Sperm production, quality, and post-copulatory performance in Australian Estrildid finches

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Dedication

To my parents, who give me wings.

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General Abstract

Birds are obligately polyspermatic, and require multiple sperm to fuse with the ovum for successful fertilisation and embryonic development. Some optimal number of functionally competent sperm need to survive and traverse the oviduct to reach the ovum during the narrow fertilisation window; though the exact value of this optimal number remains unclear. The number of sperm that reach the ovum can be determined from the perivitelline layer (PVL) that surrounds the yolk. However, this assay has been underutilised to understand variation in post-copulatory performance of sperm within and between species. I have revealed considerable variation in the number of sperm trapped on the PVL within a species, and even across a clutch in three related species: Gouldian finch (*Erythrura gouldiae*), long-tailed finch (*Poephila acuticauda*), and zebra finch (*Taeniopygia guttata*). This variation is related to species and individual differences in post-copulatory regulation of ejaculate-female and sperm-egg interactions in response to different contexts. Therefore, in experimental examinations of two of these species, I tested how different ecological, physiological and phenotypic contexts impact sperm production, quality, and performance. In the two subspecies of long-tailed finch I demonstrated how post-copulatory constraint on the number of sperm reaching the ovum potentially limits their hybridization. In zebra finches, I showed how experience and reproductive success alter the number of sperm reaching the ovum, and how ejaculate traits (sperm morphology and performance) influenced these numbers. I characterised the change in ejaculate traits across a single reproductive cycle in relation to changes in testes and circulating testosterone, and ecologically relevant high ambient temperatures. This thesis demonstrates the value of measuring ejaculate traits in concert with the sperm trapped on the PVL as a research tool. My work provides evidence that reproductive context – ecological, physiological and phenotypic – impacts ejaculate traits and post-copulatory performance of sperm, in turn influencing reproductive success.

Declaration of originality and ethics

I certify that this thesis entitled "Sperm production, quality, and post-copulatory performance in Australian Estrildid finches" is the result of my own original work. Collaborative efforts are specifically indicated in the text. This work has not been submitted for a higher degree to any other university or institution other than Macquarie University.

In addition, I certify that all information sources and literature used are properly referenced in the thesis. All photos used in the thesis are my own.

The research presented in this thesis was approved by the Macquarie University Animal Ethics Committee. Animal Research Authority: 2013/028, 2013/029, 2014/025, 2015/028.

Laura L. Hurley

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Division of labour in co-authored articles

Contribution by collaborators and co-authors of the thesis, my own percent contribution in brackets. Collaborator key: CF —
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OC – Ondi Crino, SA – Samuel Andrew, SG – Simon Griffith.

	Chapter 1	Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Concept & Planning	LH (100%)	LH (70%), KF, SG	LH (70%), SG, OC	LH (90%), SG	LH (90%), SG, KB, MR	LH (90%), MR, SG	LH (100%)
Sample Collection	–	LH (85%), KF	LH (100%)	LH (100%)	LH (100%)	LH (100%), CM[*]	–
Sample Processing	–	LH (85%), KF	LH (100%)	LH (80%), MR		LH (50%), CF[**], CM	–
Data Analysis	–	LH (95%), SA, SG	LH (100%)	LH (100%)	LH (100%)	LH (80%), MR	–
Interpretation and Writing	LH (100%)	LH (75%), SG, KF, MR	LH (90%), SG	LH (85%), SG, MR	LH (90%), SG	LH (75%), MR, SG	LH (100%)

[*] Assisted in all collections
[**] Assisted CM in validating video processing.

Chapter One

General Introduction

Fertilisation and polyspermy

Successful fertilisation requires only a single sperm to reach and penetrate the ovum. In many taxa penetration of the egg by more than one sperm causes pathological polyspermy which can trigger embryo failure (Hasson and Stone 2009). However, some taxa show non-pathological polyspermy, in that multiple sperm-egg fusions is not lethal (Hasson and Stone 2009). For example, birds are obligately polyspermic, meaning that they require multiple sperm to fuse with the ovum for successful fertilisation and embryonic development to occur (Mizushima et al. 2008, Hemmings and Birkhead 2015, Ichikawa et al. 2016). This requires that a number of sperm be present at the time of fertilisation.

Sperm that are present at the time of ovulation either fuse with the inner perivitelline layer (PVL) which surrounds the ovum, or can be trapped by the outer PVL which forms within 15 minutes of ovulation as the ovum moves through the infundibulum (the upper portion of the oviduct) (Bobr et al. 1964, Bakst and Howarth 1977). The total number of both fused and trapped sperm are highly correlated with one another (Birkhead and Fletcher 1994, Birkhead et al. 1994), scale with ovum and bird body size, and vary in similar sized birds (Birkhead et al. 1994). This variation likely represents different levels of pre- and post-copulatory selection on sperm in a given species, since PVL sperm numbers can differ with life history and even across a clutch (Lifjeld et al. 2000, Johnsen et al. 2012). Investigation of sperm-egg interactions using the PVL as a tool has been around for some time (Wishart 1987), but has largely been underutilized in understanding fertilisation success in different contexts in which post-copulatory selection may differ (Hemmings et al. 2016).

Sperm morphology and post-copulatory selection

Pre- and post-copulatory selection in birds is often viewed as an evolutionary arms race between the sexes (Parker 1984, Snook 2005, Williamson et al. 2008, Hasson and Stone 2009, Simmons and Fitzpatrick 2012), with post-copulatory selection being one of the primary drivers for evolution in sperm traits (Pizzari and Parker 2009, Rowe et al. 2015a). In

particular, a common belief is that sperm competition will favour faster swimming sperm, which are presumed to mean longer sperm (Pizzari and Parker 2009). This theory had been generally well supported in male-male competition (Kleven et al. 2009, Lüpold et al. 2009, Bennison et al. 2015), and within a male ejaculate as well (Calhim et al. 2007, Immler et al. 2008, Mossman et al. 2009). However, recent work suggests that increased speed is not explained simply by increased overall length, but a more complicated relationship between the morphological composition of the sperm (length and ratios relations of the head, mid-piece, and flagellum) (Rowe et al. 2013, Hemmings et al. 2016) which can have evolutionary implications.

Changes in sperm morphology can lead to changes in reproductive compatibility in a population, which may contribute to speciation (Rice and Holland 1997). This has been observed in sister species of *Drosophila* (Price 1997, Manier et al. 2013a, Manier et al. 2013b), sympatric species of *Asterias* sea stars (Harper and Hart 2005), and in fish (McKenzie et al. 2017). Rate of divergence in sperm can vary due to differential selection pressures from inter-male sperm competition (Pizzari and Parker 2009), the female reproductive tract (Snook 2005), and potentially the environment (Reinhardt et al. 2015). This variation is evident in that some sister species of birds that have been divergent for long periods show little change in sperm morphology (Lifjeld et al. 2013), but newly divergent species can show significant changes (Hogner et al. 2013, Rowe et al. 2015b). Therefore, studying and understanding pre- (variation in male sperm phenotype) and post-copulatory selection (which sperm reach and fertilise the egg) is key to understanding the conditions for successful reproduction within a species (Birkhead and Pizzari 2002, Birkhead and Brillard 2007).

Research on post-copulatory selection in birds has mostly been done in domesticated poultry (e.g. Birkhead et al. 1999, Donoghue et al. 2003, Pizzari et al. 2007) and species found in northern temperate climates (e.g. Calhim et al. 2009, Cramer et al. 2014, Cramer et al. 2016). Life histories in northern temperate species can vary widely in term of ecology, migration, mate competition, mate fidelity, and parental investment, but for the most part the

general population of a given species all breed at the concurrently and at the same time of year (figure 1A) (Charmantier et al. 2008, Dawson 2008, Hahn et al. 2009, Ramenofsky 2011). More specifically, researchers have hypothesised that reproductive timing is dictated by various energetically demanding life history stages a species experience (i.e. breeding, moulting, migrating) (Jacobs and Wingfield 2000, Wingfield 2008, Ramenofsky 2011). Most variation in timing of reproduction across a species is due to latitudinal effects (i.e. timing of reproduction shifts with latitudinal differences in the primary breeding cue of day length and supplementary cues such as food availability) (Sanz 1997, Helm 2009, Ketterson et al. 2015). This narrow period of reproduction as a population makes it relatively easy to compare individuals in studies investigating aspects of post-copulatory selection such as spermatic maturation and sperm competition (e.g. Lüpold et al. 2012, Laskemoen et al. 2013).

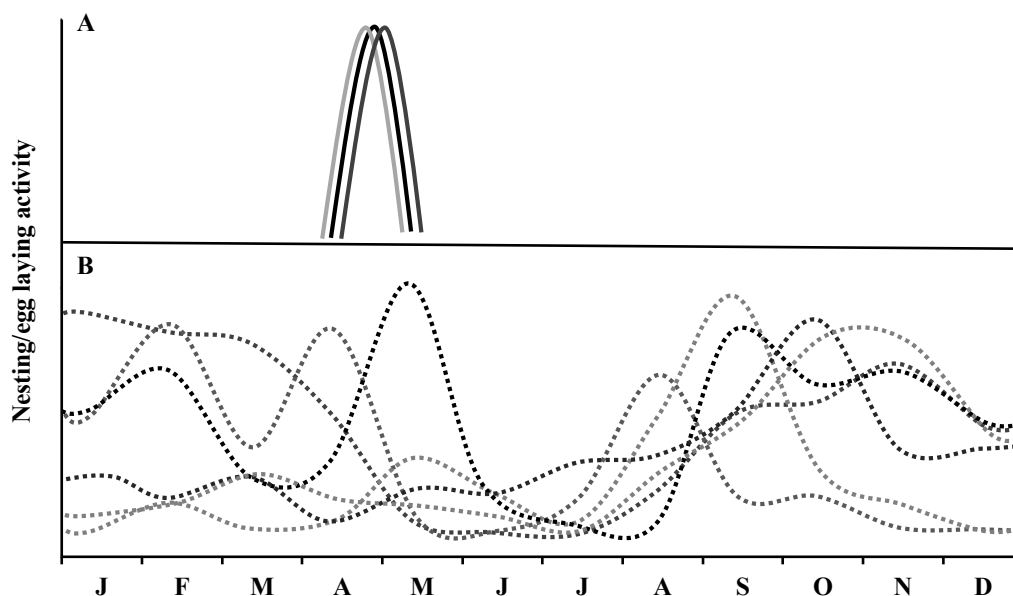**Figure 1.** Breeding activity in A) northern temperate species representative, represented by one population of great tits, *Parus major*, reflecting shift in mean egg laying date over 47 years (adapted from data in Charmantier et al. (2008) figure 1A), B) Australian representative species: six populations of zebra finch around Australia reflecting nesting percentage over one to six years (adapted from Zann (1996) figure 7.1).

Redefining the norm: Looking beyond the northern temperate zone

Comparatively little post-copulatory work has been done on species with a different breeding phenology than that typically seen in northern temperate birds (figure 1A), beyond some reproductive plasticity work on flexible breeders in the northern hemisphere (Hahn et al. 1997, Deviche et al. 2006, MacDougall-Shackleton et al. 2006). Most research on non-northern temperate species has occurred in captive zebra finches (*Taeniopygia guttata)*, under controlled conditions (e.g. Perfito et al. 2007, Birkhead 2010, Bennison et al. 2015, Hemmings et al. 2016). These birds have been domesticated for many generations, which is a concern since domestication could impact sperm morphology (Immler et al. 2012) and inbreeding can impact sperm performance and the proportion of normal sperm (Opatová et al. 2016). Studies of reproductive plasticity in wild zebra finches have been constrained in their data collection and interpretation through a lens shaped by knowledge of northern temperate bird species (e.g. Perfito et al. 2007). Beyond the zebra finch, some research on wild species examined variation in sperm traits in a few groups of African birds (Omotoriogun et al. 2016a, Omotoriogun et al. 2016b) and sperm competition in Australian Maluridae (Rowe and Pruett-Jones 2011, Rowe and Pruett-Jones 2013). Additionally, Australian Maluridae have distinct behavioural, morphological and physiological characteristics that indicate breeding condition (Rowe and Pruett-Jones 2011), which is not the case in all species. The limited amount of work done on non-temperate zone birds is a concern, because not all species demonstrate the predictable seasonal breeding phenology demonstrated in most northern temperate species.

A recent comprehensive study of bird species in Australia, showed that most bird species there breed opportunistically, not seasonally like northern temperate birds (figure 2: Duursma et al. 2017). In truly opportunistic breeding birds timing of reproduction can vary greatly, even with in the same species, with egg laying occurring over an extended period of time, with the possibility that some portion of the population continues breeding year-round, with some individuals producing multiple clutches in close succession or across the year (figure 1B) (Zann 1996, Dawson et al. 2001, Dawson 2008, Hahn et al. 2008). This is a

marked contrast to the more seasonal rigid season of northern temperate birds, with the difference in timing of reproduction classically explained that opportunistic breeders respond more to non-photic cues (i.e. rain and food) (Immelmann 1969, Zann et al. 1995, Dawson 2008, Hahn et al. 2008), while northern temperate species use photoperiod as their primary predictive cue (Dawson et al. 2001, Dawson and Sharp 2007).

The zebra finch is often described as the ultimate iconic opportunistic breeding model, opportunistic because a large portion of the population lives in unpredictable arid environments with reproduction that appears to be primarily responsive to rain cues (Zann et al. 1995, Zann 1996), and iconic since it is a common model species used in a broad range of research around the world (Griffith and Buchanan 2010). There are generally two overlooked but important points in the definition of truly opportunistic breeding birds: 1) there typically is some seasonality within these populations, it can just be highly flexible and variable within and between populations (Zann 1996, Perfito et al. 2007, Duursma et al. 2017), and 2) even if the general population is reproductively active, individuals in that population can be at different stages of reproduction from one another (Zann 1996), which is less common in northern temperate birds. In other words, in a given population not all birds are necessarily engaging in breeding all at the same time (figure 1B), a marked difference from the more reproductively constrained northern temperate birds (figure 1A). Consequently, when sampling a population at a single time point, different numbers of birds can be non-breeding, preparing to breed, nesting, laying eggs, incubating eggs, feeding chicks or fledglings, and the ratio of birds in these stages can vary widely with time of year.

