos. Male fertilisation success (i.e. proportion of paternity) was influenced by an interaction between the male mating order and female selection line. Additionally, male mating order and female line and the patterns of sperm loss from the SSTs influenced sperm proportions observed on the OPVLs. At present, the mechanisms determining these patterns are unknown; however, some suggestions are discussed below.

Sperm proportions on OPVLs

More long sperm were observed on the OPVL indicating that the long-sperm males were superior competitors in sperm competition, especially when the long-sperm male was the first male to mate with the female. This result contrasts with previous research where the last male to mate had greater fertilisation success, presumably because more sperm reached the ovum, as a consequence of last male sperm precedence (Birkhead et al. 1988b). The controlled differences in sperm total length between the competing males, and the resulting differences in swimming velocity are likely to explain these contrasting results. Males producing faster swimming sperm have a greater fertilisation success compared to males producing slower swimming sperm (e.g. Gage et al. 2004), especially across avian taxa (e.g. Birkhead et al. 1999b; Donoghue et al. 1999).

The longer, faster swimming sperm in the present study may have a competitive advantage due to three (not necessarily mutually exclusive) explanations. First, longer, faster swimming sperm may successfully traverse the physical barriers within the vagina, whereas slower swimming sperm cannot and are eventually lost from the reproductive tract. Second, more long sperm are then available to reach and enter the SSTs, and become the fertilising set of sperm, which are then excluded from any further selective processes. Third, once inside the SSTs, the longer, faster swimming sperm could be retained inside for longer periods of time (Froman 2003). Froman (2003) suggested that due to a decline in swimming velocity over time, sperm with a higher initial velocity maintain an adequate velocity for longer and avoid being flushed out of the SST by the internal current. The swimming velocity of shorter, slower swimming sperm may fall below a threshold value sooner (<25µm/s in domestic fowl: Froman 2003), resulting in sperm loss from the SSTs.

The final point discussed above may explain why there was a particularly high proportion of long sperm observed on the OPVL when long-sperm male mated first with the female. If long sperm remain in the SSTs for a longer time compared to short sperm, fewer long sperm will have been lost from the SSTs prior to clutch initiation. This means that sperm will be released while the female is laying eggs and will be present in greater proportions on the OPVL than expected under the assumptions of the passive loss model of sperm loss (Colegrave et al. 1995). An increased retention time of long sperm is not expected to prevent the shorter sperm from entering SSTs, because maximal filling of the SSTs in zebra finches is unlikely to occur (C. Bennison, pers. obs). Observations in both the yellow-headed blackbird *Xanthocephalus xanthocephalus* (Briskie 1996) and the zebra finch (Pellat 1998) suggest that SSTs mature along a spatial gradient across the UVJ (from the vagina to the uterus); therefore, there should be ample SSTs to accommodate the second (short sperm) male's sperm. This tentatively suggests that long-sperm precedence in the present study occurs because of their faster swimming velocity.

There were also more long sperm observed on the OPVL when the long-sperm male mated second: however, the proportion of long sperm was not significantly different to 0.5. One possible explanation for this pattern is as follows. Because the long-sperm male mated second, and assuming the long sperm are retained in the SSTs for longer, release of the long sperm may only occur later in the clutch; thus, the overall proportion of long sperm on the OPVL may be lower than expected. This effect may be exacerbated if the short sperm egress from the SSTs accelerates across the clutch as the swimming velocity of more sperm falls below the (unknown) threshold value required to remain in storage (Froman 2003).

The discussion above provides some explanations that fit the general patterns observed in the present study. However, the factors influencing the actual pattern of sperm proportions on the OPVL were more complex, and were determined by a significant three-way interaction between the order of male mating, female line and the number of days between the male swap and the focal egg being laid. Over successive eggs, the proportion of sperm from the second male to mate increased (as expected from the passive loss mechanism of sperm competition; Colegrave et al. 1995) when the female line and second male line *matched*, i.e. a male and female from the long line. Conversely, when the female line and second male line *mismatched*, the proportions of sperm from the second male actually decreased over successive eggs. There are a number of explanations for the decline in second male sperm proportions.

(i) If the mismatching male (the second male to mate) inseminates fewer sperm to the female, although both male's sperm are lost over time, the proportion of the matching male's sperm will be greater on each successive egg PVL (Figure 7). Strategic allocation of sperm, in terms of sperm numbers occurs in domestic fowl and depends on female attractiveness and the perceived risk of sperm competition (Cornwallis & Birkhead 2007). There is currently no data regarding the numbers of sperm transferred to females from long- and short-sperm males as collecting natural ejaculates using a dummy female (Pellat & Birkhead 1994) was unsuccessful. Data available indicate that there are no differences in the concentration of sperm in the distal portion of the SG (the mature sperm) between long- and short-sperm males (therefore the sperm available for transfer to the female), nor that males adjust the copulation rate according to the selection line of the female (N. Hemmings, unpublished data). Additional methods to determine if strategic allocation of sperm occurs in the zebra finch are discussed in Chapter 7.

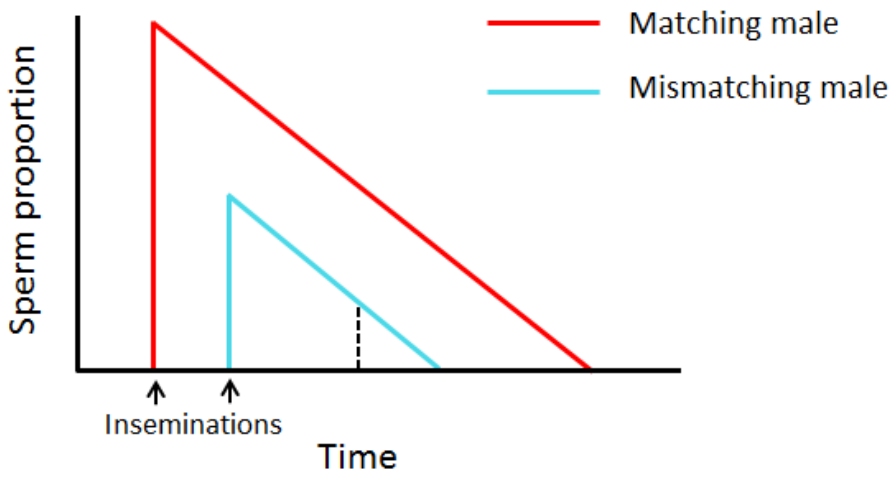


Figure 7. Schematic illustrating how the proportion of second male sperm (blue) declines over successive egg OPVLs as a consequence of the matching male (red) transferring greater numbers of sperm to the female of the same selection line. When ovulation occurs (dotted line) there will be fewer sperm from the second, mismatching male (blue) reaching the ovum.

(ii) The second possible explanation for the decreasing second male sperm proportions over successive eggs could be that sperm are released from the SSTs at different rates depending on whether or not the female and male selection line match (i.e. a long female and a long male). This could occur via three scenarios: (1) an increase in sperm release rate of the second mismatching male, (2) a decrease in sperm release rate of the matching male, or (3) both scenarios concurrently (Figure 8). This is a more complicated explanation than that of Froman (2003) who suggested that sperm were released from the SSTs as sperm velocity declined below a threshold level (Froman 2003). Pinpointing the cause of this pattern is challenging due to the lack of information regarding general sperm release rate. Rising hormone levels, particularly progesterone, have been implicated in initiating sperm release from storage (Ito et al. 2011). Detailed examination of turkey *Meleagris gallopavo* (Freedman et al. 2001) revealed that the SSTs were surrounded by smooth muscle and neural tissue, and individual SSTs may be connected to this tissue by individual nerve axons. These findings allude to the possibility of (cryptic) female control of sperm storage.

(iii) There could be a difference in the acceptance rate of sperm into the SSTs depending on whether the male and female selection lines match. For example, if a long line female accepts more sperm from a long-sperm male, regardless of the mating order, then more sperm from the matching long-sperm male could reach the SSTs and be observed on successive OPVLs compared to sperm from the mismatching (short-sperm male). When the *mismatching* male mates second, the second male sperm proportions decrease over time (Figure 9A). When the *matching* male mates second, second male sperm proportions increase over time (Figure 9B). The female may 'recognise' the sperm from the male of the same line due to similar sperm surface proteins. It is possible that these sperm will function better in a female from the same selection line, resulting in more 'matching' sperm reaching the SSTs.

The three hypotheses, however, require further empirical data to indicate which, if any, may explain the patterns observed in the present study. Methods to test hypotheses i and ii are discussed in Chapter 7, and these data could be used to infer the likelihood that hypothesis iii may explain the data.

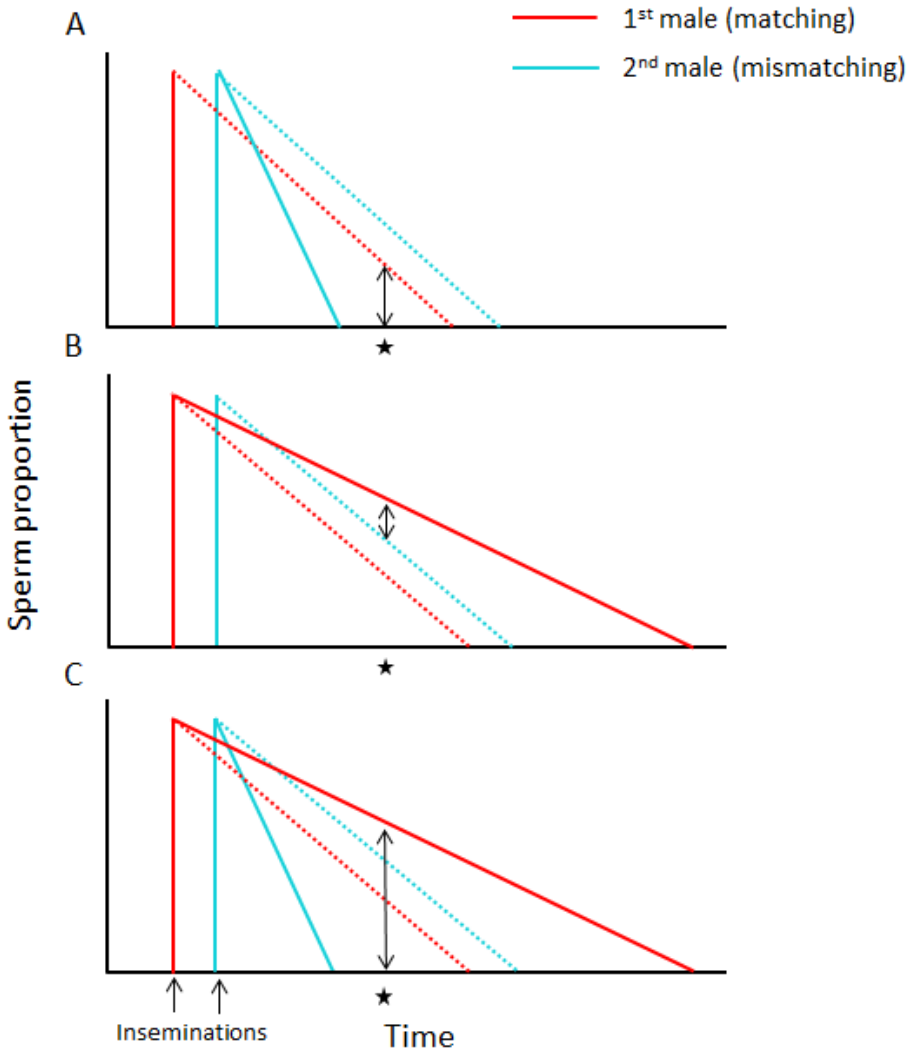


Figure 8. Schematic illustrating the proportion of sperm from the second male to mate decreases over successive OPVLs through differences in sperm release rates from the first (red) and second (blue) male to mate. The dotted lines represent the equal sperm release rate expected under the passive loss model of sperm competition (Colegrave et al. 1995). The solid lines represent the different release rate that could explain the observed decline in second male sperm proportions (see Results). The double-headed arrows illustrate that, at a given point in time (indicated by the star), there are relatively fewer second male sperm observed on the OPVL compared to the first male, and the proportion of sperm continues to decline over time. The selection line of the first male *matches* the female line; therefore, the selection line of the second male *mismatches* the female line. (A) The *mismatching* male's sperm (blue) are released at a faster rate. (B) The *matching* male's sperm (red) are released at a slower rate. (C) Both of the above occur simultaneously.