Wild zebra finches are also known for their low level of extra-pair paternity (1.7% in terms of offspring), when the average for other socially monogamous species is *ca.* 15% (Griffith et al. 2010). However, other Australian Estrildid finches show similarly low rates; 12.8% in the Long-tailed Finch (van Rooij et al. 2016)); and 8.6% in the Gouldian Finch (Bolton et al. 2017). These rates can vary in captivity, as domesticated 'wild type' zebra finches have been found to have extra-pair paternity rates of 28% (Burley et al. 1996). It is

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expected this difference in extrapair paternity could significantly impact the rate of sperm competition (Parker 1984), potentially leading to different results in experiments investigating post-post copulatory selection. Taken together, this further suggests that greater consideration should be given and care taken when trying to extrapolate and interpret findings to make generalizations between species and species with different phenology.

There are important global differences in breeding patterns including opportunistic breeding in Australia (Duursma et al. 2017) and flexible breeding in areas such as Africa (Moreau 1950) and South America (Stouffer et al. 2013). These breeding strategies are more common than research studies coming out of northern temperate zones suggest (Dawson et al. 2001). Research investigating reproductive responses in other parts of the world should put aside the lens shaped by knowledge of northern hemisphere bird reproductive patterns. Therefore, it is necessary to establish non-northern temperate zone models in, a proper context, to understand the various aspects of opportunistic breeding phenology, and how it responds to different pressures such as hybridization and climate change.
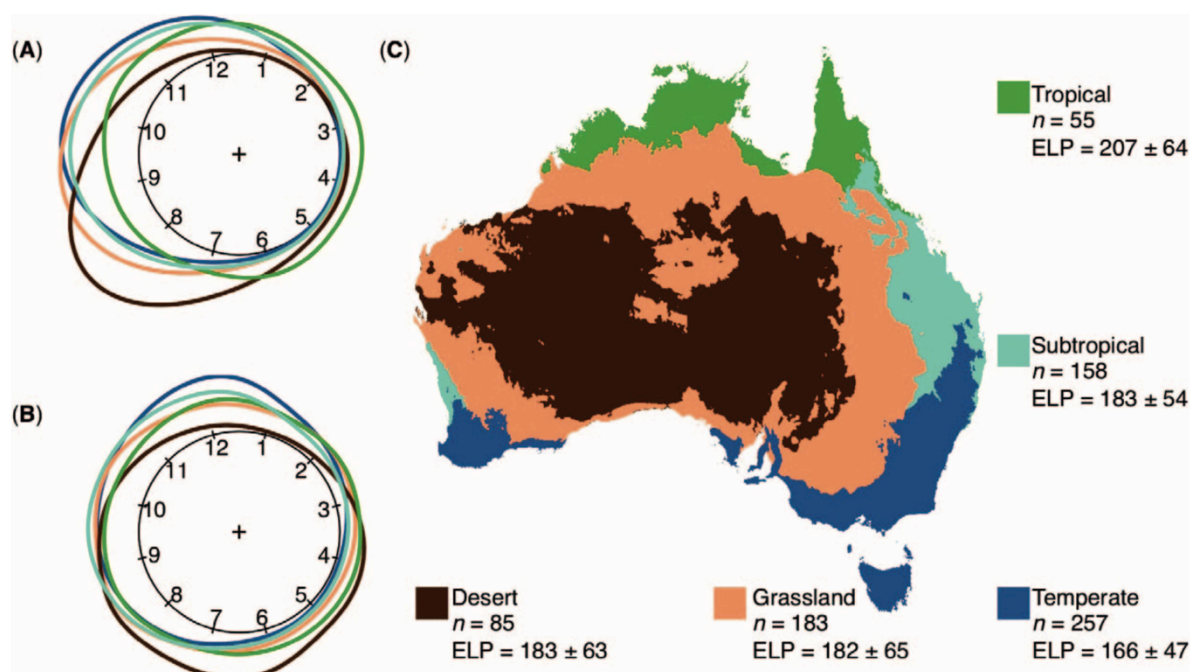
Figure 2. Timing of the length of the egg-laying period for 337 bird species within 5 Australian biomes. The egg-laying period (ELP) is the number of days between the 5th and 95th percentiles of all first-egg dates, and the confidence interval is across species in the biome. (A) Coloured lines are kernel density estimates of first-egg dates, and the numbers are the months of the year (e.g., January = 1, February = 2, etc.). (B) Coloured lines are kernel density estimates for the number of species laying eggs in each month within different biomes. (C) Map of Australian biomes and the number of species (n) and the mean egg-laying period for bird species with more than 50 observations (figure 3 from Duursma et al. 2017).

Aims and Thesis Outline

There are two main foci in this thesis. The first is an assessment of different contexts (i.e. species, hybridization, behavioural) in which post-copulatory sperm performance may change and impact the amount of sperm reaching the ovum and potentially fertility. The other is an examination of how behaviour, reproductive states, and the external environment are related to changes in sperm performance and morphology. Under the foci are several aims which take the form of specific questions. Below I describe how each chapter answers these questions. Some chapters present data that fall under both foci.

Focus one: An Assessment of how post-copulatory performance impacts the number of sperm reaching the ovum in different contexts.

Question 1: How does the number of sperm reaching the ovum differ when birds are of similar sized and in the same family of birds? Comparative work suggests that the number of sperm reaching the ovum in birds varies with body and ovum size, but there is stochastic variation in birds of similar size and same taxonomic family (Birkhead et al. 1994). Chapter Two aims to broadly assess the difference in the number of sperm that become trapped on the outer perivitelline layer (PVL) in three species of Australian Estrildid finch, and presents new data

on how similar species can vary in average number of sperm reaching the egg within a clutch, within pairs, and between pairs.

Question 2: To what extent is hybridization restricted by post-copulatory constraints? Research on post-copulatory constraint limiting hybridization between closely related sympatric or subspecies has been limited, with some studies only making conclusions based on outcomes of pairing (Veen et al. 2001, Veen et al. 2009), and others looking at sperm performance in heterospecific females (Cramer et al. 2014, Cramer et al. 2016). In Chapter Three, I assess the number of sperm reaching the ovum in different pairing combinations of the two subspecies of long tailed-finch, *Poephila acuticauda acuticauda* (yellow bill) and *P. a. hecki* (red bill), and F1 hybrids (orange bill) of the two subspecies, to investigate the potential impact of post-copulatory constraints on fertilisation success. The results reveal the long-tailed finch as an ideal model for studying divergence of sperm morphology and fertilisation success as a potential mechanism limiting hybridization.

Question 3: Does breeding experience and/or reproductive success alter post-copulatory selection of sperm? Previous experience with a partner has been shown to improve coordination between the pair and increase the number of offspring produced (Limmer and Becker 2010, Baran and Adkins-Regan 2014, Sanchez-Macouzet et al. 2014). Chapter Four investigates how a pair's breeding experience in and successfully rearing offspring as a pair, impacts post-copulatory selection on the number of sperm reaching the egg in zebra finches, *Taeniopygia guttata*.

Focus two: Determine if sperm performance and morphology change with context, and with reproductive state.

Question 4: Are differences in post-copulatory selection of sperm with experience and/or reproductive success related to variation in sperm morphology, quality, or performance?

Sperm morphology and ejaculate numbers can impact the number of sperm reaching the ovum (Bennison et al. 2015, Hemmings and Birkhead 2015, Hemmings et al. 2016). Chapter Four further investigates if sperm morphology, quality, and performance variation can explain any differences in post copulatory selection on the number of sperm reaching the ovum.

Question 5: How does reproductive organ development and output vary in an opportunistic breeder across a single reproductive cycle? Opportunistic breeding birds are categorized as species that are able to quickly respond to good periods in unpredictable environments (Hahn et al. 2008). The presumption is that they are able to quickly respond by keeping their gonads in a developed state, while more seasonal breeders tend to fully regress their gonads when they are not breeding (Dawson et al. 2001). One study of wild zebra finches, an opportunistic breeder, suggests that level of regression in these birds varies with habitat predictability (Perfito et al. 2007). Chapter Five describes how the gonads of male and female zebra finches change across a single breeding cycle, and how in males this is reflected in a change in sperm production and performance. This presents beneficial new data for interpreting reproductive patterns in a larger population when an individual's breeding condition is unknown.

Question 6: How do external environmental conditions impact sperm performance? Sperm performance is not only about genetics and morphology, but can be impacted by internal and external environmental factors, a fact that has largely ignored (Reinhardt et al. 2015). Chapter Six investigated the impact of ecologically relevant elevated temperatures on cloacal temperature and sperm morphology and motility in zebra finches, highlight the potential role of temperature in determining male fertility in birds. Given climate change induced increase in global temperatures, it is important to understand how high temperatures impact sperm performance.

Please note that the content presented in Chapter Two has been published in Auk, and Chapter Six is under review at a journal at the time of thesis submission. The formatting for these chapters has been changed minimally for aesthetics in the presentation of this thesis (i.e. figures have been married with their legends). Therefore, chapters use the following English spellings: Chapter Two – American, Chapter Six – British, and all other parts of the thesis – Australian. Additionally, other chapters are intended for submission for publication and citation style is formatted accordingly to the journal noted at the beginning of each chapter.

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Chapter Two

Variation within three species of estrildid finches in the number of sperm trapped on the perivitelline layer of the egg

Chapter Two Vignette

Physiological polyspermy, the regular and normal occurrence of multiple sperm penetration of the ovum, was first described as a normal part of avian fertilisation over a century ago (Harper 1904), but is still largely understudied and little understood. The variation in the number of sperm that reach and either penetrate or become trapped in the perivitelline layers (PVL) around the ovum has been described: 1) in a number of species, but based on a limited number of samples (Birkhead et al. 1994), and 2) in a handful of species in characterising variation across and within pairs (Johnsen et al. 2012; Lifjeld et al. 2000). In this chapter, I conduct a more in-depth investigation of how the number of sperm reaching the ovum in three Australian Estrildid finch species of approximately the same body size: Gouldian finch (*Erythrura gouldiae*), long-tailed finch (*Poephila acuticauda*), zebra finch (*Taeniopygia* guttata). In these species, I examine how the sperm that reaches the ovum varies in number across the laying order of a clutch, between pairs of the same species, and within pairs across multiple clutches. This is a descriptive chapter that provides a demonstration of the value in using the analysis of sperm trapped on the PVL as a research tool to study polyspermy by showing the natural variation in closely related species.

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RESEARCH ARTICLE

Variation within three species of estrildid finches in the number of sperm trapped on the perivitelline layer of the egg

Running head: Variation in sperm trapped on the perivitelline layer

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Abstract

Birds are physiologically polyspermic, with normal embryonic development following penetration of the inner perivitelline layer (PVL) of the ovum by multiple sperm. The PVL traps dozens to thousands of the sperm present at the time of fertilization, providing information about the number of sperm that traversed the females' reproductive tract and reached the ovum. Broadly, across avian species, the number of sperm detected on the PVL is positively related to ovum size and female body mass. However, relatively few studies have characterized the amount of variation that occurs within and between closely related species that are similar in size. Here, in three similar-sized species of estrildid finch, we characterize variation in the average number of sperm trapped by the PVL across the species, and between and within breeding pairs. The average number of PVL sperm changed significantly across the laying order in two of the species, and there was significant variation between breeding pairs in all three species. The variation in PVL sperm number was not always consistent within a pair across multiple breeding attempts (two species examined). Our data highlight the need to better understand the patterns and processes of selection that optimize and alter the number of sperm reaching the ovum within and across species.

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Introduction

Physiological polyspermy, the regular occurrence of multiple sperm penetrating the inner perivitelline layer (PVL) of the ovum, was first described as a normal part of avian fertilization over a century ago (Harper 1904). The presence of non-penetrating sperm caught on the PVL was later described (Bobr et al. 1964) and subsequently detailed to be specifically embedded in the outer PVL, which is laid down as the egg traverses the infundibulum (first part of oviduct) (Bain and Hall 1969; Bakst and Howarth 1977; Bellairs et al. 1963). The number of trapped sperm positively correlates with the number of sperm that have penetrated the ovum, and is a good proxy for the number of sperm present at the surface of the egg at the time of fertilization (Birkhead et al. 1994; Wishart 1997; Lifjeld et al. 2000). Although only a single sperm is required for successful fertilization more appear to be required for early embryo survival (Hemmings and Birkhead 2015; Mizushima et al. 2014). In studies of domesticated and wild birds the likelihood of fertilization of an ovum appears to drop significantly when the number of sperm trapped in the PVL fall below between about 30-200 (Birkhead and Fletcher 1998; Török et al. 2003; Wishart 1997). However, too many sperm can cause developmental failure (Christensen et al. 2005; Fechheimer 1981; Mizushima et al. 2008). Therefore, the number of sperm trapped on the PVL may reflect an optimal number of sperm that must be present at the time and site of fertilization to ensure normal development (Birkhead et al. 1993).

The excessive number of sperm intimately associated with successfully fertilized eggs may reflect the fact that the fertilization target area of the germinal disc is a very small target on the relatively large yolky avian egg. Consequently, it makes sense that the number of sperm on the PVL is positively related to the size of the ovum (Birkhead et al. 1994). In their inter-specific comparative study across 26 species of bird (that varied in size from Penduline Tit (*Remiz pendulinus*) to Greater Rhea (*Rhea americana*)), Birkhead et al. (1994) demonstrated that the average number of sperm trapped by the PVL varied from 29 to 164,000. However, whilst at a broader level the inter-specific variation in egg size (correlated with female body

size) certainly explains a lot of the variation in the number of trapped sperm between species, variation in across similar sized species remains to be thoroughly explained.

Intuitively it might seem that the number of sperm at the site of fertilization will be affected by the mating system and the intensity of sperm competition. In polygynous species, where females mate with multiple males and the risk of sperm competition is high, males should be favored by selection if they inseminate females with larger numbers of sperm (Parker 1984). However, it is interesting to note that in their comparative analysis, Birkhead et al. (1994) found the lowest average number of sperm per egg to be present in the Dunnock (*Prunella modularis*)*,* a species with a particularly high level of sperm competition (Birkhead et al. 1991; Burke et al. 1989). The low level of sperm present on the PVL of the polyandrous dunnock is also consistent with the idea that there may be costs of having too much sperm present at the site of fertilization. Although in polyandrous species there will be selection on males to inseminate relatively large amounts of sperm (to be competitive in sperm competition), females are likely to control either insemination, storage or transfer up to the point of fertilization, and filter out lots of sperm along the way. Thus, the overall number of sperm on the PVL in a species may therefore reflect the current resolution of a conflict between the two sexes (in species that are not completely monogamous) (Rice and Holland 1997), and each species may be at a different point of equilibrium (Hasson and Stone 2009; Morrow et al. 2002).

In addition to the variation across species, there is also likely to be variation within a single species. Whilst Birkhead et al (1994) provided an excellent overview of sperm numbers in relation to large-scale differences in body size, in most cases (20 out of 26 species), species were assayed through a sample of less than 10 eggs. Indeed, for only half of the species (13 of 26) were whole clutches sampled from more than one female and only one species had more than three clutches sampled (Birkhead et al. 1994). To date, relatively few (non-production) avian species have been characterized in relation to the variation that exists in the number of sperm found on the PVL between individuals, and across the laying order. In just four

passerine species have studies characterized variation across and within pairs and clutches with unrestricted mating: the Great Tit (*Parus major*), Blue Tit (*Cyanistes caeruleus*), Bluethroat (*Luscinia svecica*), and Tree Swallow (*Tachycineta bicolor*) (Johnsen et al. 2012; Lifjeld et al. 2000). In their study, Lifjeld et al. (2000) found that Great Tits have twice as many sperm present in their eggs in comparison to Blue Tits although the eggs are only 30% larger by volume. Additionally, the total number of sperm per egg across the laying order only varied significantly for Blue Tits, and not Great tits. However, for both species the number of sperm in eggs showed high variance within, compared to between clutches (Lifjeld et al. 2000). In an additional species the Zebra Finch (*Taeniopygia guttata*), sperm numbers in eggs were studied most extensively in pairs in which mating access was restricted and typically when they were housed as a single pair (Birkhead and Fletcher 1998; Birkhead et al. 1993). These studies found no difference in the number of PVL sperm per egg across a clutch in either domestic birds (singly housed) (Birkhead and Fletcher 1998; Birkhead et al. 1993), or wild-derived birds (the paper does not describe how these birds were housed) (Birkhead et al. 1993).

Here, we investigate PVL sperm variation in three estrildid finch species, all endemic to Australia, the Gouldian Finch (*Erythrura gouldiae*), Long-tailed Finch (*Poephila acuticauda*), and Zebra Finch. These species are of comparable body size, lay similar sized clutches (between 2 and 8, usually 4 to 6 eggs/clutch), form socially monogamous pair-bonds to breed, and demonstrate some degree of flexible breeding (Peter et al. 2006). We counted the sperm that were trapped on the outer PVL in these three species to characterize variation in sperm numbers across the laying order, both within and across pairs. This data was collected over several years, with the earliest sample processing (Gouldian Finch) mirroring the collection and quantification technique that focused on PVL sperm around the germinal disk (GD) (Birkhead et al. 2008). In the later work on the Long-tailed Finch and Zebra Finch, we adopted a more comprehensive approach and counted all sperm present on the whole PVL, while keeping a record of which were found in the GD region (merging the techniques used in

a range of previous work on songbirds by two groups: Birkhead et al. 2008; Birkhead et al. 1993; Birkhead et al. 1994; Johnsen et al. 2012; Lifjeld et al. 2000). The major goal of this paper is to provide the first comprehensive description of intraspecific variation in PVL sperm numbers of closely related species in order to highlight the variation that exists and stimulate future studies that address the relevance of that variation.

Methods

Bird Maintenance and Egg Collection

The main set of samples were collected from breeding pairs of each finch species using a forced-pair design in which a male and female were placed together in a cage and given the opportunity to breed. A small subset of samples from wild-derived Zebra Finch breeding socially in an aviary containing ~50 pairs were also collected (these individuals were either wild caught themselves, or the offspring or grand-offspring of wild caught individuals). All birds were provided with food and water *ad libitum*, as well as a nesting box and nesting material. Both the Gouldian and Long-tailed Finch occur in different forms, and due to the possibility of hetero-morphic (or subspecific) genetic incompatibility (Pryke and Griffith 2009), we restricted our work in these species to assortatively mated pairs only. A single clutch of eggs was collected from 12 pairs of Gouldians (seven black-headed pairs and five red-headed pairs). Two complete clutches of eggs were collected from 10 pairs of Long-tailed Finch (5 pairs of the yellow-billed sub-species *P. a. acuticauda*, and 5 pairs of the red billed sub-species *P. a. hecki*), and 17 pairs of domestic Zebra Finch. Lastly, a single clutch was collected from 4 pairs of wild-derived Zebra Finch. All pairs were unique, except for one male and one female Gouldian Finch that were each represented in two separate pairs. Each egg was collected on the day it was laid, weighed, and replaced with a dummy egg, until the clutch was determined to be complete (i.e. when females laid no further eggs for at least two consecutive days). Eggs were maintained at 4 °C and processed within a few weeks of laying (Gouldian Finch and Long-tailed Finch), or were frozen after collection (-20 °C and held for no more

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than a year) and then slowly thawed at 4 °C before processing (Zebra Finch). We froze these eggs for logistical reasons, as there was no time to process them fresh at the time they were collected due to other unrelated work being conducted at that time. We validated the protocol of freezing and thawing them by directly comparing some trial eggs from other pairs of Zebra Finch in a previous preliminary study. These eggs were either processed fresh or after freezing, and freezing did not appear to affect either the appearance or structure of the PVL or the sperm. Furthermore sperm numbers align with those previously reported (Table 1: Birkhead and Fletcher 1998; Birkhead et al. 1994), suggesting no reason for believing that freezing the eggs compromises the detection of sperm. The samples of the different species were collected over several years, with Gouldian Finch data being collected in 2010, Long-tailed Finch in 2013-2014, and Zebra Finch in early 2015. Although eggs were collected at different times, the average environmental conditions the birds experienced where not significantly different, and therefore, not expected to impact variation in sperm numbesr.

All work was conducted according to relevant national and international guidelines and was approved by the Macquarie University Animal Ethics Committee (Animal Research Authority 2010/054; 2013/28; 2013/29)

Egg and PVL Processing

Collection of the PVL and quantification of sperm followed previously described methods, which we adapted over the course of our study (Birkhead et al. 2008; Johnsen et al. 2012). In all cases, eggs were cut open with fine scissors and the shell and albumen discarded. The yolk was rinsed, and placed in a small weighing boat with the GD facing up. Collection of Gouldian Finch PVLs were processed as described in Birkhead et al. (2008) using a doughnut-shaped piece of filter paper (outer diameter = 6 mm, inner diameter = 3 mm) to aid in supporting the PVL while isolating and cutting out a section containing the GD. This section of PVL was then gently teased from the filter paper in PBS (Sigma, 0.01 M phosphate buffer,

0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C) before proceeding. Long-tailed Finch PVLs were processed in the same manner, but the non-GD portion of the PVL was also collected. Zebra Finch PVLs were cut approximately in half (following Johnsen et al. 2012), with the GD centered on one side of the PVL approximating the size of the 6mm diameter of the filter paper doughnut. This was easy to manage as once frozen, yolks remain semi-solid so do not deflate upon cutting the PVL as fresh yolks do.

The sections of PVL were gently washed in PBS to remove yolk, stretched flat on a microscope slide and stained with 15 µL of Hoechst 33342 fluorescent dye (Sigma, 5 µg/ml. Slides were incubated for 10 min in the dark before counting. Slides were viewed using a fluorescence microscope (Leica DM5000B) with a BP 340–380 excitation filter and a LP 425 suppression filter. The sperm on the PVL were counted by systematically scanning the slide at 200x. Independent of PVL handling method, in all species all stained sperm in the GD portion of the PVL were counted (hereafter, 'GD sperm'). In the Long-tailed Finch and Zebra Finch the sperm on the remainder of the PVL was also counted (hereafter, 'non-GD sperm') and added together to determine the total number of sperm.

We counted only the number of sperm trapped on the outer PVL, and not the number of holes in the inner PVL created when sperm penetrate. It has been demonstrated that the number of sperm trapped on the PVL is correlated with the number of holes on the inner PVL, so the PVL sperm represents an index of the total number of sperm present (Birkhead et al. 1994; Lifjeld et al. 2000).

Analysis

R (version 3.2.3) was used for analyses using *lme4* package (Bates et al. 2015). To analyze differences in a given PVL sperm number we ran generalized mixed models (GLMMs) using a Poisson distribution, summary z values are reported with p-value generated using package

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lmerTest (Kuznetsova et al. 2016). ANOVA p-values for regression coefficients were obtained using the car package which reports chi-square (X^2) with the p-value (Fox and Weisberg 2011). All models included at least egg number (in order of lay) as a fixed factor, and pair identity and row identity as a random factors. Row identity creates a unique identifier that is used for calculating residual variance (Nakagawa and Schielzeth 2013). For interspecies comparison of GD sperm species identity was added as a fixed factor. In Long-tailed Finch and Zebra Finch models, clutch was added as a fixed factor. In Long-tailed Finch and Gouldian finch, colour was added as a fixed factor to start, but was dropped from model if not significantly different. In these models the random effect of pair identity had random intercepts as well as slopes by using an interaction with the order effect of clutch (Nakagawa and Schielzeth 2013; Johnson 2014). Residuals were visually inspected for normality and homogeneity. Variation between eggs was assessed using pairwise comparisons using Tukey contrasts, but as consecutive change between eggs was of interest the main models were releveled to obtain p-value contrast to egg of interest. For each model the Intraclass correlation coefficient (ICC) for pair identity and the marginal R^2 for the fixed effects were calculated along with conditional R^2 of the total model, using the *MuMIn* package (Bartoń 2016). The results of the random factor are reported below as ICC. All ± are standard error of the mean (SEM) unless otherwise noted.

Results

Interspecific Variation in PVL Sperm Number

There was not a significant difference among species in the average number of sperm observed on the GD portion of the PVL (GD sperm; $X^2_2 = 0.80$, $p = 0.67$; Table 1). However, how GD sperm varied across the laying order did differ ($X^2_4 = 11.7$, $p = 0.02$). We did not have data on PVL Total sperm (sperm on the entire PVL) from the Gouldian Finch, but in the Zebra Finch and Long-tailed Finch we found a similar non-significant difference between the

species in total number of sperm per egg ($X^2_1 = 0.40$, $p = 0.52$).

Intraspecific Variation in PVL Sperm: Variation Within and Between Clutches

Gouldian Finch. 41 eggs from 10 clutches of 10 unique pairs were used in the analysis below. Originally, 53 eggs were processed from 12 whole clutches (11 females, one bred separately with two males). The female paired twice had no sperm on any of her eggs. Therefore, these two clutches (5 eggs in total) were removed from the subsequent analysis. Restricting analysis to only the first 5 eggs of a clutch removed a further 7 eggs from the analysis.

GD Sperm. The number of GD sperm per egg averaged 57.85 ± 10.58 (range, 0 to 295) (Table 1). Two clutches contained one egg with no GD sperm, but these occurred in different positions in the laying order. Overall, the number of GD sperm changes significantly over the clutch ($X^2_4 = 11.2$, $p = 0.02$) primarily due to an increase from egg 1 to 2 ($z = 3.03$, $p = 0.02$) followed by a decrease from egg 2 to 3 ($z = -2.21$, $p = 0.03$) (Figure 1). Random factor variation showed there was no consistent pattern of change in the number of GD sperm across the laying order between pairs (ICC = 0.58). This could be driven primarily by two pairs that had higher than average sperm counts (Figure 2A). These same two pairs also appeared to drive the high variance in the number of sperm per egg especially in their fifth egg which had more than twice the number of GD sperm than other eggs in the laying order, with GD sperm counts of 202 and 295 sperm in the fifth eggs (SEM = 50.24 vs. SEM ranging between 8.97 an 20.23 in first four eggs) (Figure 2A).

Long-tailed Finch. 90 eggs from 20 clutches, 2 clutches from each of 10 unique pairs, were used for the analysis below. There was no significant effect of head colour on number of sperm per egg (GD sperm: $X^2_1 = 1.18$, $p = 0.27$, total sperm: $X^2_1 = 0.31$, $p = 0.58$), and was therefore dropped form all analyses.

GD Sperm. The number of sperm on the PVL per egg averaged 64.13 ± 6.81 in the GD region (range 0 to 304, n = 81). The number of GD sperm per egg differed significantly across

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the clutch ($X^2_4 = 13.1$, $p = 0.01$), showing an increase from first to second egg ($z = 2.16$, $p = 0.03$) significantly decreasing again by egg 4 ($z = -2.86$, p < 0.01) (Figure 1). This pattern was common in most, but not all clutches (Figure 2B). Only two clutches, from separate pairs, contained eggs with no GD Sperm, but at different points in laying sequence. The number of GD sperm and non-GD sperm were significantly, positively correlated (r = 0.776, n = 86, $p = 0.001$: Figure 3A).

The variance in number of GD sperm across clutches, was mostly partitioned between pairs (ICC = 0.62), but within pairs there was not a significant change between the first and second clutch ($X^2_1 = 2.66$, $p = 0.10$). The difference between pairs appears to be largely driven by a few pairs (see below).

Total Sperm. The total number of sperm trapped on the PVL per egg averaged 134.96 ± 13.57 (ranging 0 to 527, n = 97), which varied significantly with egg number ($X^2_4 = 14.6$, p < 0.01). As with GD sperm, there was a significant increase from the first to second egg (z = 2.51, p = 0.01), which significantly decreased again with egg 4 (z = -2.55, $p = 0.01$), The total number of sperm did not differ within pairs ($X^2_1 = 1.09$, $p = 0.30$), with variance mostly divided between pairs (ICC = 0.68) (Figure 4). Relatively high numbers of sperm (pair 6) and relatively low numbers of sperm (pair 1 and 5) could be largely driving this difference between pairs (Figure 4).