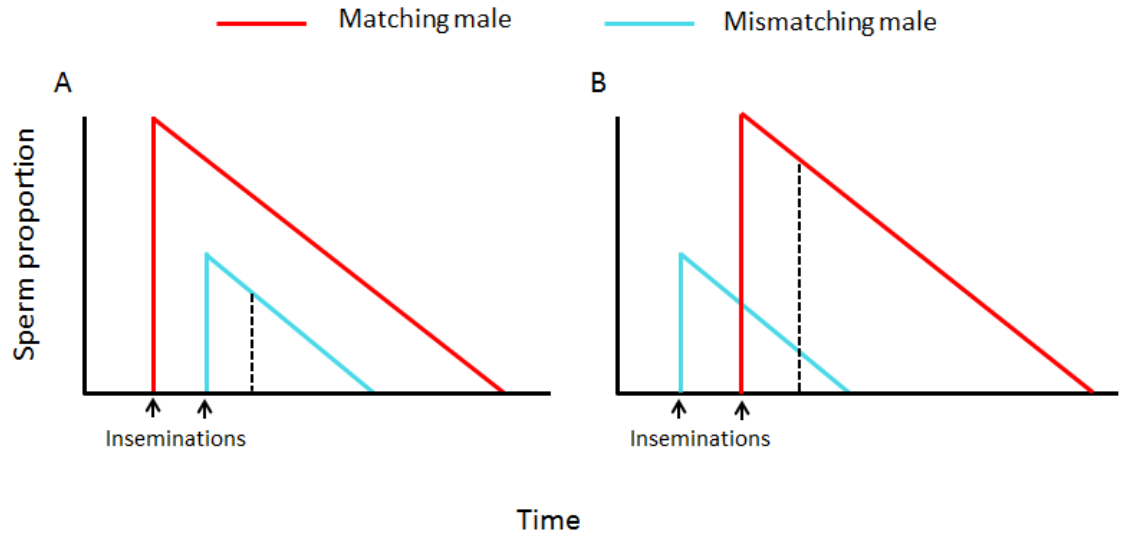


Figure 9. Schematic illustrating how a difference in acceptance rate of sperm to the SSTs may determine sperm proportions on the OPVLs. (A) If sperm from the matching male (red) enter the SSTs in greater numbers compared to the second, mismatching male's sperm (blue), the proportion of second male sperm on a given egg PVL (dotted line) is lower, and will decrease over time. (B) When the matching male mated second, the higher proportions of sperm accepted into the SSTs result in greater proportions of his sperm on a given OPVL (dotted line), and this proportion will increase over time.

Proportions of paternity

Overall, the proportion of paternity by the second male to mate (P2) was similar to the sperm proportion results (discussed above). The long-sperm male sired more embryos compared to the short-sperm male, regardless of mating order. As more long sperm reached the ova generally, this result suggests that, following prior selection of sperm at the SSTs, fertilisation success (i.e. P2) was determined according to which male's sperm reached the ova in greater numbers (the 'raffle' model of sperm competition: Parker 1990).

Examining the results in more detail revealed that P2 was determined by an interaction between the selection line of the male and female. The third variable (the number of days between the male swap and the focal egg being laid) did not affect P2, probably because around one third of all clutches examined, a single male (9 out of 30) sired all embryos. In the remaining clutches, there were no patterns of paternity across the clutches, in contrast to

observations in domestic fowl where poor quality, low mobility ejaculates fertilised ova early on in the clutch (Pizarri et al. 2008).

The interaction between male and female line determining P2 was intriguing because the long-sperm male sired the greatest proportion of P2 in three out of the four male – female mating combinations. In the fourth combination (long female mated to a short- then long-sperm male), both males gained a similar P2 – around 0.5. Interactions between male and female selection line determining P2 have been demonstrated previously (Miller & Pitnick 2002). In this study, male and female flies were artificially selected for a divergence in sperm, and seminal receptacle (SR) length, respectively. In sperm competition, the long-sperm males gained greater paternity when mated to females with the longest SRs, suggested to occur by the longer sperm maintaining a superior position in the SR and increasing fertilisation success (Miller & Pitnick 2002).

However, the result of the present study is in contrast to Miller & Pitnick's (2002) result, because the long-sperm male achieved the lowest P2 when mated to the long line females. If coevolution of some reproductive characters between the male and female (not necessarily sperm length and SST dimensions – although this has not yet been tested) had occurred in the present study, then a balanced P2 response might be expected; for example, where the long-sperm males were competitively superior when mated to long line females in both mating combinations.

The unbalanced response described above may indicate that the interaction could be a spurious result. Additional evidence indicating this interaction may not be reliable is the pattern of second male sperm proportions in the male – female mating combination where the P2 was approximately 0.5 for both males. In this combination, the second male sperm proportions (from the long-sperm male) increased (as would be expected) over successive eggs; therefore, the expectation may be that the long-sperm male should gain high P2. The female, on average, began to lay eggs around three days after the male swap had taken place. Assuming there are differences in the length of time the sperm are retained in the SSTs, this could mean that the long sperm are only exiting the SSTs later in the female's breeding cycle, and can only fertilise ova later in the clutch.

The long sperm advantage

This study has experimentally demonstrated that longer (and faster swimming) sperm have an adaptive advantage in a competitive context compared to shorter, slower sperm. This long-sperm advantage is clear in the number of sperm that reach, and eventually fertilise the ovum. Because there were no differences in measures of sperm quality between the long- and short-sperm males, the competitive success of the long-sperm males is attributed to the differences in sperm length, and the correlated increase in swimming velocity. This study also demonstrated how male competitive success is not determined simply by a 'race to the egg' between the sperm of the rival males; instead, the female is likely to exert control, possibly through sperm storage, on the numbers of sperm from each male that reach the ova, which finally determine the outcome of sperm competition.

Chapter 7

General Discussion

7.1: Overview

In this thesis I have demonstrated that applying strong artificial selection on total sperm length produced a marked divergence between the three selection lines, in terms of the final sperm phenotype (Chapter 3) and corresponding genetic architecture (Chapter 4). This selective breeding regime has highlighted that zebra finch sperm may fall into one of two sperm morphotypes (Chapter 3): (1) sperm with a midpiece that comprises the majority of the flagellum, and (2) sperm where the midpiece is relatively short compared to the flagellum.

Because sperm morphology and swimming velocity are significantly heritable (Birkhead et al. 2005; Mossman et al. 2009) and genetically covary (Mossman et al. 2009), sperm swimming velocity was also subject to strong directional selection as a consequence of artificial selection (Chapter 3). Examining the differences in swimming velocity of sperm from the three selection lines uncovered a (generally) correlated response between sperm morphology and swimming velocity, whereby longer sperm swam faster. However, interestingly, the longest sperm suffered a decline in velocity due to these sperm having an extremely long tail (Chapter 5). In other words, directional selection for high sperm motility may impose stabilising selection on sperm length.

In a competitive fertilisation trial, males producing longer, and faster swimming sperm had a competitive advantage compared to males producing shorter and slower sperm (Chapter 6). This link between sperm morphology and reproductive success via swimming velocity suggests that male competitive success may also be under directional selection.

Throughout this thesis, I have discussed the factors that may be important in explaining the specific patterns observed in each chapter. Consequently, in this section, I synthesise the overall body of work while focusing on some additional issues, and then discuss future research that will help clarify our understanding of the factors that determine, individual fertilisation success.

7.2: How applicable are these results?

Although the results in this thesis are derived from zebra finches subject to artificial selection, my results are likely to be applicable to zebra finches in general. The range of sperm lengths reported in Chapter 3 are comparable to unselected birds' sperm lengths (Birkhead et al. 2005;

Mossman et al. 2009), and the phenotypic associations between sperm components are similar to those observed in wild zebra finches (Immler et al. 2012). Given that the phenotypic associations (Chapter 3) describe the genetic relationships between sperm components (Chapter 4), it is probable that wild zebra finches also exhibit at least two sperm morphotypes across males (Chapter 3). The patterns of sperm motility, and consequently, fertilisation success reported in this thesis may then also occur in the wild, although detecting these patterns may be difficult due to naturally low sperm competition intensity. It is more difficult to generalise these results across other species of passerine birds given the range of mating strategies evident, but there are similarities between the tentative relationships regarding morphology, swimming velocity and fertilisation success in this study and others (e.g. Laskemoen et al. 2010), suggesting that, alongside other factors, sperm form and function are implicitly involved in male reproductive success.

The selective breeding regime comprised three selection lines; replicate lines were not possible for logistical reasons. While this may not be ideal when characterising a selective response, we had prior knowledge of the underlying heritability of the trait under selection and were confident that divergence would occur strongly in the expected directions. Due to this prior knowledge, the primary motivation in carrying out the selective breeding regime was to breed a large number of males producing extremely long and short sperm, with which to carry out the competitive fertilisation trial, in order to obtain ample replication.

7.3: Additional correlates of selection: 'known unknowns'

As a consequence of the selection experiments, there may also be unintended selection on the male, i.e. by changes to other phenotypic traits that genetically covary with the selected sperm components. These could include: (i) other aspects of sperm quality, such as sperm longevity, (ii) alterations in the chemical constituents (non-sperm component) of the seminal plasma or transparent fluid of the ejaculate, (iii) general morphology, and (iv) the reproductive behaviour of the males. With regard to the above examples, no differences were found between the selection lines for a number of sperm quality parameters (Chapter 6 & Appendix A10), except for, obviously, sperm morphology and swimming velocity. One interesting trend was that increased sperm longevity was associated with higher sperm concentrations (Chapter 6). Whether this was due to higher concentrations of sperm reducing the ability of sperm to swim (as there were many more sperm in a given volume of media) and conserving the sperm energy 'budget' for a longer period of time, or a greater amount of seminal plasma providing a more favourable environment for sperm survival is unknown. Seminal plasma is added to the

sperm as they move through the male reproductive tract, and transparent fluid is added to sperm just prior to ejaculation (Fujihara 1992). Because the cocktail of proteins and chemicals in the seminal plasma of domestic fowl *Gallus gallus domesticus* influence sperm quality, especially swimming velocity (Ashizawa & Okauchi 1984; Cornwallis & O'Conner 2009), these components could also be a target of selection to enhance fertilisation success. Although similar proteins may be ubiquitous across birds, little is actually known about the non-sperm components of the passerine ejaculate. There could also be trade-offs between the investment in sperm production and seminal fluid production depending on the exact competitive situation experienced. If sperm are not required to survive for a long periods, i.e. at the end of the breeding season, it may be prudent to increase sperm numbers in the ejaculate at the expense of the seminal fluid.

Finally, with regard to whether other morphological and behavioural traits could be affected by artificial selection, the only differences detected in the zebra finch were that the short-sperm males were skeletally smaller (as measured by the length of the left tarsus: Appendix A1), and these males also had a lower testes mass, both before and after correcting for body mass. Given that the concentration of the sperm in the seminal glomera of short-sperm males did not differ from that of long-sperm males (Chapter 6 Section 6.3), and that we detected no difference in copulation rate between the long- and short-sperm males (N. Hemmings 2013, unpublished data), the difference in testes mass was unlikely to affect the competitive ability of the short-sperm males. The interesting question, however, is whether changes to the internal testes structure could contribute to the difference in testes mass between the selection lines, and ultimately affect sperm production (discussed further in Section 7.3).

The possible unintended correlates of selection discussed above are only one set of 'unknowns'. There are also many post-insemination processes that occur inside the female that remain poorly understood. For example, although sperm motility may determine which sperm reach the female's sperm storage tubules (SSTs), the process by which sperm actually enter the SSTs is unclear. Nor is it known if further female mediated sperm selection occurs inside the SSTs.

Remarkably, although the infundibulum is the site of fertilisation, we know almost nothing about sperm behaviour in this part of the oviduct. Once released from the SSTs the sperm appear to move passively up the tract, aided by oviductal contractions, towards the ovum (Fujihara et al. 1993). Depending on the orientation of the ovum in the infundibulum, motility may be important determining which sperm reach the germinal disc. Once the sperm have

entered the ovum by hydrolysing holes in the inner perivitelline layer (IPVL), creating a 'halo' of holes around (but not directly above) the germinal disc (Bramwell & Howarth 1992; Steele et al. 1994; Birkhead et al. 1994), the process by which sperm reach the female pronucleus is unclear, and it is possible that further selection of sperm could occur at this point.

Overall, the 'unknowns' discussed above are only a minority of the additional processes that could impact on male fertilisation success. Regarding the results outlined above, and in consideration of some of the above 'unknowns', a number of future directions are now discussed.

7.4: Future directions

An additional, unexpected consequence of artificial selection could be a coevolutionary response between aspects of male and female reproductive anatomy. A well-cited example of such a coevolutionary response is that between the sperm length and the dimensions of the female seminal receptacle (SR) in Drosophila (Miller & Pitnick 2002), where males with the longest sperm are most successful when mated to females with the longest SRs. The results presented in Chapter 6 indicated a male – female interaction influencing the outcome of sperm competition. However, the observed pattern is not indicative of a clear coevolutionary response between male and female reproductive anatomy (as was observed by Miller & Pitnick 2002), because in the zebra finch, long-sperm males achieved the highest P2 in three out of four mating combinations (refer to Chapter 6). To investigate this result further, the SST dimensions from females from the long and short selection lines should be obtained and compared to the distribution of sperm lengths produced by males from each selection line. Following the method in Birkhead & Hunter (1990), the reproductive tracts of female zebra finches in breeding condition would be removed on the day of clutch initiation, and a representative sample of SST lengths measured from across the uterovaginal junction from several primary mucosal folds (Pellat 1998).

In Chapter 6, I described a three-way interaction that determined the change in sperm proportions over time on the outer perivitelline layer (OPVL) of each successive egg in the clutch, which was dependent on the specific male – female mating combination. To recap briefly, when the female and the second male selection line matched, there was an increase in second male sperm proportions over successive eggs, as expected from the passive loss model (Colegrave et al. 1995). However, when the female and second male selection line mismatched, there was a decrease in second male sperm proportions. A number of possible

explanations for these patterns were outlined in the discussion in Chapter 6, two of which are explained here: (i) that sperm allocation by males differed according to the selection line of the female, and (ii) that sperm are released from the SSTs at different rates depending on the whether the selection line of the female and second male to mate match or mismatch. Methods by which these two hypotheses might be distinguished are described briefly below.

(i) Differences in sperm allocation. A direct method testing this hypothesis is to allow long and short sperm males to copulate with females from both the long and short selection lines, and use a platinum wire loop to scoop out the entire ejaculate from the cloaca of the female (as described by Birkhead & Fletcher 1995a). The ejaculate must be collected quickly before sperm are dispersed or ejected, and with minimum distress to the female. If successful, this technique would provide a meaningful estimate of sperm allocation by males to different females, as sperm numbers in natural ejaculates are repeatable for a given male (see Birkhead & Fletcher 1995b). An indirect method to establish if differential sperm allocation occurs could be counting the absolute number of sperm reaching each ovum (via PVL counts; see below), and combining these data with the ejaculate transfer data. This could provide insight into whether pre- or post-storage processes determines the proportions of sperm on the PVL.

(ii) Differential release rate of sperm from the SSTs. This could be assessed in two stages using similar methods to those described in Chapter 6. The first stage of this study would be a non-competitive fertilisation trial. A male (e.g. the short-sperm male) would be paired with a female (e.g. from the short selection line) and allowed to copulate freely until clutch initiation (the day that the first egg is laid), after which the male and female would be separated while the female completes the clutch. Dissecting each egg (Birkhead et al. 2008; Chapter 6 Section 6.2), and counting both the number of holes and sperm on the PVL provides a measure of the number of sperm reaching the ovum, and an estimate of the rate of loss of sperm from the SSTs over successive eggs (Wishart 1987; Brillard 1993). When no sperm are observed on the OPVL (14 d post-insemination; Birkhead et al. 1989), the trial is repeated as above, and the short-sperm male is then paired to a female from the long selection line. Exactly the same procedure is then carried out using a long-sperm male, resulting in four clutches of eggs from four fertilisation trials as follows: (i) short male x short female, (ii) short male x long female, (iii) long male x short female, and (iv) long male x long female.

The second stage of the experiment would be competitive fertilisation trial, similar to that described in Chapter 6, where a pair of long- and short-sperm males mated sequentially (for 3 d per male) with a female (e.g. from the long selection line), who would produce a clutch of

eggs. The same male pair would then be mated with the same female, but in the alternate order, to produce a second clutch of eggs. In this additional experiment, the procedure would be repeated exactly as above, except that the same male pair would also be mated to a female from the short selection line. In total, four clutches of eggs would be obtained from each set of trials per male pair and dissected as described above. This experiment has increased sensitivity to detect a male x female influence on sperm loss from the SSTs, due to the balanced experimental design.

Finally, I suggest some further future directions, that build on the work conducted in the thesis and involve increasing our understanding of the: (i) role of the sperm midpiece, (ii) changes in testes internal structure with varying sperm morphology, and (iii) genomics of sperm competitive success.

(i) Given that the absolute midpiece length of zebra finch sperm had minimal effects on sperm swimming velocity, the question of the exact role of the midpiece in this species remains. The patterns are unclear across the literature; across passerine birds (Rowe et al. 2013), species with larger midpieces produce greater amounts of adenosine triphosphate (ATP), but swimming velocity is unaffected by ATP production. However, in the domestic fowl *Gallus domesticus* (Froman & Feltmann 1998), sperm mobility phenotype was determined by the amount of ATP, but mitochondrial helix length was unrelated to ATP content. As there are significant differences in sperm structure between passerine and non-passerine sperm (Jamieson 2007) and the selection pressures experienced will be species-specific, intra-specific studies of the above relationships are likely to be the most enlightening. Exploiting the variation in sperm design through the differences in each morphotype's midpiece: tail ratio may provide insights into how the ATP 'budget' of a sperm per unit length affects sperm function.

(ii) The amount of spermatogenic tissue in the passerine testes, and thus the ability of the testes to produce sperm are associated with sperm competition intensity (Lüpold et al. 2009c). Sperm competition has also selected for an increase in the dimensions of the seminiferous tubules to produce larger sperm (Lüpold et al. 2009c). Differences in the internal cellular organisation of the testes determining the efficiency of spermatogenesis are influenced by sperm competition intensity (Lüpold et al. 2011). Currently, little is known about the spermatogenic cycle in passerine birds, especially within a single species. Given the extreme variation in zebra finch sperm length (Birkhead et al. 2005; Chapter 3), where the longest sperm are twice the length of the shortest sperm, this species may provide a suitable model to

determine the duration of the spermatogenic cycle, but also to understand how the internal structure of testes adapts to accommodate such variable length sperm. Such a study may also provide insights with regard to the reduction in testes mass observed in short-sperm males.

(iii) The results presented in this thesis demonstrate that artificial selection on sperm morphology resulted in a change in sperm swimming velocity and consequently fertilisation success. Given the wealth of genomic resources now available for the zebra finch (Stapley et al. 2008, 2010; Dawson et al. 2010; Warren et al. 2010) combined with the full pedigree and the suite of morphological measurements of the males from this, and previous studies (e.g. Birkhead et al. 2005; Mossman et al. 2009), the logical next step (currently in progress) is to locate the regions of the chromosomes, and ultimately the loci that determine both sperm morphology and sperm swimming velocity. Similar research has been conducted in other species (e.g. stalk eyed flies *Cyrtodiopsis dalmanni*: Johns & Wilkinson 2007). Regions of the genome where influential loci are expected to be located vary from autosomal to sex-linked locations (see Simmons & Moore 2009 for a comprehensive review), which could suggest complex polygenic control over phenotypes that affect competitive success.

7.5: Concluding remarks

The future directions described here will aid our understanding of male fertilisation success by establishing how sperm total length is associated with three levels of the reproductive physiology of the male; through changes in the genome, the sperm and the testes. Sperm competitive ability is not a simple phenotypic trait because trait expression also depends on the female environment in which sperm compete. This means that understanding how male and female factors interact through each level of male reproductive physiology is likely to prove crucial for predicting the outcome of sperm competition in internally fertilising species.

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Appendices

Appendix A1 – the effect of artificial selection on body mass, bill colour and tarsus length

During data collection for Chapter 3, three additional morphological measurements were obtained for each bird to establish whether artificial selection on sperm total length had additional unexpected effects. Body mass (to the nearest 0.01g) was recorded for every chick on day 0, 5, 10, 20, 30, 40 and 100 d post hatching. Left tarsus length (to the nearest 0.01mm) and bill colour (ordinal scale 0-6) were recorded at sexual maturity (see Chapter 2 Section 2.2).

The effect of artificial selection on body mass was tested using linear mixed models (LMMs) with sex, line and cohort fitted as fixed effects. Bird ID nested within family was included as a random effect to control for multiple measures of body mass per individual, and for any associated family effects that could affect body mass, such as poor parental care. The effect of artificial selection on bill colour was investigated using separate Kruskall-Wallis tests, which tested for differences between the three selection lines and the three cohorts. Sex differences in bill colour were tested using a Wilcoxon test. The effect of artificial selection on tarsus length was tested using a LMM with sex, line and cohort as fixed effects. Family was included as a random effect to control for differences in parental care that could affect tarsus length via differences in chick growth in individual clutches.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package, lme4 (Bates et al. 2012) and languageR (Baayen 2011).

Results

Selection line, cohort and sex all had no effect on body mass (Table A1.1; all $p > 0.05$). There was no change in bill colour across the lines or the cohorts (Figure A1.1 & Table A1.2; both $p > 0.05$). As expected, sex had a significant effect on bill colour with males having darker bills (higher scores). Males from the L- and S-line had a small but significant increase and decrease in tarsus length respectively, compared to the I-line males (Table A1.3; $p = 0.0290$ and 0.0053 respectively). Birds in cohort 2 generally had slightly longer tarsi ($p = 0.0053$). Male birds also had longer tarsi than female birds ($p = 0.0004$) as expected. Overall, artificial selection for sperm total length is associated with a change in tarsus length, specifically an increase and decrease in L- and S-line tarsus length respectively. This represents a change in skeletal size in

these two lines. However, these differences are not expected to affect sperm performance, as there is no evidence of an association between sperm traits and phenotypic traits such as bill colour (Birkhead & Fletcher 1995) or body condition (Birkhead et al. 1999).

Table A1.1. Results of LMMs[1] analysing the effect of selection line, cohort and sex on body mass. Body mass was not affected by any variable.

	Estimate	HPD (lower, upper)	pMCMC
Intercept[2]	10.52	---	---
Sex: male	-0.20	-0.45, 0.07	0.12
Cohort 1	0.26	-0.08, 0.60	0.14
Cohort 2	0.14	-0.19, 0.46	0.41
Line L	0.13	-0.20, 0.46	0.42
Line S	0.10	-0.22, 0.44	0.53

[1]Data are based on 7643 observations from 1129 birds divided between 222 families.
[2]The intercept is the intermediate line, which all other variables are compared to in the model.

Table A1.2. Results of individual tests of the effect of selection line, cohort and sex on bill colour[1]. There was a significant effect of sex on bill colour. Significant results ($p < 0.05$) are in bold.

	X^2 [a] W[b]	p
Cohort	0.1576[a]	0.9242
Line	1.00[a]	0.6057
Sex	5640[b]	**<0.0001**

[1]Data are based on 1054 birds.
[a]Kruskall-Wallis test.
[b]Wilcoxon 2 sample test.

Table A1.3. Results of LMM analysing the effect of selection line, cohort and sex on mean tarsus length[1]. Mean tarsus lengths were increased and decreased in the L- and S-line respectively, compared to the I-line, and were also longest in cohort 2. Male birds also had longer mean tarsus lengths compared to females. Significant results ($p < 0.05$) are in bold.

	Model estimate	T	95% HPD (lower, upper)	pMCMC
Intercept[2]	16.63	---	16.50, 16.78	---
Line: L	0.14	2.19	0.04, 0.25	**0.0290**
Line: S	-0.17	-2.61	-0.28, -0.07	**0.0092**
Cohort 2	0.19	2.80	0.05, 0.27	**0.0053**
Cohort 3	0.08	1.17	-0.05, 0.17	0.2403
Sex: male	0.11	3.58	0.05, 0.17	**0.0004**

[1]Data are based on 1049 birds divided between 220 families.
[2]The intercept is the intermediate line, which all other variables are compared to in the model.