Paired domestic Zebra Finch. 154 eggs from 34 clutches of eggs were used, 2 clutches each from 17 pairs, in the analysis below.

GD Sperm. The average number of GD sperm per egg was 51.10 ± 3.71 (range 1 – 234, n = 154), which positively correlated with non-GD sperm (r = 0.647, n = 102, $p = 0.000$: Figure 3B). The number of GD sperm did not differ significantly across the clutch ($X^2_4 = 3.74$, $p = 0.44$) (Figure 1), but there was significant variation in GD sperm numbers within pairs ($X^2_1 = 7.12$, $p < 0.01$). There was also a high degree of variation between pairs (ICC = 0.63).

Total Sperm. The average total number of sperm was 106.31 ± 6.54 (range, $3 - 489$, n $= 154$), and did not change significantly across the clutch ($X^2_1 = 3.63$, $p = 0.46$). However, there was a significant difference within pairs ($X^2_4 = 5.83$, $p = 0.02$). Variance between pairs was again high (ICC $= 0.61$) (Figure 5).

Social wild-derived Zebra Finch. 20 eggs from 4 clutches were used.

GD Sperm. In 20 eggs, produced by four pairs in a social setting, the average number of GD sperm was 65.6 ± 8.46 (range, $1 - 153$) (Table 1). Average number of GD sperm did not differ across clutch ($X^2_4 = 1.73$, $p = 0.79$), but did vary between pairs (ICC $= 0.60$).

Total Sperm. The average number of total sperm was 109 ± 14.20 (range, $3 - 228$), did not differ across clutch ($X^2_4 = 2.06$, $p = 0.73$), but did vary between pairs (ICC $= 0.52$) (Figure 5).

Comparison to paired domestic Zebra Finch. Although the sample size is low for the wild-derived Zebra Finch sample, there was no significant difference in the number of sperm between these and the domestic Zebra Finches as sampled above, in either the pattern of variation across a clutch in GD sperm numbers ($X^2_4 = 3.99$, $p = 0.40$) or total sperm numbers ($X^2_4 = 3.37$, $p = 0.49$), nor in average per egg PVL sperm in either the GD ($X^2_1 = 1.24$, $p = 0.26$) or total number of sperm ($X^2_1 = 0.14$, $p = 0.71$). The variance in GD and total number of sperm was lower when the wild-derived and domestic Zebra Finches were pooled (respectively: ICC $= 0.44$ and 0.40).

Discussion

We did not find significantly different numbers of sperm trapped in and around the germinal disc (i.e. GD sperm) of the PVL across the three species of estrildid finch. Despite there being no significant difference between species, on average Long-tailed Finches had 11% more GD sperm than Gouldian Finch, and 20% more than Zebra Finches. The pattern in the number of

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GD sperm per egg across the laying order did vary across species. The Gouldian Finch and Long-tailed Finch both exhibited a significant change in the number of PVL sperm across the laying order of a clutch, while there was no such pattern in the Zebra Finch. Furthermore, we found significant variation across and within pairs in our sample that are interesting and demand further explanation. Within pairs of Long-tailed Finch the number of sperm on the PVL had a reasonable level of consistency across two breeding attempts, but there was no such consistency in the Zebra Finch. These patterns held true in the Long-tailed Finch and Zebra Finch regardless if we looked at GD sperm alone or at the total number of sperm on the PVL. Given that the number of sperm present on the PVL seems likely to influence the fertilization success of eggs (Birkhead and Fletcher 1998), this variation may contribute to differences in the fitness of different individuals or pairs. Indeed, we found some pairs that had no sperm present on the PVL in some, or all of their eggs and these would have been infertile, and reduced the reproductive output of these pairs. Future work should focus on the factors that influence the variation in the number of sperm on the PVL of eggs both within and between pairs, and also across species. This work can be approached from a number of different angles, including how male and/or females and the environment act separately and in conjunction to influence sperm function.

One direct factor potentially contributing to variation in PVL sperm is the rate of pair copulations, as limited copulations may lead to declining sperm numbers across the clutch due to sperm loss (Birkhead and Fletcher 1998; Birkhead et al. 1993; Lifjeld et al. 2000). In our study birds were not restricted in any way from mating, and the highly variable number of sperm across the clutch suggests that sperm availability was not declining. However, it would certainly be interesting to explore the variation in the rate of copulation across pairs and species to determine the extent to which it may help to explain the variation that we have characterized in the number of sperm on the PVL.

Although we may expect that the rate of copulation would be influenced by the rate of genetic polyandry with in a species, there is no obvious direct link between the rate of genetic

polyandry in these species, and the number of sperm found on the PVL. All three species have relatively low rates of extra-pair paternity compared to the level seen in other socially monogamous passerines – *ca*. 15% (Griffith et al. 2002). The level of extrapair paternity in terms of offspring are 2% in the Zebra Finch (Griffith et al. 2010); 12.8% in the Long-tailed Finch (van Rooij et al. 2016)); and 8.6% in the Gouldian Finch (Bolton et al. 2017). Regardless, most of the pairs we studied were breeding in a context (single pairs to a cage) that prevented multiple matings. An obvious remaining question is the extent to which a different social context, and access to more matings, may affect the number of sperm on the PVL. In the relatively limited sample of Zebra Finch pairs that were breeding in a social context in an aviary, and in which the females may have mated with multiple partners, we found similar numbers of sperm on the PVL (to those in cages). However, more work is needed to evaluate how differential social situations may impact PVL sperm, especially in species with higher rates of extrapair paternity.

One example of how social situation may alter the number of sperm reaching the ovum comes from previous work on Gouldian Finch that found sperm morphology varies over time, with sperm length varying in relation to the social competitive context (Immler et al. 2010). Such morphological variation could alter the ability of one male's sperm to out compete a potential rival's sperm or a female's selection of a type of sperm, even in the absence of competition between multiple males (Immler et al. 2010; Pryke et al. 2010). Sperm morphology can change across ejaculates, and likely varies within a species depending on the amount of sperm competition faced (Birkhead and Immler 2007; Calhim et al. 2007; Immler et al. 2010). In perhaps the most intensively studied species, the Zebra Finch, it has been reported that the sperm morphology is consistent within an individual male (Birkhead and Fletcher 1995; Birkhead and Immler 2007; Birkhead et al. 2005). However, even then, the variation between males might affect the degree to which their sperm gets onto the PVL of the eggs laid by their partner. Two recent studies in Zebra Finch, have demonstrated this: First, Bennison et al. (2015) showed that longer sperm are more likely than short sperm to reach the

egg, which translated into a higher likelihood of paternity. Next, Hemmings et al. (2016) demonstrated that it is not just length, but the overall morphological composition of sperm that impacts the success of sperm in reaching the ovum. This may explain the variation in PVL sperm numbers we saw between pairs, but not the within pair variation of the Zebra Finches. More comprehensive studies are needed to understand such variation, and how and why the amount of sperm varies over the laying order of a clutch, as seen here in the Long-tailed Finch and the Gouldian Finch, and previously in the Blue Tit, Bluethroat and Tree Swallow (Table 1). Is it simply a by-product of the amount of sperm inseminated and varying copulation rates over the fertile period, changing sperm morphology, or does it reflect changes in male or female reproductive physiology?

Our study almost doubles the number of species for which a reasonable sample of eggs and clutches have been examined with respect to sperm numbers on the PVL (see Table 1). It is unwise to make too much from a comparison across this relatively low number of species, beyond the fact that the estimates within the different Zebra Finch studies are remarkably consistent, whilst those for both the Great Tit and Blue Tit vary a lot between studies / populations. The latter might reflect genuine spatial or temporal differences across populations, or might just represent sampling effects combined with a high variance amongst pairs, and provides an interesting target for further work. This is further supported by a comparison of the standard error estimates across the different species, estimated with roughly similar sample sizes (at both the egg and clutch level). This does suggest that perhaps the level of variance in the number of sperm varies across species, being particularly high in the Tree Swallow, and particularly low in the three estrildids.

Last, further research is needed into characterizing interactions between sperm and the environment (internal and external) (i.e. sperm ecology) as it could be interacting or impacting on factors discussed above. For example, some behavioral work has focused on the importance of behavioral compatibility of socially monogamous partners (Adkins-Regan and Tomaszycki 2007; Mariette and Griffith 2012), as well as perhaps the experience of mating

partners (Komdeur 1996; Ollason and Dunnet 1978), both which could influence pre- and post-copulatory sperm selection. Future studies of socially monogamous species should examine the extent to which the number of sperm on the PVL relate to behavioral measures of partner quality such duration of the pair bond, coordination over other activities such as nest building or parental care, and perhaps also traits such as courtship or nest building rituals that could serve to coordinate the pair physiologically at around the time of fertilization. Furthermore, sperm quality may also be affected by intrinsic qualities such as female or male age (Santos et al. 2013; Santos et al. 2015), extrinsic environmental factors such as heat (Ashizawa et al. 2010; Ashizawa and Sano 1990; Vallverdu-Coll et al. 2016), or a combination of both as with diet and toxins (Eid et al. 2006; Møller et al. 2014; Vallverdu-Coll et al. 2016). Future studies can either address these different ideas directly, or start narrowing down the likely possibilities by examining the extent to which variation in sperm numbers on the PVL is driven by males, females, or an interaction between the two. One of the key reasons for doing so is because between 3.8 - 24% of avian eggs fail to hatch (Spottiswoode and Møller 2004), and gaining a better understanding of fertilization processes is likely to play an important role in understanding the determinants of that failure. Further investigation of the number of PVL sperm are likely to provide additional insight into the mechanisms through which post-copulatory isolating mechanisms can occur both within and between species (i.e. Pryke et al. 2010; Veen et al. 2001). For example, assessing the number of sperm on the PVL after matings in different contexts (i.e. with con and heterospecific males), may help us to understand at which point cryptic female choice may be taking place. Is it upstream of the egg, such as through the biased selection of sperm for storage or through filtering along the reproductive tract, or is it largely the result of interactions between egg and sperm? Further work on the ability of males and females to optimize the number of sperm on the PVL, is also likely to provide new insight into the forms of selection that might affect the relative reproductive success of different pairs and individuals within a population, adding an important new component to the study of sexual selection.

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Table 1.

Summary statistics for sperm numbers on the perivitelline layer (PVL) of the first 5 eggs in clutches from Gouldian Finch, Long-tailed Finch, and Zebra Finch (domestic paired and wild-derived social breeding), and similar data (where applicable) from other species in the literature (references: a) this study; b) Birkhead et al. (1993); c) Johnsen et al. (2012); d) Lifjeld et al. (2000)). All report total sperm on the outer PVL, except Lifjeld et al. (2000) whose total also includes holes in the inner PVL in partial (~3 eggs/clutch) and full clutches).

| Species | Number of eggs (clutches) | mean number per egg ± SE | | | Change across order of lay | Ref |
		GD sperm (range)	non-GD sperm (range)	Total sperm (range)		
Gouldian Finch	41 (10)	57.10±10.57 (0-295)	-	-	Y	a
Long-tailed Finch	90 (20)	64.13±6.81 (0-304)	70.82±7.59 (0-350)	134.96±13.57 (0-527)	Y	a
Zebra Finch domestic (paired)	159 (34)	51.10 ± 3.71 (1-234)	55.21±3.26 (1-262)	106.31±6.54 (3-489)	N	a
Zebra Finch wild derived (social)	20(4)	65.6 ± 8.46 (1-153)	43.4 ± 7.41 (2-126)	109 ± 14.2 (3-228)	N	a
Zebra Finch domestic (paired)	< 48(8)	-	-	87.4 ± 28.9	N	b
Zebra Finch wild derived (unknown)	< 50(10)	-	-	119.9 ± 43.6	N	b
Bluethroat	68(12)	-	-	72.4 ± 109.3 (5–826)	Y	c
Tree Swallow	71(17)	-	-	358.5 ± 543.7 (13–4283)	Y	c
Blue Tit	103(10)	-	-	244.9 ± 128.5 (23-711)	N	c
Blue Tit (partial clutch)	35(13)	-	-	185 ± 21	-	d
Blue Tit (full clutch)	72(8)	-	-	128 ± 15	Y	d
Great Tit (partial clutch)	52(21)	-	-	293 ± 35	-	d
Great Tit (full clutch)	35(4)	-	-	407 ± 107	N	d

Figure 1
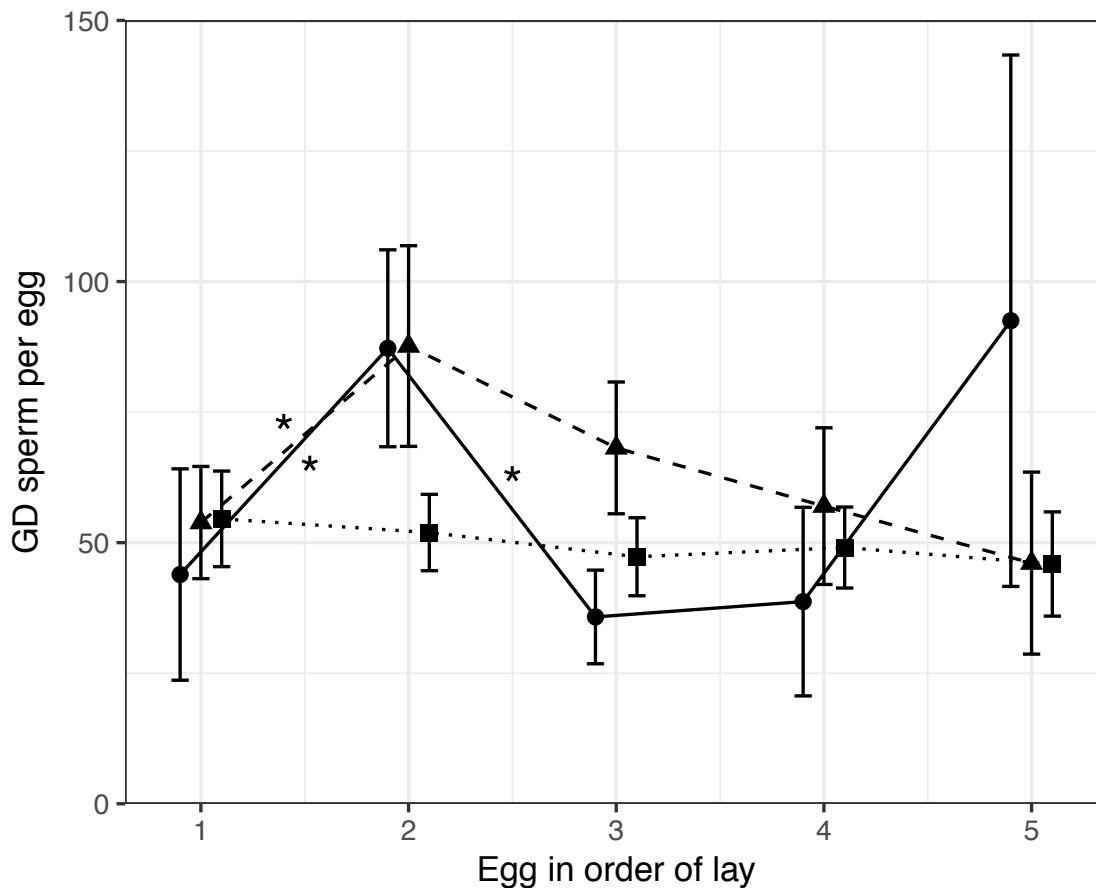
Figure 1. The average number of sperm per egg on the perivitelline layer (PVL) in the germinal disk region (GD sperm) across the first 5 eggs in a clutch, (circle-solid line) Gouldian Finch, (triangle- dashed line) Long–tailed Finch, (square-dotted line) Zebra Finch. Points are shifted over egg number to clarify overlapping points. The mean and confidence intervals were constructed on square root scale, but converted back to original units for plotting. Therefore, errors= 95% confidence interval on the square root scale calculated cell by cell. Asterisk (*) next to lines denotes significant change in average GD sperm numbers between sequential eggs in given species.