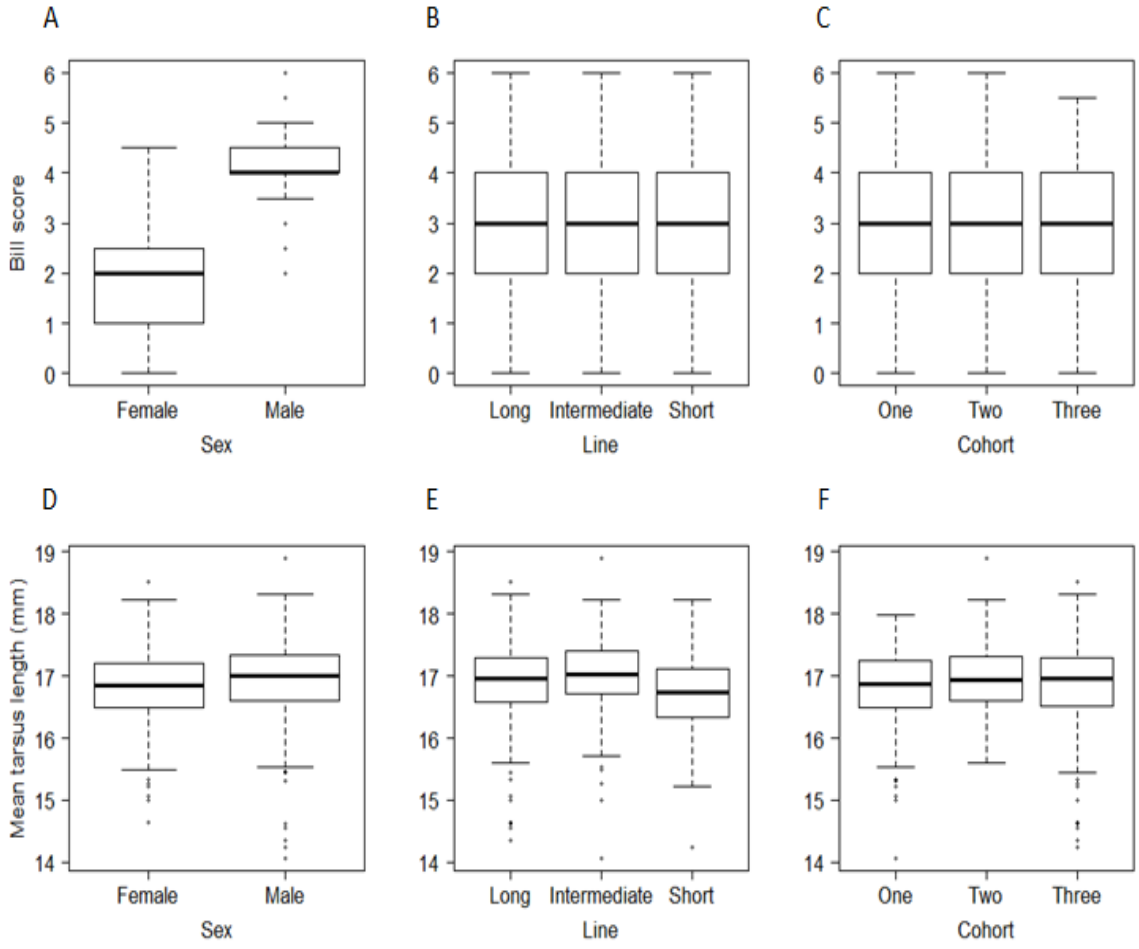

Figure A1.1. The effect of sex, selection line and cohort on bill colour score (A – C) and mean tarsus length (D – F). (A) Males had darker bills compared to females but there was no effect of selection line (B) or cohort (C) on bill colour. (D) Males had longer tarsi compared to females. (E) The S-line had shorter tarsi compared to the L- and I-lines. (F) Cohort 1 had longer tarsi compared to cohort 2 and 3. In all plots the black horizontal lines across the boxes are the median values.

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Appendix A2 – a simulation to establish the minimum number of sperm required for an accurate estimate of morphological variation per male

In Chapter 2 the protocol for the set-up and management of the three selection lines was described (Chapter 2 Section 2.2), and the results from this artificial selection experiment are presented in Chapter 3.

In order to determine the sperm length phenotype of each male zebra finch throughout the three years of this selection experiment, a representative sample of the sperm from each male must be measured. As more sperm per male are measured, the accuracy of the estimate increases, although with diminishing returns. Simulations were carried out to estimate the minimum number of measured sperm that provides an accurate estimate of sperm component dimensions per male, using an R script kindly provided by S. Calhim (Calhim et al. 2011), which builds on methods described in Pattarini et al. (2006).

Fifteen sperm were measured from 25 male zebra finches (as described Chapter 2 Section 2.4.2). The simulation used the maximum number of sperm measured, i.e. 15, as the most accurate estimate of the sperm component value per male. This estimate was compared to estimates of sperm components given by 1, 2, 3 - 15 sperm. Linear regressions of the sperm components value from the subsamples against the accurate estimate of these values were carried out using each male as a data point, producing R squared (R^2) values indicating the proportion of variance captured by measuring 1,2,3 - 15 sperm. A re-sampling approach (resampling 1000 times) gave the mean ± S.D for each R^2 values. This was carried out for each component of sperm morphology: (i) head, (ii) midpiece, (iii) tail, and (iv) total length. These values were plotted for each sperm component (Figure A2.1) showing how the value of R^2 increased as the number of sperm measured per male increased.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package, and the package gplots (Warnes et al. 2012).

Figure A2.1 A-D illustrates that measuring 5 sperm per male provided an extremely high value of R^2 (greater than 95%) resulting in an accurate estimate of each component of sperm morphology (see also Birkhead et al. 2005). Therefore 5 sperm were measured per male zebra finch to obtain an accurate estimate of sperm morphology for each male, which are used in analyses included in Chapter 3 and 4.

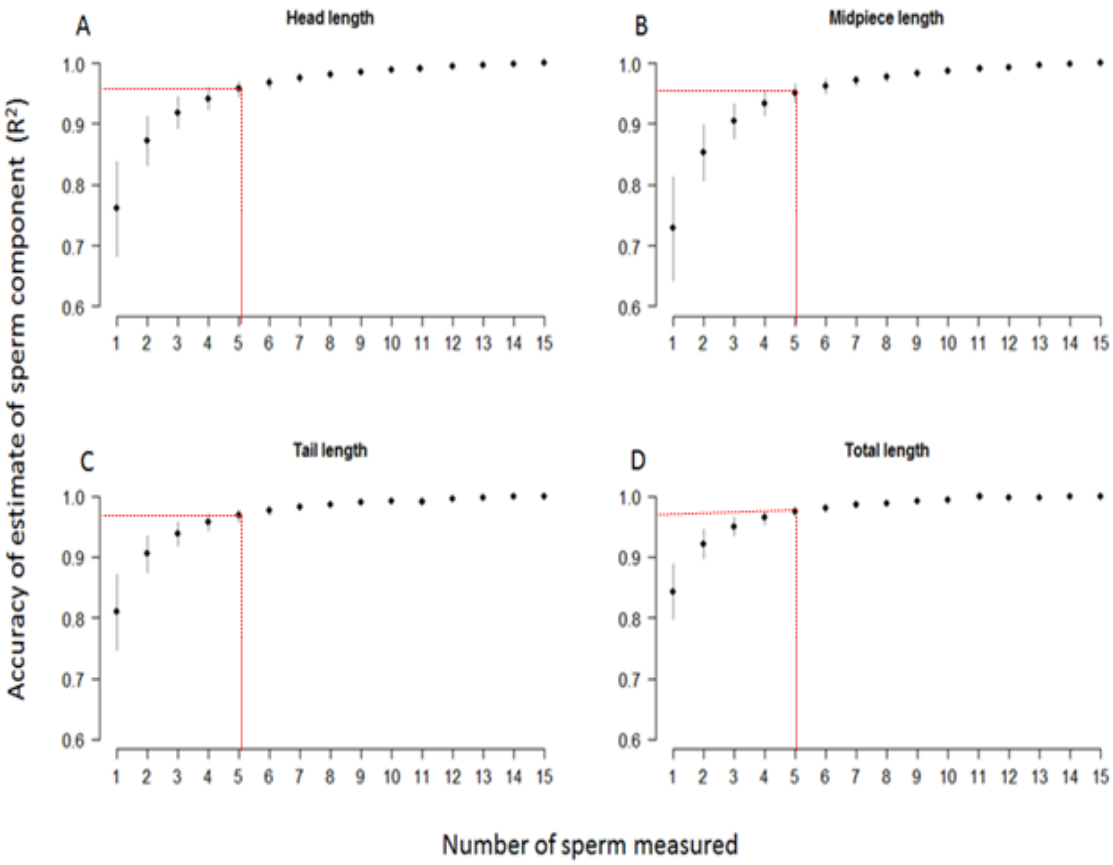

Figure A2.1. The change in accuracy of the estimate of each sperm component with increasing sampling size. The grey bars represent 95% confidence intervals around the mean. The accuracy of each component: head (A), midpiece (B), tail (C) and total length (D) were greater than 95% when 5 sperm were measured from each male (red dotted lines).

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Appendix A3 – the effect of different frame rates on three kinematic parameters of sperm

In Chapter 5, sperm motility assays using computer assisted sperm analysis (CASA: see Chapter 2 Section 2.4.1) were carried out on male zebra finches (n = 144), and the influence of sperm morphology on swimming speed was described. Due to an error in the communication between the CASA software and the microscope camera, the motility parameters of a subset of males (n = 42) were recorded at 37fps instead of 50fps (the details of the original software settings are in Chapter 2 Section 2.4.1).

It is unlikely that the small difference in frame rate (between 37 and 50fps) would significantly affect the magnitude of the kinematic parameters (average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL)). Recording at 37fps as opposed to 50fps provides 37 pieces of location information for each sperm as opposed to 50 locations per sperm. The VSL values of a particular sperm should be unchanged, because the starting and ending position of the sperm is the same regardless of the recording frame rate. An increasing frame rate merely provides more precise information regarding the shape of the path of the swimming sperm.

Unfortunately, it was not possible to re-analyse the subset of motility recordings at the correct frame rate because samples of live sperm were required to obtain video recordings at both 37 and 50fps to understand if the kinematic parameters at each frame rate were comparable. As it was deemed inappropriate to sacrifice zebra finch males for this trial, we decided to use samples of canary sperm because sperm can easily be obtained by cloacal massage (Burrows & Quinn 1937). Here, the evidence presented demonstrates that changing the frame rate from 37 to 50fps does not significantly affect the values of the kinematic parameters, and that all data from all male zebra finches can be analysed together for Chapter 5.

Sperm were obtained from 11 male canarys *Serinus canaria domestica* by cloacal massage (Burrows & Quinn 1937). Each sperm sample was divided into two aliquots and stored at room temperature (approximately 20^{o}C). Sperm were diluted using warmed (38^{o}C) Ham's F10 media to an appropriate concentration for CASA, and then videos of swimming sperm were recorded following the protocol described in Chapter 2 Section 2.4.1. One aliquot was used for recording for each frame rate (37 and 50fps), and frame rate recording order (i.e. 50fps recorded first or

second) was randomised across males. Estimates of VAP, VCL and VSL were obtained for every sperm from each male.

Linear mixed effect models (LMMs) were used to analyse the effect of frame rate on each of the three kinematic parameters. Frame rate (37 or 50fps), recording order (50 fps recorded first or second) and the interaction between them were included as fixed effects. Bird ID was included as a random effect to control for multiple measures of sperm per male.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package, lme4 (Bates et al. 2012) and languageR (Baayen 2011).

There was no significant effect of the interaction (assessed using log likelihood tests – data not shown) between frame rate and recording order for VAP, VCL or VSL; therefore, the interaction was removed from the models. The frame rate also had no significant effect on the estimates of VAP, VCL or VSL (Table A3.1 & Figure A3.1); however; sperm samples recorded second (regardless of the frame rate) had significantly lower estimates for all three kinematic parameters, presumably because the sperm quality (i.e. motility) declined over time (Table A3.1 & Figure A3.1).

Table A3.1. The effect of frame rate on three kinematic parameters[1]. The frame rate did not affect any kinematic parameter; however, each parameter declined significantly when the video recordings were made second. Significant values ($p < 0.05$) are in bold.

Kinematic parameter	Explanatory variable	Model estimate	HPD 95% interval (lower, upper)	pMCMC
VAP	Intercept	61.16	49.07, 72.52	---
	Frame rate	2.31	2.31, -2.39	0.344
	Order	19.75	-19.75, -24.51	**0.0001**
VCL	Intercept	74.86	61.29, 88.27	---
	Frame rate	3.17	-2.16, 8.75	0.254
	Order	-22.06	-28.17, -17.23	**0.0001**
VSL	Intercept	52.84	41.69, 63.88	---
	Frame rate	-0.27	-4.70, 4.81	0.910
	Order	-17.42	-21.81, -12.97	**0.0001**

[1]All results were produced LMM. Models estimates, higher posterior density intervals (HPD) and p values were produced using Markov chain Monte Carlo (MCMC) sampling in the R package languageR (Baayen 2011).

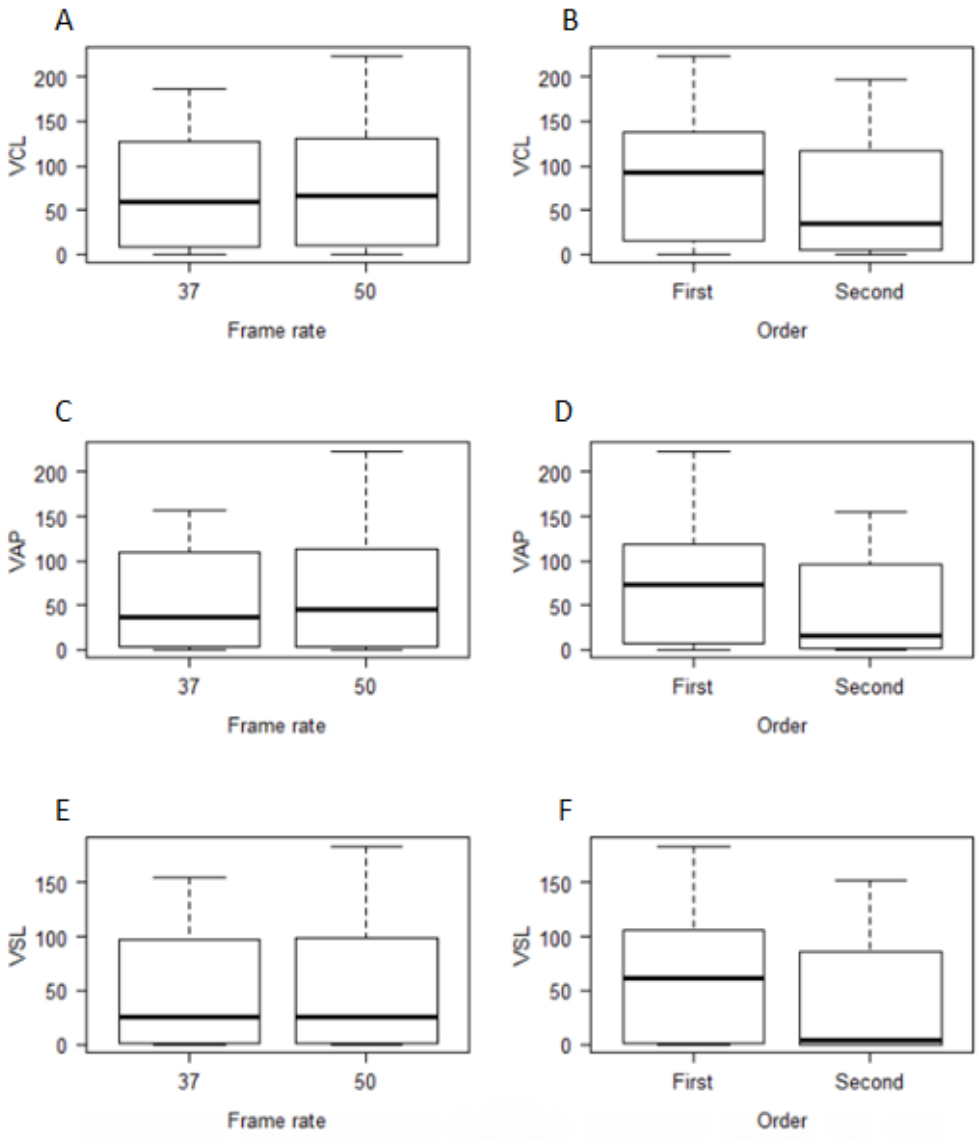

Figure A3.1. The difference in kinematic parameters at two frame rates (37 and 50fps). There was no difference in estimates between the two frame rates in VCL (A), VAP (C) or VSL (E). However, the order in which video recordings were made had significant effects on all kinematic parameters VCL (B), VAP (D) and VSL (F) with a significant reduction in all kinematic values observed when recordings were carried out second.

Due to the lack of difference in all three kinematic parameters when recorded at either 37 or 50fps, motility data from all males was combined to make the dataset that was used in analyses in Chapter 5. Even though this trial was not carried out on zebra finch sperm, the similarity in basic sperm structure across passerine species mean that these results are appropriate to be generalised to the zebra finch. Of course, when possible, this trial will be repeated using sperm from zebra finches.

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Appendix A4 – comparison of the sperm data in Mossman et al. (2009) and the present study

In Chapter 5, a quadratic relationship between three measures of sperm morphology (tail, flagellum and total length) and sperm swimming velocity was demonstrated, i.e. sperm velocity increased with increasing length of sperm components up to a certain value before declining. A previous study (Mossman et al. 2009) using the same population of zebra finches (albeit different birds) demonstrated a linear relationship between those particular sperm components and swimming velocity, such that long sperm swam faster. This major difference between the two studies was surprising.

This appendix presents summary statistics for the data from Mossman et al.'s (2009) study and the data from zebra finches produced by the selective breeding regime (Chapter 3) and analysed in Chapter 5. This was carried out to understand why the present study found a quadratic relationship between some sperm components and swimming velocity (Chapter 5).

The descriptive statistics of the two data sets reveal that the datasets are very similar (Table A4.1). The data range of the two datasets is comparable, so the difference in conclusions between the two studies is not a consequence of the selective breeding regime (Chapter 3). However, in the present study there were numerically more males analysed with extreme phenotypes, i.e. very long and very short sperm, producing a bimodal distribution (Figure A4.1), compared to the normal distribution of Mossman et al.'s (2009) dataset. This could have facilitated the detection of a quadratic relationship between sperm length and swimming velocity in the present study.

Table A4.1. Descriptive statistics for the data from Mossman et al. (2009) and from the present study (data from males analysed in Chapter 5), where the mean sperm component value per male was obtained using five and ten sperm respectively.

Sperm component (μm)	Mean ± S.D	
	Mossman et al. 2009 (n_{males} = 108)	**Present study** (n_{males} = 148)
Head	11.08 ± 0.57	11.17 ± 0.63
(range)	(9.92 – 12.62)	(9.49 – 12.82)
Midpiece	30.91 ± 4.54	30.85 ± 4.51
(range)	(15.60 – 37.89)	(16.11 – 39.80)
Tail	22.32 ± 7.77	24.02 ± 9.33
(range)	(8.28 – 48.85)	(9.55 – 46.74)
Total	64.24 ± 5.96	66.05 ± 7.40
(range)	(48.20 – 79.23)	(49.57 – 79.76)

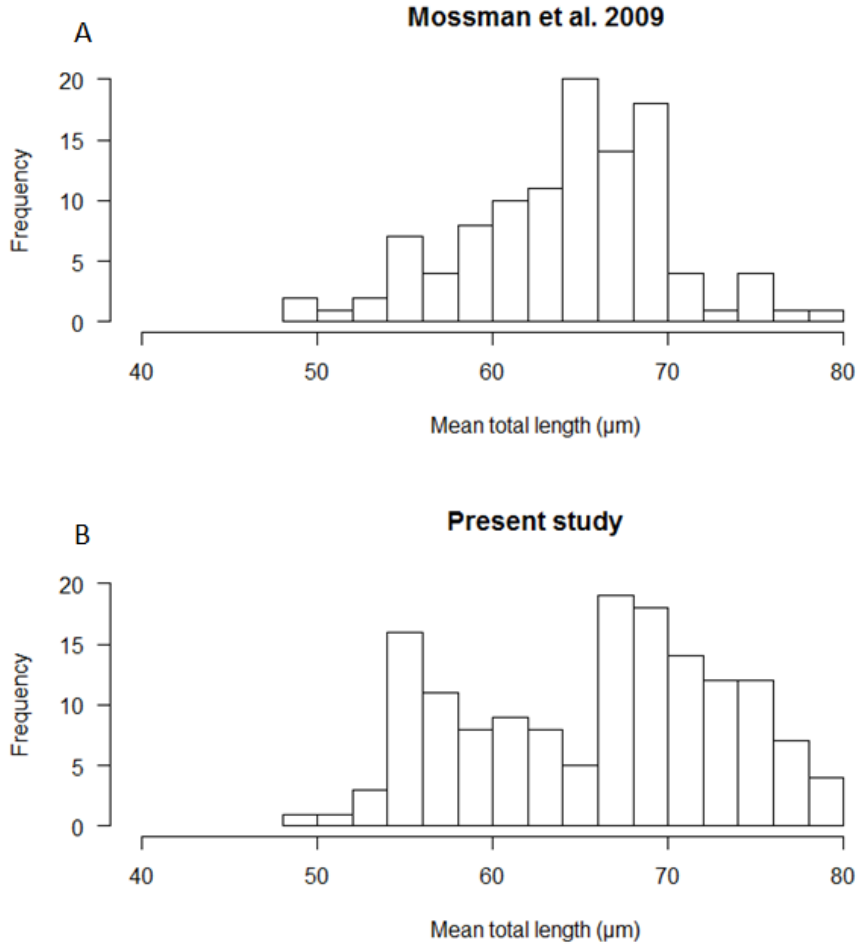

Figure A4.1. Distribution of mean sperm total length used to investigate the association between sperm length and velocity in the zebra finch. Data are from (A) Mossman et al. (2009) and (B) the present study. Although the ranges of the two distributions are similar, there are greater numbers of the extreme sperm lengths in the present study. The distribution of sperm lengths in the present study is bimodal, as a consequence of the selective breeding regime.

References

Mossman, J., Slate, J., Humphries, S. & Birkhead, T.R. (2009). Sperm morphology and velocity are genetically co-determined in the zebra finch. *Evolution* **63** (10): 2730-2737.

Appendix A5 – the effect of selection line on kinematic parameters and sperm dimensions on sperm swimming velocity (PC1) in two subpopulations of sperm

The analyses conducted in Chapter 5 investigated the effect of selection on sperm motility using three subpopulations of sperm; the total population, the fastest 10% and the fastest single sperm. Because the patterns of motility were broadly similar across these three subpopulations of sperm, the plots were included in this appendix for brevity. See Chapter 5 Section 5.2 for details of the statistical models used, and Chapter 5 Section 5.3 for the associated results tables for all three subpopulations of sperm.

The effect of selection line on the kinematic parameters across the fastest 10% (Figure A5.1) and the fastest single sperm (Figure A5.2) subpopulations were qualitatively similar, such that the S-line sperm had significantly lower values of all parameters compared to the L- and I-line sperm, with the exception of LIN and STR (Chapter 5 Table 3).

The effect of the dimensions of individual sperm components (head, midpiece, tail, total length, flagellum: head ratio and midpiece: tail ratio) on sperm swimming velocity (PC1) across the fastest 10% (Figure A5.3) and the fastest single sperm (Figure A5.4) subpopulations were also broadly similar. Sperm with longer heads swam faster; however, midpiece length was not associated with swimming velocity. Tail length, total length, flagellum: head ratio and midpiece: tail ratio significantly determined swimming velocity via quadratic relationships, such that swimming velocity increased until a threshold values was reached, after which, velocity decreased (Chapter 5 Table 4).

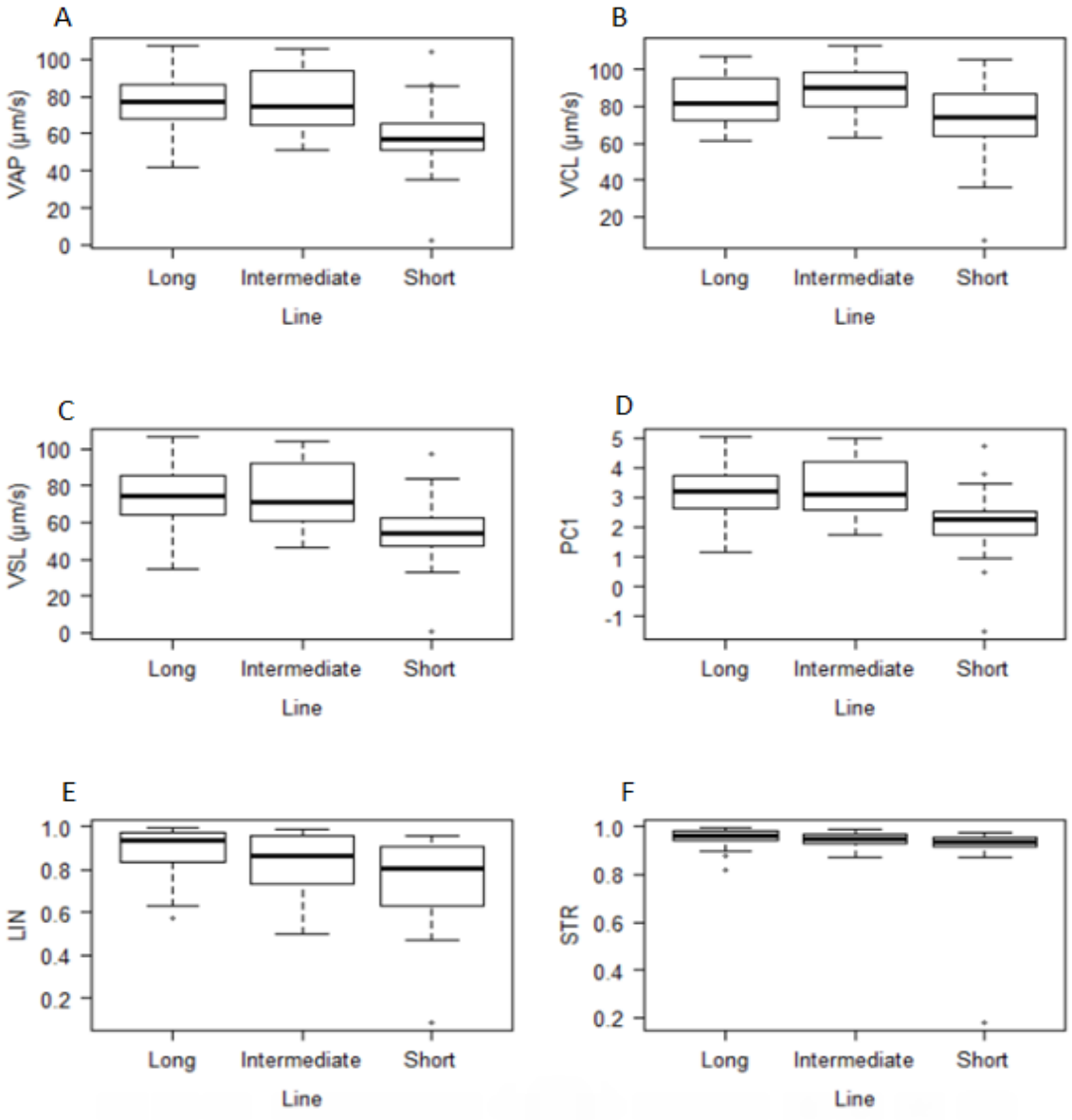

Figure A5.1. The effect of selection line on six kinematic parameters of zebra finch sperm for the **fastest 10% of sperm**. VAP (A), VCL (B) and VSL (C) were analysed using Principle Components Analysis (PCA) to give (D) an index of sperm swimming velocity (PC1). LIN (E) and STR (F) are given by VSL/VCL and VSL/VAP respectively. Median values are represented by the thick black line across each boxplot. (A-D): the S-line has significantly lower values for the kinematic parameter. (E-F): there are no significant differences between the lines for either STR or LIN.