Figure 2
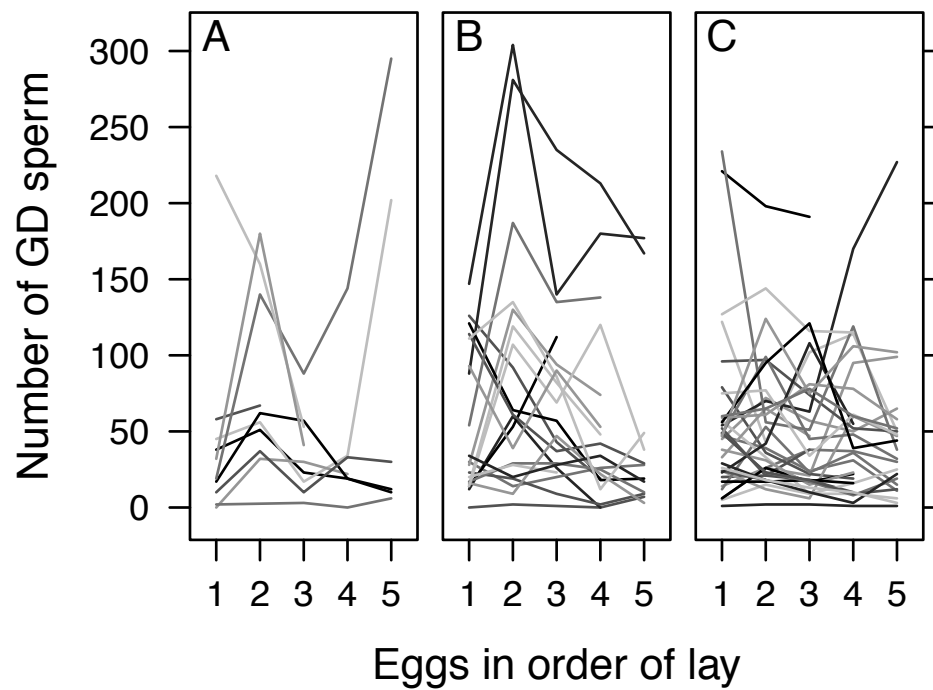
Figure 2. The number of sperm trapped in the perivitelline layers (PVL) in the germinal disk region (GD sperm), across the laying order in clutches of (**A**) Gouldian Finch, (**B**) Long–tailed Finch, (**C**) Zebra Finch. The first five eggs only are illustrated, with each line representing a unique clutch.

Figure 3
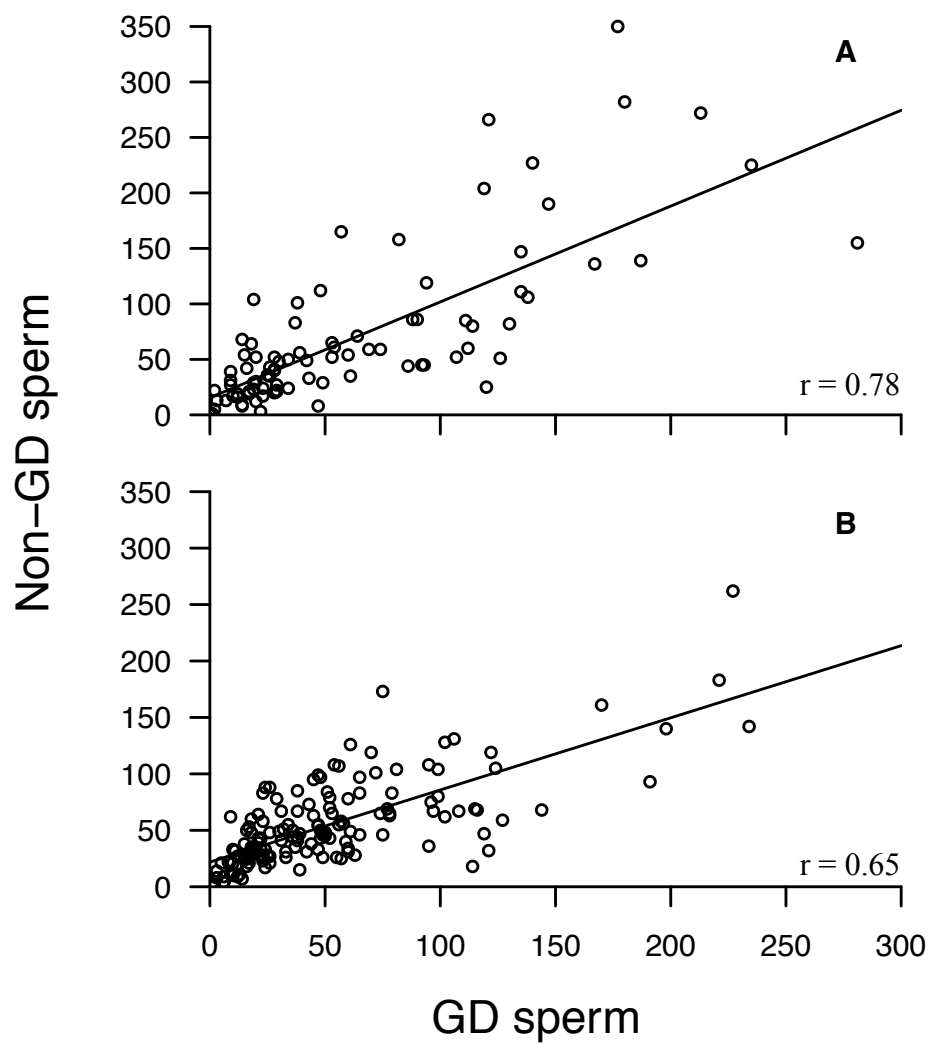
Figure 3. The relationship between the number of sperm trapped on the perivitelline layers (PVL) in the area of the germinal disk (GD) and those in other parts (non-GD) of the PVL in the same egg in (**A**) Long–tailed Finch, (**B**) Zebra Finch.

Figure 4
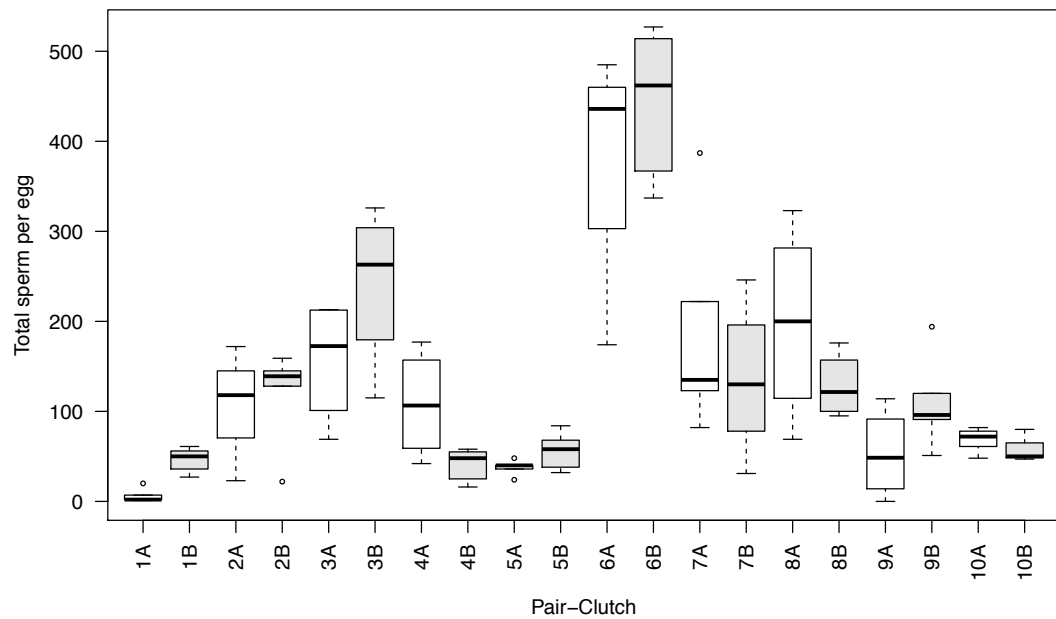
Figure 4. Boxplot distribution of total sperm per egg across two clutches of eggs from 10 pairs of Long-tailed Finch. Each pair's clutch is represented by: clutch 1 = #A and white box, clutch 2 = #B and grey box. Whiskers represent scores outside middle 50% quartiles, points represent outliers 1.5 times the interquartile range above the upper quartile and below the lower quartile range.

Figure 5
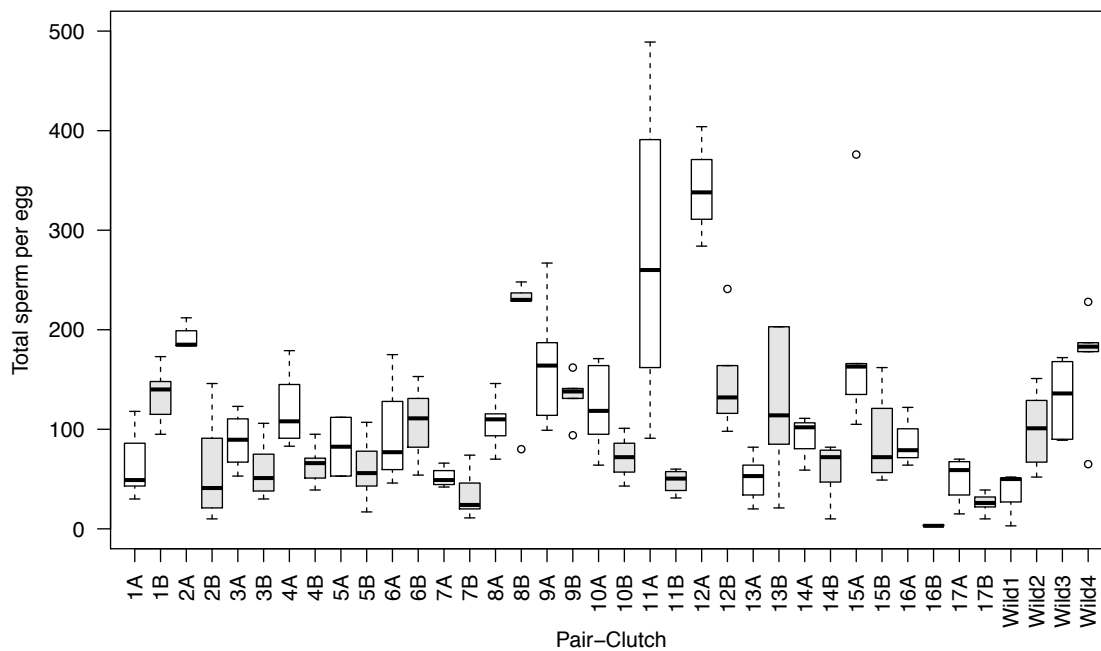
Figure 5. Boxplot distribution of total sperm per egg across two clutches of eggs from 17 domestic pairs and one clutch each from 4 pairs of wild-derived Zebra Finch (denoted: Wild#). Each domestic pair's clutch is represented by: clutch 1 = #A and white box, clutch 2 = #B and grey box. Whiskers represent scores outside middle 50% quartiles, points represent outliers 1.5 times the interquartile range above the upper quartile and below the lower quartile range.

Chapter Three

Post-copulatory constraints to hybridisation across the two

subspecies of the long-tailed finch

Chapter Three Vignette

After a general exploration of variation in the number of sperm reaching the ovum in three different Australian Estrildid finch species, I now focus on just one to investigate what perivitelline layer sperm numbers can tell us about post-copulatory constraint on hybridisation. The long tailed-finch, *Poephila acuticauda* diverged into two subpopulations 0.3 (Jennings & Edwards 2005) to 0.57 Ma (Singhal et al. 2015): yellow billed *P. a. acuticauda* in the west, and red billed *P. a. hecki* in the east. This has led to a significant divergence in their sperm morphology (Rowe et al. 2015). However, these populations have since come into contact again 21 to 24 kya (Reeves et al. 2013a, b, Fitzimmons et al. 2013), forming a narrow hybridization zone where orange billed individuals persist (figure 1: Griffith & Hooper 2017). This zone has remained narrower than would be predicted by neutral diffusion with natal dispersal (Griffith & Hooper 2017). Therefore, this chapter investigates if post-copulatory pre-zygotic sperm selection impacts the number of sperm reaching the ovum in different pairings of the three bill colours, possibly constraining hybridisation by limiting reproductive success.
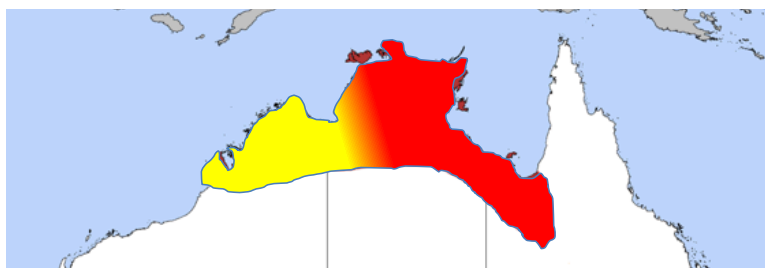
Figure 1. Approximate distribution in northern Australia of long-tailed finch by bill colour: yellow billed *P. a. acuticauda* in the west, and red billed *P. a. hecki* in the east, and orange billed hybridization zone (approximated from Higgins et al. 2006, Griffith & Hooper 2017).