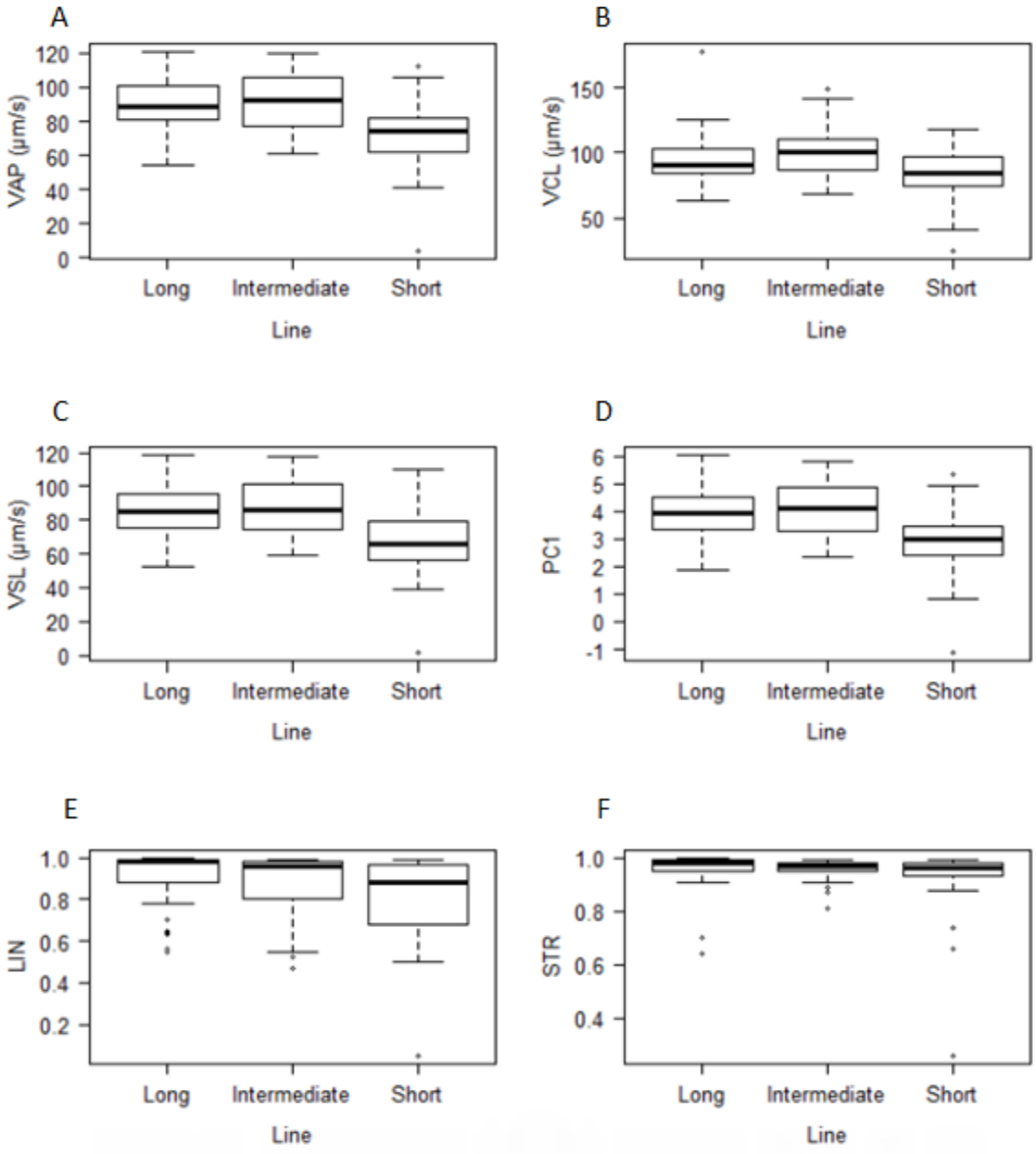

Figure A5.2. The effect of selection line on six kinematic parameters of zebra finch sperm for the **fastest single sperm**. VAP (A), VCL (B) and VSL (C) were analysed using PCA to give (D) an index of sperm swimming velocity (PC1). LIN (E) and STR (F) are given by VSL/VCL and VSL/VAP respectively. Median values are represented by the thick black line across each boxplot. (A-D): the S-line has significantly lower values for the kinematic parameter. (E-F): there are no significant differences between the lines for either STR or LIN.

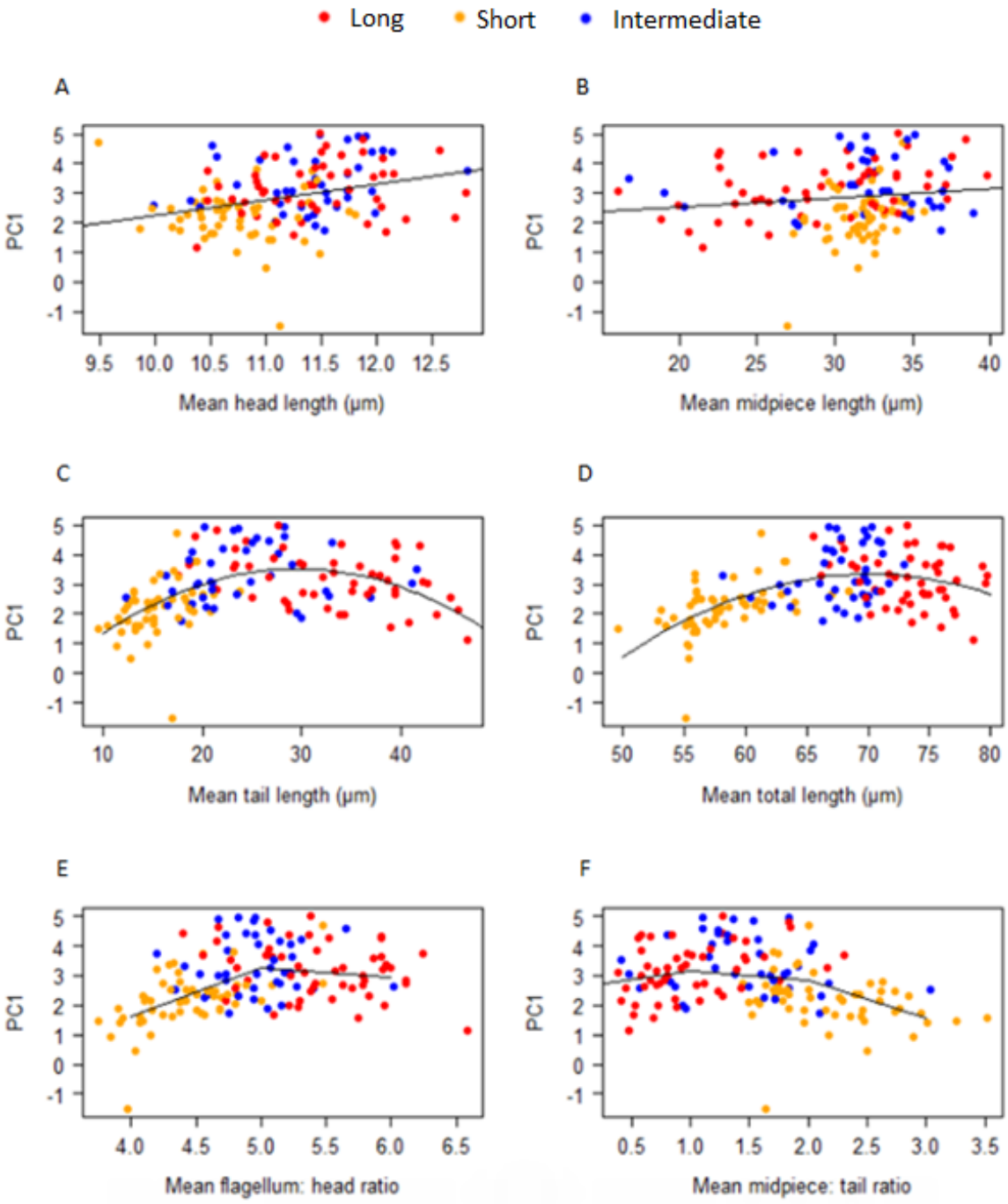

Figure A5.3. The relationship between sperm swimming speed (PC1) and six measures of sperm morphology for the **fastest 10% of sperm**: head (A), midpiece (B), tail (C), total length (D), flagellum: head (E) and midpiece: tail (E). The coloured points represent the 3 selection lines: long (red), intermediate (blue) and short (orange). Each point represents data from a single male zebra finch (n = 144). Each sperm component had a significant effect on sperm swimming speed, with the exception of midpiece length (B) where the relationship was marginally non-significant.

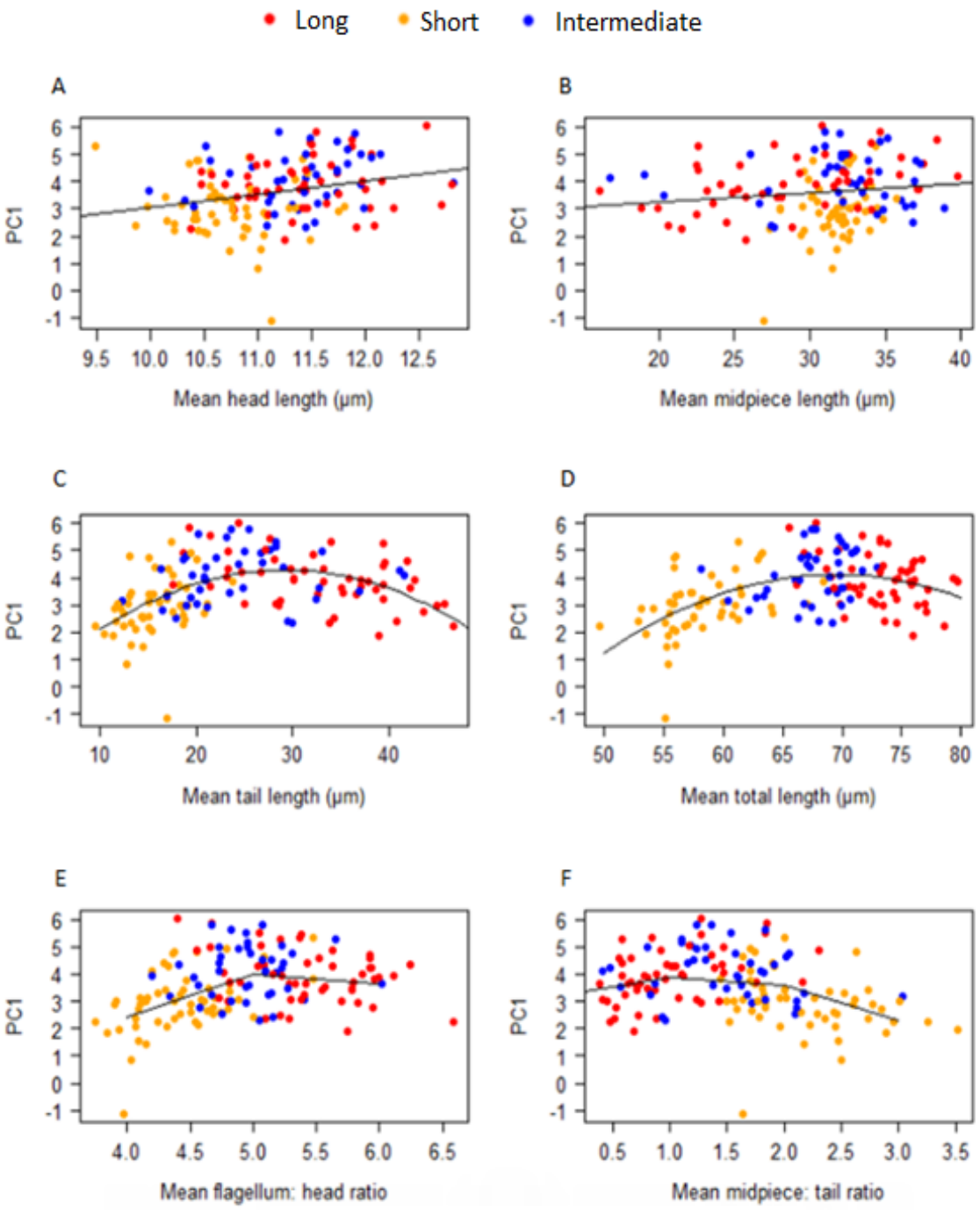

Figure A5.4. The relationship between sperm swimming speed (PC1) and six measures of sperm morphology for the **fastest single sperm**: head (A), midpiece (B), tail (C), total length (D), flagellum: head (E) and midpiece: tail (E). The coloured points represent the 3 selection lines: long (red), intermediate (blue) and short (orange). Each point represents data from a single male zebra finch (n = 144). Each sperm component had a significant effect on sperm swimming speed, with the exception of midpiece length (B) where the relationship was marginally non-significant.