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RESEARCH ARTICLE

Post-copulatory constraints to hybridisation across the two subspecies of the long-tailed finch

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Running title: Post-copulatory constraint to avian hybridisation

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Abstract

Most work on the driving forces behind speciation in internal fertilizing vertebrates has focused on pre-copulatory barriers to hybridisation. Substantially fewer studies have investigated post-copulatory mechanisms that impact fertilization success across species barriers. Here we used various pairings of the two subspecies of long tailed-finch, *Poephila acuticauda acuticauda* (yellow bill) and *P. a. hecki* (red bill), and F1 hybrids (orange bill), to investigate the potential impact of post-copulatory constraints on fertilisation success. We utilised the fact that in birds a portion of sperm that reach the ovum are trapped by the outer perivitelline layer (PVL) around the ovum, are highly correlated with and therefore represent the number of sperm that arrive at the site of fertilisation. We found no significant differences in the number of sperm trapped in pure red or yellow pairings, but a significant and asymmetrical difference when the pure birds were hybridised. Fewer sperm from yellow males reached red female ova compared to the opposite mixed pairing or pure pairs. Further, regardless of pairing type, backcrossed pairs (F1 orange birds paired with red or yellow individuals) always had less sperm on the PVL and therefore reaching the ovum. Interestingly, in crosses between two F1 orange hybrids PVL sperm numbers were equivalent to those in pure red or yellow pairs. None of the significant differences that we observed in sperm numbers on the egg membranes were explained by differences in egg measures (egg mass, yolk mass or egg volume). Our study demonstrates that the long-tailed finch is an ideal model for studying divergence of sperm morphology and fertilisation success as a potential mechanism limiting hybridisation.

Introduction

The speciation process often starts with divergence between two populations, often due to geographic isolation (Winker, 2010). When there is a sufficient level of divergence between forms, a variety of mechanisms will be under selective pressure to maintain a reproductive barrier between them. There has been much research on changes in the traits that isolate species before hetereospecific pairs mate (pre-copulatory isolating mechanisms). For example, in closely related bird species there are often clear differences in colouration (Greig et al., 2015, Veen et al., 2009) and song (Greig et al., 2015, Zann, 1976). There has been less focus on post-copulatory isolating mechanisms, particularly in birds, although sperm is one of the most variable cell types known, with trait selection coming from both pre- and post-copulatory responses (Pitnick et al., 2009a). The rate of divergence in sperm can be variable, rapid or slow; this is likely due to differential selection pressures from inter-male sperm competition (Rowe et al., 2015a, Pizzari & Parker, 2009), the female reproductive tract (Snook, 2005), and potentially the environment (Reinhardt et al., 2015). This variation is evident in that some sister species that have been divergent for long periods show little change in sperm morphology (Lifjeld et al., 2013), but newly divergent species can show significant changes (Rowe et al., 2015b, Hogner et al., 2013).

Changes in reproductive compatibility between populations may contribute to speciation (Rice & Holland, 1997), which can be seen by looking at compatibility between sister species of various taxa. In sister species of *Drosophila* females show conspecific over heterospecific precedence influences sperm's fertilisation success (Manier et al., 2013a, Manier et al., 2013b, Price, 1997). Sympatric species of *Asterias* sea stars show differential preference for conspecific over heterospecific sperm (Harper & Hart, 2005). Further, a recent study in subspecies of Atlantic killifish, *Fundulus heteroclitus*, demonstrated that while fertilisation did not differ, hatching success and developmental rates differed depending on the direction of the cross (McKenzie et al., 2017).

Despite the divergence seen in sperm morphology in birds (Hogner et al., 2013, Rowe et al., 2015b), relatively little work has investigated the differential use of conspecific and heterospecific sperm in this taxon. Mate choice and paternity studies of sympatric species of *Ficedula* flycatchers in their contact zone (Veen et al., 2001, Veen et al., 2009) and sympatric colour morphs of Gouldian finch (Pryke et al., 2010) suggest that there are postcopulatory processes in place favouring males of the same genetic background as the female. If females are mediating sperm competition (between different types of male) this can occur at any point after copulation and before the formation of the zygote (Birkhead & Brillard, 2007, Pitnick et al., 2009b). In closely related bird species, early selection in the vagina does not appear to be a limiting factor, as heterospecific vaginal fluids do not significantly impact sperm performance (Cramer et al., 2014, Cramer et al., 2016). In more divergent avian species, there can be unidirectional or asymmetric hybridisation (e.g. canary X bullfinch is typically only successful if the male is the canary) (Birkhead & Van Balen, 2007), suggesting that selection occurs in storage preference of sperm similar to that seen in the *Drosophila* (Price, 1997). This selection would directly impact the sperm that is available for fertilisation, which can be investigated by looking at which sperm reaches and penetrates the ovum (Bennison et al., 2015, Hemmings et al., 2016).

Birds are obligately polyspermatic, requiring more than one sperm to penetrate the inner perivitelline layer (PVL) to ensure successful development of embryos (Hemmings & Birkhead, 2015). Therefore, a number of sperm must be present in the 15-minute window between ovulation and the outer PVL formation around the ovum (Sasanami et al., 2013), which traps any unfused sperm (Bakst & Howarth, 1977, Bobr et al., 1964). To best coordinate sperm number and availability, female birds hold sperm in sperm storage tubules (SSTs) (reviewed in Sasanami et al., 2013). SSTs sperm uptake selection remains poorly understood. The intrinsic motility (Froman, 2003) and morphology (Briskie & Montgomerie, 1993, Pitnick et al., 2009a) of the sperm likely play a large role in determining which sperm are stored and reach and fertilise the ovum. The latter can be determined by looking at which

sperm are trapped by the PVL (Hemmings et al., 2016, Bennison et al., 2015, Birkhead et al., 1999).

To investigate the potential limiting effects of variation in sperm morphology on maintenance of subspecies, we examined the impact of hybridisation on the number of sperm reaching to ovum in the long-tailed finch, *Poephila acuticauda*. These are an Estrildid finch found in the Northern savannah of Australia, which occur in two divergent phenotypic subspecies: yellow billed *P. a. acuticauda* in the west, and red billed *P. a. hecki* in the east (Higgins et al., 2006). Divergence between these subspecies likely occurred during the aridification of Australian continent as recently as 0.3 Ma (Jennings & Edwards, 2005) up to 0.52-0.57 Ma (Singhal et al., 2015), with a secondary reconnect occurring 21 to 14 kya ago after the Last Glacial maximum when climate conditions warmed and the monsoon was re-established (Reeves et al., 2013a, Reeves et al., 2013b, Fitzsimmons et al., 2013). This has created a central hybridisation zone where orange billed individuals are present (Griffith & Hooper, 2017). The two subspecies populations were apparently separated long enough for significant change in sperm morphology to have arisen (Rowe et al., 2015b). However, the hybridisation zone has remained narrow (~150km) and more geographically abrupt in transition than one would predict with neutral diffusion over generations given their natal dispersal distance (based on bill colour admixture: Griffith & Hooper, 2017). This suggests that selection is occurring to maintain the two distinct subspecies. Unlike other species that have been the focus of similar work (Cramer et al., 2016, Lifjeld et al., 2013, Hogner et al., 2013), long-tailed finches breed easily in nest boxes in captivity, which makes them an ideal system to test how well sperm from one subspecies is able to fertilise the eggs of another. We therefore tested for differences in the total number of sperm on the PVL membrane between pairs of pure colours (red with red or yellow with yellow), mixed crosses between the two subspecies (one red and one yellow individual), backcrossed (one F1 hybrid orange with a red or yellow individual), and orange (pairing of hybrid individuals) pairings to see if postcopulatory mechanisms are impacting the maintenance of the narrow hybridisation zone.

Materials and methods

Study System and set-up

42 breeding pairs of long-tailed finches were formed using 26 females and 24 males over 7 months, between February-June and September-October 2014. Individuals were wild-caught or F1 generation pure colour (red or yellow) subspecies or F1 pure hybrid (henceforth orange: produced from red and yellow pairing). Pairs were placed alone in an outdoor aviary (4.1 x 1.85 x 2.24 m) with four nest boxes, and provided with nesting material and *ad libitum* food and water, with daily supplemental foods (green pea mixtures and Queensland fly pupae, *Bactrocera tryoni*) to encourage breeding.

Four categories of pairs were formed: pure colour (red only or yellow only pairs), mixed (one red and one yellow individual), backcrossed (one orange with a red or yellow individual), and orange (pairing of hybrid individuals). Care was taken to ensure no inbreeding. The mixed and backcrossed pair types were balanced so that each sex was represented by each colour type. For example, for we tried to set up a red female-yellow male mixed pair for every yellow female-red male mixed pair. Therefore, we ended up with 9 colour pairing types (female colour listed first, final number of pairs): red-red (n=5), yellow-yellow (n=6), yellow-red (n=5), red-yellow (n=6), orange-yellow (n=3), yellow-orange (n=3), orange-red (n=5), red-orange (n=6), orange-orange (n=3). All colour pairings are known to produce viable young (L.L. Hurley, unpublished observation).

All work was conducted according to relevant national and international guidelines and was approved by the Macquarie University Animal Ethics Committee (Animal Research Authority 2013/28).

Egg collection and PVL collection and processing

Pairs were allowed to produce two clutches of eggs. Eggs were collected on the day they were laid and replaced with a dummy egg. Eggs were maintained at 4ºC until the clutch was

complete. Eggs were then either processed within a few weeks (first half of study), or were frozen and then slowly thawed at 4ºC before processing (second half of study). The freezing process was validated prior to implementation, and does not impact the results of sperm counts.

Analysis of sperm on the outer perivitelline layer (PVL) followed previously described methods (Birkhead et al., 2008, Johnsen et al., 2012). In all cases, eggs were measured – length and width of all eggs to the nearest 0.1 mm – then cut open with fine scissors and the shell and albumen discarded. The yolk was rinsed, dried across filter paper, and cut approximately in half with the germinal disc (GD) centred on one half. The PVL was removed and gently cleaned of yolk using a hair loop in PBS (Sigma, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25°C).

Both halves of PVL where stretched flat on a microscope slide and stained with 15 µL of Hoechst 33342 fluorescent dye (Sigma, 5 µg/ml), and incubated for 10 minutes in the dark before reading. The sperm on the PVL were counted by systematically scanning the slide at 200X using a fluorescence microscope (Leica DM5000 B) with a BP 340–380 excitation filter and a LP 425 suppression filter. The number of sperm on each half were counted and summed for the total number of sperm. We counted only the number of sperm trapped on the outer PVL, and not the number of holes in the inner layer created when sperm penetrate. It has been demonstrated that the number of sperm on the outer membrane is correlated with the number of holes on the inner membrane, so taken alone the number of outer membrane sperm represent an index of the total number of sperm present at fertilisation (Birkhead et al., 1994, Lifjeld et al., 2000).

Data analysis

We first did a preliminary analysis of the four categories of pairs to look at overall differences in number of eggs laid, egg mass, egg volume, yolk mass, and total PVL sperm separately between group types. Then we looked at the specific influence of the pairing types on these

same measures. To test fixed effects, we began with a two-way interaction with category or pair type with clutch number. Egg number in order of lay was included as a covariate to look at variation across clutch. We removed the interaction if it was not significant. Models contained a random intercept for male and female identity (to account for the use of some individuals in multiple pair types) and pair identity. To correct for over dispersion in total sperm on PVL membrane, data row number was also added as a random factor. Count data (PVL sperm and number of eggs laid) were run in Poisson log link models.

All statistical analysis was performed using R (version 3.3.3; R Core Team, 2017) *lme4* package (Bates et al., 2015) with significance estimate via *lmerTest* (Kuznetsova et al., 2016). Poisson model ANOVA p-values were obtained using the *car* package which reports chi-square (X^2) with the p-value (Fox & Weisberg, 2011). Post hoc testing was done by iteratively changing reference levels for variables with significant interactions or main effects. To establish how strongly measures between group vary we calculated coefficient of variation (CV) for each response variable visually examining overlap of confidence intervals (CI) we calculated using 1000 bootstrap samples and corrected for bias. Figures were constructed using ggplot2 (Wickham, 2009), and modelling assumptions (normality and heterogeneity of variance of residuals) were assessed visually (following Zuur et al., 2009). All tests were two-tailed and considered significant at $\alpha < 0.05$. Data presented are mean ± standard error or 95% CI for most values.

Results

PVL sperm numbers

Overall total PVL sperm by category (e.g. pure, mixed, backcross, orange) did not change significantly between the first and second clutch ($X^2_1 = 0.38$, p = 0.54), but PVL sperm across the laying order did ($X^2_6 = 34.4$, p < .001). Pure (z = 1.98, p = 0.05) and mixed (z = 2.24, p = 0.03) pairs had significantly more total PVL sperm on average per egg than backcrossed pairs

(figure 1), with no significant difference between other groups. Although backcrosses had the highest coefficient of variance (CV = 1.09, *95% CI* = 0.87 to 1.37) it only was significantly higher than orange x orange crosses (CV = 0.60, *95% CI* = 0.47 to 0.78).

Within pairs, only orange-yellow pair types (female colour always listed first) showed a significant difference in the average total PVL sperm per egg in clutch 1 to clutch 2 ($z = -3.96$, $p < 0.001$), but all except orange-red backcross pairs and red-red pairs showed a declining trend between clutches (figure 2). Contrast of average total PVL sperm per egg by colour pairing types (figure 2) revealed a significant difference between mixed types, with red-yellow pairs having less sperm in both clutch 1 ($z = -2.00$, $p = 0.05$) and clutch 2 ($z = -2.24$, $p = 0.03$) than yellow-red pairs. There was no significant difference in average PVL sperm per egg between pure colour pairs in clutch 1 ($z = -0.12$, $p = 0.90$) or clutch 2 ($z = -0.07$, $p = 0.95$). Orange-red pairs had less sperm than orange-yellow pairs in clutch 1 ($z = 2.41$, $p = 0.02$) and this was the only difference between backcross pairs average PVL sperm per egg. Red-orange pairs also had significantly higher variance then all other pair types (CV = 1.62, *95% CI* = 1.31 to 2.10) with orange-red pairs having the lowest (CV = 0.60, *95% CI* = 0.46 to 0.77), but only significantly different from pure pairs. Orange-orange pairs also had significantly less variation (CV = 0.60, *95% CI* = 0.46 to 0.78) than red-red (CV = 1.06, *95% CI* = 0.92 to 1.23) and yellow-yellow pairs (CV = 0.97, *95% CI* = 0.81 to 1.13).

Egg measures

There was no significant difference in the number of eggs produced by category ($X^2_3 = 3.12$, $p = 0.37$) or clutch ($X^2_1 = 0.003$, $p = 0.37$). All pairs produced two clutches of at least 3 eggs, with an average by category of around 5.0 ± 1.0 eggs except for mixed pairs that averaged 4.2 ± 1. This was driven by red-yellow pairs that averaged 4.0 ± 0.95 eggs per clutch. So clutch size may be a real difference, but power to detect limited by sample size. Egg mass did not differ by pair type ($F_{3, 23.8} = 0.75$, $p = 0.53$) or clutch ($F_{1, 349.7} = 2.02$, $p = 0.16$). Egg volume did not differ by pair type ($F_{3, 22.3} = 0.88$, $p = 0.46$) or clutch ($F_{1, 351.1} = 1.41$, $p = 0.24$) either, but

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there was a significant interaction between pair and clutch ($F_{1, 351.4} = 3.93$, $p = 0.01$). This was due to a significant increase in the egg mass observed in pure pairings ($t = 2.00$, $p = 0.05$), while all other groups showed a non-significant decrease in mass. Yolk mass also differed between pairing categories ($F_{3, 27.5} = 6.56$, $p < 0.01$), as backcross pairs had significantly heavier yolks than all others. This is primarily due to most other pairs in both clutches having significantly lighter yolks than both red-orange pair clutches (clutch 1: pure and mixed ca. $t = 2.35$, $p = 0.02$; clutch 2: all except orange-yellow ca. $t = 3.50$, $p < 0.01$) and orange-red pairs clutches (clutch 1 all except orange-yellow: ca. $t = 3.70$, $p < 0.01$; clutch 2 all: ca. $t = 3.50$, $p < 0.01$).

Discussion

Our results showed that when pairing types were categorised, backcross pairs (F1 orange crossed with a pure bill colour individual) generally have sigificantly lower number of sperm trapped on the PVL than pure pairs (matching red or yellow individuals) and or mixed pairs (a red paired with a yellow individual) (figure 1). Additionally, backcrossed pairs showed more variance in PVL sperm numbers per egg than orange pairs (both pairs F1 hybrids). Pure coloured pairs did not significantly differ in sperm numbers on the PVL from each other or orange-orange pairs (figure 2). When divided into individual pairing types neither of the pure colour types differed from any of the backcross pairing types, but the results from the categorised comparisons suggests this is from our low sample size. Within mixed pairings, we found asymmetrical differences in the amount of sperm on the PVL with respect to the sex of the red and yellow birds. Red females paired with yellow males had less sperm on average, on the PVL, than the opposite colour pairing. Further orange males had less sperm that make it to the ovum when they were paired with a pure colour female, especially red females. There was no major difference in egg measures that explains the differences in total sperm number, since generally PVL sperm number scales with ovum and body size (Birkhead et al., 1994). Overall,

this suggests that red females are more selective that yellow females against sperm coming from a heterosubspecific male, with red females paired with orange males having the highest coefficient of variance of any pair type. Although, overall orange individuals seem to undergo high pre-zygotic post-copulatory selection of sperm when paired with a pure colour individual regardless of sex, the number of PVL sperm per egg tended to decrease in the second clutch in pure by orange pairing types, but only significantly in orange-yellow pairings (figure 2). These variations in post-copulatory sperm selection could therefore contribute to a reduced reproductive success of hybrid individuals, thus reducing admixture between the two subspecies, and maintaining the hybridisation zone, and isolation of the two subspecies.

The asymmetrical acceptance of sperm in mixed pairs could be related to the direction of divergence of the subspecies. Molecular evidence suggests that *P.a. acuticauda* (yellow bill) diverged from *P.a. hecki* (red bill) (Jennings & Edwards, 2005), with yellow males have less variable and significantly longer and narrower sperm morphology than red (Rowe et al., 2015b). This suggests that the yellow founding population was small, which is supported by some molecular evidence (Rollins et al., 2012), with sperm morphology representing the upper range of the red's morphological variation. There is strong genetic basis for sperm length, so a small population would put heavy selection in favour of the longer – less variable – sperm length (Birkhead et al., 2005, Simmons & Moore, 2009). Fertilisation success has been linked to variation in sperm length, potentially due to differential storage success of the various sperm types (Bennison et al., 2015). The sperm storage tubule size is positivly correlated with sperm length (Briskie & Montgomerie, 1993, Pitnick et al., 2009a), so divergence of sperm traits in separate populations could lead to fertilisation incompatibilities with secondary contact (Howard et al., 2009). Evidence in *Drosophila* support this idea (Lüpold et al., 2012, Manier et al., 2013a, Manier et al., 2013b). Therefore, they asymmetric selection we are seeing in mixed pairs is potentially caused by red females less able to store longer yellow male sperm, while yellow females are more able to accept shorter red sperm.

Future work could investigate the processes underlying the apparent asymmetric selection we have seen by quantifying the dominate morphology of sperm that reach the ovum in each pure pair in comparison to the average normal morphology available in the male's ejaculate, similar to recent work in the zebra finch (Bennison et al., 2015, Hemmings et al., 2016). Potentially the majority of yellow male sperm morphology falls outside the range of that red females can store, but the higher variability in red male sperm morphology means yellow females can store and use a wider morphology range of sperm. Alternatively, the sperm storage tubules of yellow females may not have diverged as rapidly or to such a degree as the yellow males sperm has (Briskie & Montgomerie, 1993), so there is not as strong a selection on their storage of sperm from red males. Regardless if it is change in sperm, sperm storage or both, it is clear there is an impact on sperm reaching the ovum, which can alter fertility and potentially reproductive success and limit hybridisation expansion. This potential constraint suggested by reduced PVL sperm numbers was more evident in backcross pairs.

The sperm morphology in orange males has not yet been determined, but could differ depending on the colour pairing of the parents since they both can influence the sperm traits of their offspring (Birkhead et al., 2005, Reinhardt et al., 2015, Simmons & Moore, 2009). In addition, hybridisation may impact the quality of the sperm produced (i.e. the amount of abnormal sperm), which could alter sperm performance (Opatova et al., 2016), reduce the number of sperm available for fertilisation (Gage et al., 2006), and alter reproductive success (Chapter 4). Despite our small pairing group sample sizes (n=3-6 pairs), our findings suggest these changes appear to increase post-copulatory selection when F1 orange hybrids breed with pure individuals, regardless of sex or colour pairing type, but not with another orange bird. The general reduction in sperm reaching the egg in most backcross pairs supports this idea, but needs further investigation. Therefore, the limited spread of the hybridisation zone (Griffith & Hooper, 2017) may reflect the limited success an orange hybrid individual has with a pure colour individual compared to with another hybrid. We know that all pairing types produce offspring (L.L. Hurley, personal observation), but further work is needed to determine how

difference in total PVL sperm numbers translates into overall reproductive success in these pairs. Reduced success of backcross and orange pairs compared to pure pairs, would also explain the limited expansion of the hybridisation zone.

The aim of this study was to investigate the long-tailed finch as a potential model for studying how fertilisation success contributes to the formation and maintenance of subspecies and species. We found asymmetrical post-copulatory selection on sperm in mixed paired birds, and a significant reduction in sperm reaching the ovum in backcrossed pairs regardless of pairing type. The latter suggests the importance of the post-copulatory pre-zygotic barriers in reducing the spread of hybridisation between species. Further work is needed to expand these findings and to understand the mechanism behind these results, but the long-tailed finch provides an ideal model to address these questions in the controlled setting of captivity.

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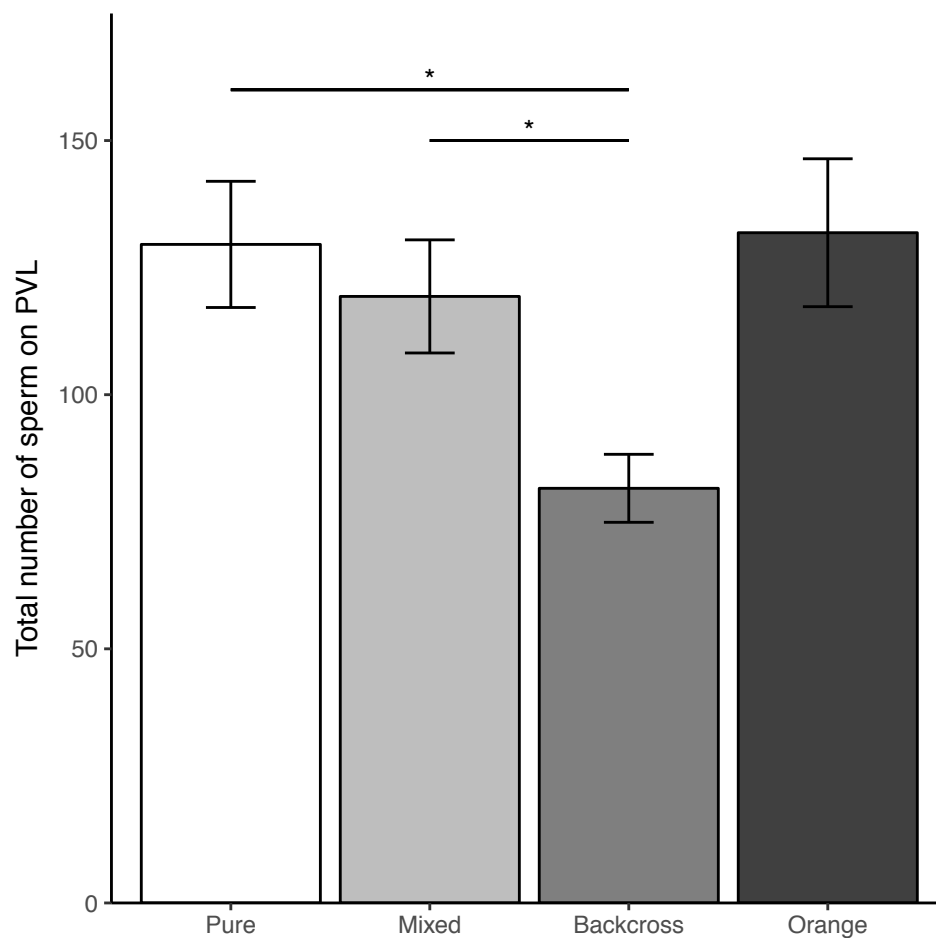
Figure 1.

Figure 1. Total number of sperm by pairing category: pure colour (white fill: red or yellow only pairs), mixed (light grey fill: one red and one yellow individual), backcrossed (mid-grey fill: one orange with a red or yellow individual), and orange (dark grey fill: pairing of hybrid individuals). Error bars ± SE. Asterisk indicates significant difference (p < 0.05).

Figure 2.
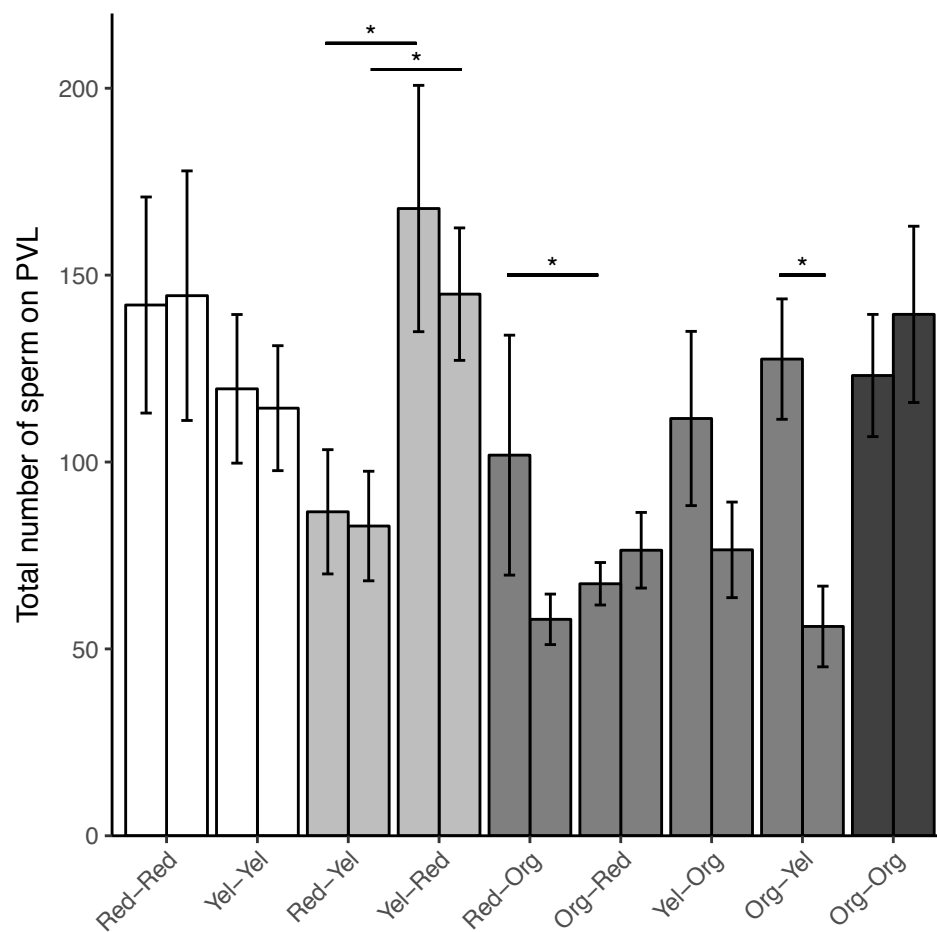
Figure 2. Average number of total number of sperm per egg in two clutches produced by each pairing type. First bar represents first clutch, second bar second clutch produced by pairs. Pairing types (female colour first): Pure coloured (white fill) red-red or yellow-yellow, hybrid (light grey fill) red-yellow or yellow red, backcross (mid-grey fill) red-orange, orange-red, yellow-orange, orange-yellow, or pure hybrid (dark grey) orange-orange. Error bars ± SE. Asterisk indicates significant difference ($p < 0.05$).