Appendix A6 – the evidence for a quadratic relationship in Mossman et al. (2009)

In Chapter 5, a quadratic relationship between three measures of sperm morphology (tail, flagellum and total length) and sperm swimming velocity was demonstrated, i.e. sperm velocity increased with increasing length of sperm components up to a certain value before declining. This was in contrast to Mossman et al. (2009) who reported a linear, positive association between tail, flagellum and total length and swimming velocity. Appendix A4 demonstrated that the range of sperm lengths analysed in Mossman et al. (2009) and the present study were similar; therefore, the artificial selection experiment (Chapter 3) was unlikely to be the cause of the different conclusions.

A subset of the data from Mossman et al. (2009) was reanalysed in order to understand why different patterns were found in Mossman et al. (2009) and the present study. The raw data and PC1 scores for each individual sperm in Mossman et al's (2009) dataset were not available, so the re-analysis was carried out using each male's mean curvilinear velocity (VCL), which was highly correlated to male mean PC1 (Mossman 2008). The analysis was conducted on sperm total length only. A linear model (LM) was constructed with VCL as the response variable and total length (the mean values per male) as the single explanatory variable. In order to assess whether or not a quadratic relationship was present in the data, the model was fitted with and without a polynomial explanatory variable, and the models compared using log-likelihood tests with one degree of freedom.

The analysis was performed using R version 2.15.1 (R Development Core Team 2012) using the base package.

The relationship between total length and VCL was best described by the model that included the polynomial term (Table A6.1), suggesting that the longest sperm showed a slight decrease in swimming velocity, similar to the results presented in Chapter 5.

Table A6.1. Results of LMs analysing the effect of sperm total length on swimming velocity (VCL). Data are from Mossman et al. (2009). The relationship between sperm total length and VCL was described best by the model with the polynomial variable, i.e. there is a quadratic relationship between sperm length and swimming speed. Significant results are in bold ($p < 0.05$).

Model	**Sperm component**	**Estimate ± S.D**	**t**	**$F_{1,106}$[a] $F_{2,105}$[b]**	**Adjusted R^2**	**p**
Linear	Total length[a]	0.311 ± 0.066	4.69	22.08	0.165	**<0.0001**
Polynomial	Total length[b]	4.161 ± 0.845	4.93	---	---	**<0.0001**
	Total length[b]	-0.031 ± 0.007	-4.57	23.55[1]	0.297[1]	**<0.0001**[1]

Comparison of the linear and polynomial model

	Residual d.f	**F**	**p**
Linear	106		
Polynomial	105	20.88	**<0.0001**

[1]This output relates to the complete model containing both the linear and the polynomial variable

References

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Appendix A7 – artificial insemination of female zebra finches

In Chapter 6 we referred to our attempt to conduct sperm competition experiments using heterospermic artificial insemination, and explained that it was not possible to use this method. This was because the proportion of fertile eggs following artificial insemination (AI) was much lower than expected and the sperm numbers observed on the outer perivitelline layer (OPVL) were extremely low. Continuing with this method would not have produced enough data to accurately determine the effect of sperm morphology on fertilisation success.

A number of trial AIs were conducted by N. Hemmings prior to the start of the experiment using the same protocol described below, with the exception that only the sperm of a single male was inseminated. Although sperm numbers on the OPVL were low, they did not give us cause for undue concern at that point.

Here, the methods and summary results table are presented for both trials: (i) AI using a heterospermic mixture, and (ii) AI using sperm from a single male. The results are not discussed as the number of successful inseminations was too few to yield insights into the outcome of sperm competition.

Artificial insemination procedure

The female zebra finches used in these experiments were trained to receive AI by holding the female on her back with the head down to expose the cloaca, every day for two weeks before the start of the trial. Inseminations were carried out by blowing gently on the cloaca, so it opened wide, and placing a few microliters of phosphate buffered saline (PBS) on the lip of the cloaca using a Gilson pipette. Females were then selected for use in the actual AI experiment if they produced fertile eggs from these trial AIs.

To ensure that all females to be used in the AI experiment were in breeding condition, each female was allowed to lay and incubate a clutch of eggs. These eggs were infertile because the female had been paired to a male using a double cage set up (Chapter 6 Section 6.2), where physical contact between the male and female was not possible. The incubated eggs were removed from groups of females on the same day to synchronise the onset of egg lay in those particular females. This was important so that multiple females were inseminated with the

heterospermic mixture from a single male pair to maximise the data from each male pair and to detect female effects on fertilisation success.

Nest boxes were checked daily, and artificial inseminations were conducted on the day that the second egg was laid. Occasionally females may produce only a single egg (N. Hemmings, pers. comm), and this would mean no further data could be collected. The laying of the second egg indicated that the female would produce a whole clutch. All inseminations were performed in the afternoon between 1500 and 1730 h GMT to avoid any additional bias due to time of day in relation to oviposition (Birkhead et al. 1995). A maximum of three females, at least one of which was from both the long and short selection line, were inseminated with the sperm from one pair of males (one long- and one short-sperm male). Inseminating only three females with each sperm mixture ensured that enough sperm were inseminated to maximise the chance of successful fertilisation.

Preparation of zebra finch sperm for artificial insemination

The pairs of male zebra finches (one from each selection line) were humanely killed by cervical dislocation. Each male pair was euthanized and dissected sequentially, and in alternate order to minimise the time period between death and sperm extraction and to control for any effects of dissection order on fertilisation success. The first seminal glomerus (SG) dissected was stored in PBS at room temperate until the second dissection was complete. The average time between the start of the first and second dissection was 25 minutes. The left SG was dissected as described in Chapter 2 Section 2.3.4. A diluted sample of sperm (approximately 10µl) was collected for sperm quality assays (Chapter 2 Section 2.4.1 – 2.4.4).

Sperm were squeezed out from the remaining distal third portion of each SG into 10µl of PBS to make a concentrated sperm solution. The sperm solutions were then mixed together by stirring with a pipette tip to create a heterospermic mixture. Previous mixing trials had showed this mixing method to be reliable (repeatability of proportions of long and short sperm in each subsample = 0.97; J. Thompson, unpublished data) and also avoided any possible damage to the sperm from other mixing methods such as aspiration or centrifugation.

Female zebra finches were artificially inseminated with the heterospermic mixture on the day that the female zebra finch laid the second egg of the clutch. Four microliters of heterospermic mixture were placed on the cloaca of each female using a Gilson p20 pipette and sterile tips. The female was held until the drop of sperm had been taken up by the cloaca. This procedure

was repeated with another 4µl of the same sperm mixture 2 minutes later, to maximise the number of sperm inseminated without putting an excessive amount of sperm solution on the cloaca at any one time. Larger volumes of sperm solution are not taken up into the cloaca as fully as smaller volumes (N. Hemmimgs, pers. comm).

Results

Nine pairs of male zebra finches (one male producing long sperm and one male producing short sperm) were used for AI with 22 different females. Usually 2 females (one female from each of the long and short selection lines (Chapter 2 Section 2.2) were inseminated with each heterospermic mixture, unless there was a high volume of sperm in which case an additional female was inseminated. Sixty-seven eggs were laid in total, of which 9 were fertile (13.4%). Sperm were observed on the OPVL of 7 eggs (10.4%). However, in each case there was, remarkably, only a single sperm on the OPVL. Two eggs were too developed to examine the OPVL for sperm but it is unlikely, considering the extremely low sperm numbers found across those clutches, that the sperm numbers on these OPVLs would have been high. Table A7.1 shows summary data from these trials. We also provide a table for comparison from the initial AI trials using the sperm from only 1 male (Table A7.2). These data highlight the extremely low fertility rate of eggs (due to low number of sperm reaching the ovum) produced by AI in zebra finches, yet also indicate extremely efficient use of sperm in the cases where fertilisation was successful with only 1 sperm observed on the OPVL (Birkhead & Fletcher 1998).

Table A7.1. Descriptive data on the number of fertile eggs produced, and sperm numbers on the OPVLs for AIs using a heterospermic mixture. Sixty-seven eggs were laid across the females (n = 20). The mean number of sperm per OPVL was extremely low.

Number of fertile eggs laid per clutch	Number of clutches (n =22)	% Females	Mean sperm per egg
No fertile eggs	15	68.2	0.04
1 fertile egg	6	27.3	0.18[a]
1+ fertile egg	1	4.5	0.67

[a]Two eggs were too developed to examine the OPVL for sperm.

Table A7.2. Descriptive data on the number of fertile eggs produced, and sperm numbers on the PVLs for AIs using a sperm from a single male. Eighty eggs were laid across the females (n = 18). The mean number of sperm per egg was low, although slightly greater than observed during the heterospermic artificial inseminations (Table A7.1).

Number of fertile eggs laid per clutch	Number of clutches (n =30)	% Females	Mean sperm per egg
No fertile eggs	16	53.3	0.06
1 fertile egg	2	6.7	0.44
2 fertile eggs	8	26.7	2.89[a]
3+ fertile eggs	4	13.3	2.87[b]

[a]One egg was too developed to examine the OPVL for sperm.
[b]Two eggs were too developed to examine the OPVL for sperm.

References

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Birkhead, T. R. & Fletcher, F. (1998). Sperm transport in the reproductive tract of female zebra finches (*Taeniopygia guttata*). *Journal of Reproduction and Fertility* **114** (1): 141-145.

Appendix A8 – The DNA extraction protocol: Ammonium acetate precipitation

This protocol was used to extract DNA from blood and tissue and was supplied by A. Krupa (2010; The University of Sheffield).

The only alterations to this protocol were step 3 and step 16 when extracting DNA from embryonic tissue. We obtained a sample by aspiration using a Gilson p20 pipette and sterile tips, as tissue quantities were often low and were comprised of flakes of tissue. Twenty microliters of T_{10} $E_{0.1}$ was added to the DNA because of the low concentration of DNA.

1) Add 250µl Digsol buffer and 10ul Proteinase K (10mg/ml) in a 1.5ml flip-top tube. Keep on ice.

2) Centrifuge blood sample at 13,000rpm for about 1 min (necessary to pellet the sample).

3) Remove sample from ethanol with toothpick and blot onto tissue. When dry, transfer the toothpick into the tube and jiggle to dislodge. Remove toothpick and place in bleach

4) Vortex, wrap the rack in tissue and elastic band and place in rotating oven at 55^{o}C (3 h) or 37 ^{o}C (overnight).

5) Once digested (straw colour) add 3000µl 4M ammonium acetate to each sample

6) Vortex several times over a period of at least 15 mins at room temperature to precipitate the proteins.

7) Centrifuge for 10 minutes at 13,000rpm

8) Aspirate supernatant (clear liquid containing the DNA) into clean labelled 1.5ml flip-top tubes (discard the gunky protein stuff which usually pellets on the bottom although could be floating on the top).

9) Add 1ml 100% ethanol

10) Invert tubes gently several times to precipitate DNA

11) Centrifuge for 10 minutes at 13,000rpm

12) Pour off ethanol taking care not to lose DNA pellet

13) Add 500µl 70% ethanol and invert several times to rinse pellet

14) If the pellet dislodges from the bottom of the tube centrifuge for 5 minutes at 13,000rpm

15) Pour off ethanol in a smooth movement and stand tubes upside-down on clean tissue (approximately 30-60 mins)

16) Once fully dry add approximately 100µl T_{10} $E_{0.1}$ (the amount added is dependent on size of pellet)

17) Flick sample to dislodge pellet

18) Place tubes in a waterbath for 30 mins (37^{o}C or 65^{o}C degrees) to dissolve pellet (flicking every 10 mins)

19) Store at –20^{o}C (long term) or 4^{o}C (short term)

Appendix A9 – the effect of male selection line on embryo gender

In Chapter 6, all embryos produced during the fertilisation trials were genotyped using a multiplex of microsatellite markers (Dawson et al. 2010). One of these markers was a sex marker (Z-002E) and was genotyped to investigate if there was: (i) a gender bias in embryos produced by the long and short sperm males, and (ii) any interacting effect of the parental selection lines on embryo gender.

A chi squared test was initially used to detect if there was an association between male and female line. Data were also modelled using generalised linear mixed models (GLMM) with male and female line, and the interaction between them as fixed effects. Trio ID (the trios related to the female and male pairs used in the sperm competition experiment in Chapter 6) was included a random effect as there were multiple embryos resulting from each trio.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package and lme4 (Bates et al. 2012).

The proportions of male and female embryos produced by males from each line are shown in Table A9.1. There was no association between male selection line and embryo gender (χ^2 = 1.72, d.f = 1, p = 0.19. There was no evidence of an interaction between male and female selection line determining embryo gender (Table A9.2).

Table A9.1. The counts and proportions of the gender assignment of embryos produced in the sperm competition experiment (Chapter 6). One hundred and sixty five embryos were assigned both paternity and gender.

Male Line	**Embryo Gender**	
	Males (proportion)	**Females (proportion)**
Long	58 (0.53)	51 (0.47)
Short	23 (0.41)	33 (0.59)

Table A9.2. Results of the GLMM analysing the effect of parental selection line on embryo gender. There was no effect of either male or female line on the gender of the embryo.

	Model estimate ± S.E	**z**	**p**
Female line	-0.201 ± 0.402	-0.499	0.617
Male line	-0.737 ± 0.478	-1.542	0.123
Male line * Female line	0.498 ± 0.685	0.727	0.467

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Appendix A10 – General morphology and sperm quality between the selection lines

As part of the data collection for the sperm motility analyses in Chapter 5 (for methods of the assay see Chapter 2 Section 2.4.1), two additional sperm quality assays and morphological data were collected (see below). This appendix investigates whether or not there are consistent differences between the three selection lines (long, intermediate and short) in general body morphology, and the proportion of viable sperm, and sperm with a normal morphology in a larger data set (compared to that of Chapter 6) that would have greater power to detect any differences.

Immediately prior to being euthanized, the following measurements were collected from each male zebra finch (Chapter 2 Section 2.3.4): body mass (to the nearest 0.01g) and the left tarsus length (a crude measure of skeletal size; Birkhead et al. 2006) to the nearest 0.01mm and bill colour (ordinal scale 0-6). During dissection, the left and right testes were removed, excess tissue and the epididymis removed, before being blotted dry and weighed to the nearest 0.001g. Sperm were obtained by dissection (Chapter 2 Section 2.3.4) for sperm quality assays (normal morphology and viability: Chapter 2 Section 2.4.3 - 2.4.4).

The effect of selection line on left tarsus length, body mass and testes mass were analysed using linear models (LMs). Selection line was a three level explanatory variable (long, intermediate or short). The mass of the left and right testes were added together to give a combined mass. Because testes mass may vary with body mass (e.g. across mammals – Kenagy & Trombulak 1986), body mass was included as a covariate in models examining the effect of selection line on testes mass (Garcia-Berthou 2001). The effect of selection line on bill colour was assessed using a Kruskal-Wallis test.

The effect of selection line on sperm swimming velocity (PC1) was assessed using LMs with male selection line as a fixed effect (see Chapter 5). The effect of selection line on the proportion of viable sperm and morphologically normal sperm was analysed using generalised linear models (GLMs), with a binomial error distribution and a logit link function, with male selection line as an explanatory variable. Data were modelled as the number of ‘successes’ or ‘failures’ (number of live sperm/number of dead sperm, and number of normal sperm/number of abnormal sperm) incorporated into the response variable ‘y’ that retains sample size information. This model was compared to the null model without the inclusion of selection line

using log-likelihood tests to obtain the significance of the male selection line effect. The strength and direction of associations between the proportion of viable sperm, the proportion of normal sperm and sperm swimming velocity were assessed using Pearson's correlations.

All statistical analyses were performed using R version 2.15.1 (R Development Core Team 2012) using the base package.

Results

Neither body mass nor bill colour was affected by male selection line (Table A10.1). Males from the S-line had a small but significant decrease in left tarsus length compared to the L- and I-line (Table A10.1). The combined testes mass (controlled for body mass) was also marginally significant, with lighter testes in the S-line compared to the L- and I-line (Table A10.1). There were no differences in the proportion of viable sperm, nor the proportion of sperm with normal morphology (either overall normality or in terms of abnormalities of specific sperm components) between the three selection lines (Table A10.2).

The proportion of viable sperm and sperm with normal morphology were significantly positively correlated (Table A10.3). PC1 (swimming velocity) was also correlated with the proportion of sperm with normal morphology, so faster swimming speeds were observed when there were more 'normal' sperm (Table A10.3). Abnormal morphology, especially a missing tail or bent midpiece would be expected to have detrimental effects on motility and sperm function.

Table A10.1. Results of models analysing the effect of selection line on four morphological traits in male zebra finches. Significant values ($p < 0.05$) are in bold.

Morphological measure	**Mean ± SD**			**F** [a,b,c] **X^2** [d]	**p**
	Long	**Intermediate**	**Short**		
Body mass (g)	17.11 ± 2.00	16.96 ± 2.13	17.75 ± 2.63	1.82[a]	0.166
Testes mass (g)	0.046 ± 0.012	0.045 ± 0.012	0.042 ± 0.012	-2.05[b]	**0.042**
Left tarsus (mm)	16.99 ± 0.53	16.78 ± 0.70	16.58 ± 0.66	5.01[c]	**0.008**
Bill colour	4.51 ± 0.58	4.38 ± 0.58	4.42 ± 0.53	1.08[d]	0.584

[a]the analysis was carried out using LM, d.f = 2,155.
[b]the analysis was carried out using LM, d.f = 3,154. Body mass was included in the model as a covariate.
[c]the analysis carried out using LM, d.f = 2,149.
[d]the analysis was carried out using a Kruskal-Wallis test, d.f = 2.

Table A10.2. Results of GLMs analysing the effect of selection line on the proportion of viable sperm and the proportion of sperm with normal morphology. Normality was assessed using all data, and then according to specific categories. Means were calculated from the raw data. There were no significant differences detected between the selection lines in any of the sperm quality parameters. See footnotes for details of sample sizes.

Sperm quality parameter	Mean ± S.D			Difference in deviance	p
	Long	Intermediate	Short		
Viability[1,2]	0.69 ± 0.17	0.67 ± 0.19	0.69 ± 0.17	-0.320	0.9963
Normality[1,3]	0.71 ± 0.11	0.69 ± 0.14	0.69 ± 0.15	-5.908	0.8766
Head[1]	0.92 ± 0.05	0.90 ± 0.10	0.90 ± 0.07	-12.258	0.6587
Midpiece[1]	0.89 ± 0.09	0.89 ± 0.06	0.91 ± 0.08	-0.383	0.2214
Tail[1]	0.85 ± 0.10	0.84 ± 0.11	0.82 ± 0.11	-8.335	0.8164
Head and midpiece[1]	0.96 ± 0.06	0.96 ± 0.05	0.97 ± 0.04	-14.432	0.5634
Head and tail[1]	0.99 ± 0.01	0.99 ± 0.02	0.99 ± 0.01	-0.268	0.9766
Midpiece and tail[1]	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.02	-8.002	0.2104
Production errors[1]	0.99 ± 0.05	0.98 ± 0.07	0.97 ± 0.08	-33.736	0.4437

[1]The significance of the effect of selection line was assessed from GLMs then using ANOVA comparing models with and without line. The differences in deviance and p values from this test are presented.

[2]Viability mean values are proportions. N = 144.

[3]Normal morphology mean values are proportions of overall normal morphology, and then of each specific category or combinations of categories. N = 148.

Table A10.3. Correlations between measures of sperm quality. Data for all males analysed in Chapter 5 with data for each parameter were used (n = 144). Significant correlations are in bold and ** indicates where p <0.01.

	Normality[1]	**PC1**
Viability[1]	**0.22****	0.13
Normality[1]	---	**0.25****

[1]Data are proportions and were arcsine transformed prior to analyses.

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Appendix A11 – Number of sperm and embryos unassigned to either potential sire

In Chapter 6, the results of the sperm competition experiment were described, where long- and short-sperm males competed to fertilise ova of females. The competitive success of each male was quantified by determining (i) the numbers of sperm reaching the egg and observed on the outer perivitelline layer (OPVL), and (ii) the numbers of embryos sired by each male. However, not all sperm could be assigned to a particular male, and the paternity of some embryos could not be determined. Here, the data regarding these unassigned sperm and embryos are presented for each clutch from the individual trios to demonstrate that there was no systematic bias resulting in the long-sperm advantage observed in Chapter 6.

Sperm proportions on the OPVL

The most frequent reason preventing sperm from being assigned to a particular male was that sperm were unable to be measured accurately. This occurred when the OPVL was very thick and opaque or when the sperm were obscurred by debris. The sperm tail is the most difficult component to measure, esecially the ends of the tails of the longest sperm. Erring on the side of caution, sperm were not assigned to a male unless clearly measured. However, hypothetically, it is likely that any bias introduced in the analysis due to the inclusion of unclear sperm would be in favour of the short sperm, as the whole tail would not be measured and the sperm may be assigned to the short-sperm male. This means that the magnitude of the long-sperm advantage in Chapter 6 would be conservative. It is also unlikely that debris obscurring sperm would cause bias to the calculated sperm proportions as obstruction of all sperm, regardless of length, would be random across the OPVL. Therefore, I am confident that the numbers of unassigned sperm shown in Table A11.1 did not affect the general conclusions presented in Chapter 6.

Unassigned embryos

There was some variation in the percentage of unassigned embryos across the dataset (Table A11.2), with some families having greater unassigned paternity than others. This may be due to insufficient genetic material collected from the tiny embryos and lower quality DNA due to the 48 h incubation time (part of the experimental methods: Chapter 6 Section 6.2). However, these issues were unavoidable because a longer incubation to obtain larger embryos would

have degraded the OPVL to such an extent that reliable sperm measurements would be impossible. In additon, prior to carrying out the paternity analysis (Chapter 6 Section 6.2), some loci did not amplify properly and contained null alleles, reducing the number of loci that could be used in the paternity analysis. Despite these slight difficulties, I am confident in the overall conclusion (that long-sperm outcompete short-sperm) because the results of the paternity dataset match the conclusions of the more statistically powerful sperm proportion dataset.

Table A11.1. Summary table of the numbers (N), range and percentage of unassigned sperm across 18 clutches of eggs produced in the sperm competition experiment.

Trio ID	Clutch	Number of eggs	Unassigned sperm across whole clutch		
			Range	N	%
100	1	6	1-5	7/102	6.9
104	1	7	1-5	17/78	21.8
105	1	9	1-8	15/53	28.3
105	2	7	1-12	35/119	29.4
106	1	4	4-15	19/126	15.1
106	2	6	4-7	21/204	10.3
108	2	5	1-14	26/151	17.2
110	1	8	2-31	85/416	20.4
110	2	6	2-6	26/231	11.3
112	1	7	3-9	26/139	18.7
112	2	8	1-8	28/256	10.9
113	2	6	3-9	32/171	18.7
114	1	4	3-23	42/219	19.2
114	2	6	10-23	97/300	32.3
115	1	5	2-6	16/70	22.9
115	2	2	1	1/13	7.7
120	1	6	1-23	71/319	22.3
120	2	6	1-3	12/116	10.3
123	1	7	1-4	14/83	16.9
124	1	6	1-2	4/98	4.1
124	2	7	1-6	11/125	8.8
125	2	3	2-29	56/229	24.4
128	1	10	3-13	46/334	13.8
128	2	12	1-19	72/358	20.1
130	1	8	1-4	19/228	8.3
130	2	8	3-11	38/195	19.5
132	1	6	2-12	45/258	17.4
132	2	8	1-11	28/168	16.7
134	1	2	0	0/6	0
134	2	7	2-14	51/215	23.7

Table A11.2. Summary of the numbers of embryos that were unable to have paternage assigned at ≥80% confidence.

Trio ID	Clutch	Embryos		
		Known paternity	Unknown paternity	% unknown
100	1	6	0	0
104	1	5	2	28.6
105	1	5	0	0
105	2	5	2	28.6
106	1	6	0	0
106	2	4	0	0
108	2	4	1	20.0
110	1	5	1	16.7
110	2	8	0	0
112	1	4	4	50.0
112	2	6	2	0.25
113	2	4	2	0.33
114	1	4	0	0
114	2	6	0	0
115	1	5	0	0
115	2	2	0	0
120	1	5	1	16.7
120	2	2	4	66.7
123	1	5	2	28.6
124	1	5	1	16.7
124	2	7	1	12.5
125	2	3	0	0
128	1	7	3	30.0
128	2	9	3	0.25
130	1	8	0	0
130	2	7	1	12.5
132	1	7	1	12.5
132	2	6	0	0
134	1	3	0	0
134	2	8	0	0