Chapter Four

Impact of reproductive experience and success on sperm quality and the number reaching the perivitelline layer of the egg in zebra finches

Chapter Four Vignette

Following the findings that the number of sperm on the perivitelline layer (PVL) can differentially vary across a clutch, within pairs, and between pairs in related species, and that sperm numbers on the PVL show significant and asymmetrical differences depending on how subspecies are paired, I now investigate how experience and successful reproduction impacts sperm reaching the ovum. Here I take a step back to also look at sperm performance and morphology in the male ejaculate, and how both can impact the success of the sperm reaching the ovum (Hemmings et al. 2016, Kleven et al. 2009). For this work I return to the zebra finch, *Taeniopygia guttata*, a species that demonstrates improved reproductive output as they gain experience with a partner (Adkins-Regan and Tomaszycki 2007, Crino et al. 2017) and when they have a strong, well coordinated, pair bond (Mariette and Griffith 2012, Ihle et al. 2015).

My interest in investigating how experience and reproductive success may alter ejaculate sperm traits and sperm reaching the ovum was sparked by two side projects I worked on (see appendix 2). The first looked at the reproductive and hormonal consequences of divorce, showing the benefit of repeated experience with the same partner (Crino et al. 2017). The second looked at variation in reproductive success across captive populations of zebra finch, noting that only 64% of females set-up to breed did so successfully (Griffith et al. 2017). The following chapter investigates how the ejaculate-female and sperm-egg interactions change with experience, and how they affect or are effected by reproductive success.

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Impact of reproductive experience and success on sperm quality and the number reaching the perivitelline layer of the egg in zebra finches

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Abstract

Previous experience with a partner can improve reproductive coordination between a pair and increase the number of offspring produced. Using virgin zebra finch, we investigated how experience (i.e. multiple reproductive attempts with same partner) and pair reproductive success (on first attempt, a later attempt, or never) influenced several factors including: the initiation of a reproductive attempt, investment in the egg by females, sperm motility, the number of sperm trapped on the egg's outer perivitelline layer, and ultimately hatching success. We gave forced-paired birds multiple opportunities to breed, but found that only 70% of pairs successfully reared chicks. We found that the experience of breeding together increased egg investment regardless of whether chicks were reared successfully. Males in pairs that bred successfully showed decreased sperm swimming speed over time, while it increased in birds that were never successful, but not enough to cause a significant difference in speed between groups. However, after rearing chicks, pairs that were successful on their first breeding attempt had a significantly higher proportion of motile sperm. Males in pairs successful on their first breeding attempt also had a higher proportion of morphologically normal sperm, and tended to have longer sperm compared to pairs that were never successful. Successful pairs showed a considerable decline in the average number of sperm reaching the ovum after having successfully reared chicks, while those pairs that were never successful, demonstrated higher average numbers of sperm on the egg membrane across attempts. This pattern coincided with unsuccessful pairs having a higher rate of hatching failure, mostly due to early embryo death. We suggest that an improvement in reproductive success over time spent with a particular partner and reproductive experience is not related to behavioural compatibility alone, but to changes in ejaculate-female and sperm-egg interactions, which in turn affects the viability of eggs.

Introduction

In socially monogamous bird species, coordination between the male and female appears to be important for reproductive success. Pairs that are synchronised or coordinated, in terms of their physiological state (Riechert et al., 2014), behaviour (i.e. provisioning rates, allopreening responsiveness, etc: Spoon et al., 2006, Bebbington & Hatchwell, 2016), and even personality (Fox & Millam, 2014, Gabriel & Black, 2012), appear more likely to reproduce successfully. In several species, there is strong support for the idea that breeding experience (i.e. repeated breeding attempts with the same or a previous partner) positively influences a range of fitness measures including: earlier initiation of laying dates (Adkins-Regan & Tomaszycki, 2007, Sanchez-Macouzet et al., 2014), the production of more eggs (Limmer & Becker, 2010), or heavier eggs (Crino et al., 2017, Schweitzer et al., 2014), higher hatching success (Limmer & Becker, 2010), and fledging success (Baran & Adkins-Regan, 2014, Limmer & Becker, 2010, Sanchez-Macouzet et al., 2014). Moreover, breeding experience with the same partner is thought to strengthen the pair bond, improve synchrony and reproductive success (Fowler, 1995, van de Pol et al., 2006).

In zebra finches, *Taeniopygia guttata*, 36% of all females fail to reproduce in a captive, experimental setting (Griffith et al., 2017). One explanation for this failure is the quality of the partnership, which is often measured in terms of coordination. The value of coordination to improve reproductive success has been shown at behavioural levels (Mariette & Griffith, 2012, Ihle et al., 2015), with respect to the acoustic communication between the partners (Boucaud et al., 2017, Elie et al., 2010), and at the hormonal level (Smiley & Adkins-Regan, 2016a, Smiley & Adkins-Regan, 2016b). Furthermore, forced repairing or the establishment of a new pair bond, can have a negative impact on reproductive success and the quality of resulting chicks (Adkins-Regan & Tomaszycki, 2007, Crino et al., 2017, Sanchez-Macouzet et al., 2014, Schweitzer et al., 2014). One aspect of coordination that has not been directly investigated, however, is how it impacts fertilisation success, which is driven by an interaction between the male's ejaculate at the time of the fertile period and the number of sperm that

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arrive at the site of fertilisation. Issues with too many or too few sperm reaching the ovum could partially account for the higher hatching failure rate in asynchronous pairs (Mariette & Griffith, 2012).

The number of sperm reaching the ovum in birds likely reflects an interaction between male sperm function and female usage of sperm (Birkhead & Fletcher, 1998, Birkhead et al., 2008, Parker et al., 2002). Birds are obligately polyspermatic (requiring more than one sperm to penetrate the ovum), so successful fertilisation is a balance of enough sperm reaching the ovum to ensure fertilisation and development of the embryo (Hemmings & Birkhead, 2015, Birkhead et al., 1993, Birkhead & Fletcher, 1998), but not so much to cause abnormities that cause early developmental failure (Christensen et al., 2005, Fechheimer, 1981, Forstmeier & Ellegren, 2010). The number of sperm reaching the ovum can be assessed by counting the number of sperm that have penetrated the ovum's inner perivitelline layer (PVL: the membrane surrounding the yolk) or are trapped by the outer PVL (Bakst & Howarth, 1977, Bobr et al., 1964), values which tend to be highly correlated (Birkhead et al., 1994, Birkhead & Fletcher, 1994). This number varies between species, scaling with body and ovum size (Birkhead et al., 1994) and potentially life history (Johnsen et al., 2012), and both within species and within an individual clutch (Chapter 2, Johnsen et al., 2012, Lifjeld et al., 2000). The factors underlying this variation in sperm numbers within species and individuals, and the impacts that it may have on reproductive success are unknown, but may reflect an optimum level between too many and too few sperm to achieve fertlisation and ensure successful embronic development (Ichikawa et al., 2016, Forstmeier et al., 2014).

To date, the majority of research addressing the factors underlying the number of sperm reaching the ovum (directly or via fertilisation success) has examined the role of sperm quantity (using an index of motility generated by a specialized analyzer: Parker et al., 2002), or sperm phenotype (Hemmings & Birkhead, 2015), the number of matings the pair engage in and when they occur (Bennison et al., 2015, Hemmings et al., 2016), and the usage of the available sperm (Birkhead et al., 1993, Birkhead & Fletcher, 1998). In contrast, as pairs gain

experience and successfully reproduce these measures might change within, but this has not been examined.

In this study, we investigated how breeding experience and success of pairs over time alters the number of sperm reaching the PVL of the egg, sperm performance, and sperm quality (proportion normal morphology). In addition, we investigated how breeding experience and success impacted timing of egg-laying, female investment in eggs, and hatching success. To do this we force-paired male and female zebra finches that had no prior breeding experience. The design of the study is ecologically relevant in this species because they naturally produce multiple clutches within a year and typically remain paired for life (Zann, 1996), and methodologically important to understand as most captive studies use force pair design (Griffith et al. 2017). Given that the social bond in the zebra finch is an important determinant of reproductive success (e.g. Ihle et al., 2015, Mariette & Griffith, 2012), we predicted that the number of sperm on the PVL would change as pairs gained experience and breed successfully to reflect better the species' optimal sperm number (enough for fertilisation and development, without causing abnormalities), and that this change may reflect a change in sperm quality and performance over time. Additionally, we predicted that breeding experience would lead to an increase in egg size and a reduction in the time taken for a pair to initiate egg-laying (when given the opportunity), which would be consistent with previous studies in this species (Adkins-Regan & Tomaszycki, 2007, Crino et al., 2017, Schweitzer et al., 2014), and has been noted in other species (Sanchez-Macouzet et al., 2014).

Methods

General procedures and experimental design

Twenty pairs of male and female virgin zebra finches, all between 12 -18 months of age, were force-paired and placed in outdoor aviaries (4.1 x 1.85 x 2.24 m) located on the campus of Macquarie University from October 2014 – April 2015. Each aviary was visually separated from neighbours and fitted with 4 nest boxes and nesting material. Birds were provided with

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food and water *ad libitum*, as well as a daily supplement (green pea mix and hard-boiled egg). All procedures were conducted according to relevant national and international guidelines and were approved by the Macquarie University Animal Ethics Committee (Animal Research Authority 2013/29).

The experiment consisted of three phases (figure 1): 1) egg collection and sperm sampling, 2) breeding and chick production, and 3) collection of third clutch and sperm sampling. During phase 1 we collected the first two clutches of eggs that each pair produced (clutch 1 and 2) for an analysis of PVL sperm number. For each clutch, we also collected a sperm sample on the day that the third egg was laid/collected, to standardize sampling collection (see below for details of sperm sampling). During phase 2, pairs were allowed four attempts to lay a complete clutch and rear the resulting chicks. When pairs successfully reared this first brood, they were allowed to rear a second brood, which always yielded successful fledglings, before moving to phase 3. Pairs that were unsuccessful in rearing chicks from their fourth clutch automatically moved to phase 3 after the failure of their fourth attempt. Finally, during phase 3, we collected the next laid clutch of eggs (clutch 3) for the PVL sperm analysis and again collected a sperm sample on the day the third egg of this clutch was laid.

During phases 1 and 3, all nest boxes where checked daily for evidence of nest building or egg laying until complete clutches were produced. Eggs were collected on the day of lay and replaced with a dummy egg. Dummy eggs were left in the nest for 5 days after the final egg of the clutch was laid and then removed to promote the production of a new, replacement clutch. During phase 2, once a clutch was complete the nest box was left alone until the day prior to expected hatching (~12 d after 4th egg laid: Gilby et al., 2013, Zann & Rossetto, 1991), when it was checked daily until all eggs had hatched. Eggs that failed to hatch were dissected and scored for fertility and embryo age at time of developmental failure (scoring based on: Murray et al., 2013, Birkhead et al., 2008) to approximate the cause of failure. Fledglings were removed from the aviary when the youngest was 32 days of age.

From this point onwards, we will define success as a pair having reared chicks to fledge, with those that were successful on the first breeding attempt in phase 2 categorized as 'first' and those successful on subsequent breeding attempts (i.e. attempt 2-4) categorized as 'later'. Finally, pairs that did not successfully rear any chicks hatched to fledgling will be categorized to as 'never' successful (figure 1).

Egg collection and processing

Eggs were collected and weighed (to nearest 0.001g) on the day they were laid. Eggs were initially maintained at 4°C until the entire clutch was collected, after which they were frozen and stored at -20°C until processing for PVL sperm. To process eggs, we first slowly thawed them at 4 °C, and then measured egg length and width (to the nearest 0.01 mm) using digital callipers and calculated egg volume as, volume $= 0.51 \times$ length $\times$ breadth^2 (Hoyt, 1979). Eggs were then carefully cut open with fine scissors and the shell and albumen discarded. The yolk was rinsed, dried by gently rolling it across filter paper, and then weighed (to nearest 0.001g). The germinal disc (GD) was then visualized and the yolk was cut approximately in half with the GD centred on one side (following Johnsen et al., 2012). Both PVL sections were then separated from the yolk and washed in phosphate buffered saline (PBS) using a hair loop. The washed sections were then stretched flat on a microscope slide and stained with 15 μL of Hoechst 33342 fluorescent dye (Sigma, 5 μg/ml), which binds DNA and permits counting of individual sperm heads. Slides were incubated in the dark for 10 min before sperm were counted using a fluorescence microscope (Leica DM5000B) with a BP 340–380 excitation filter and a LP 425 suppression filter. All stained sperm on both halves of the PVL were counted as a total number by systematically scanning the slide at 200x.

Sperm sampling and analysis

Fresh sperm samples were obtained by cloacal massage (Wolfson, 1952), and were immediately used to assess sperm motile performance (swimming speed and proportion of

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motile sperm). Samples were collected on the day the third egg was laid, in order to standardize sampling conditions, as sperm motility performance appears to show relatively low within-male repeatability in passerine birds (Birkhead & Fletcher, 1995, Cramer et al., 2015). Briefly, sperm were collected and immediately diluted in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd.) pre-heated to 40ºC (the approximate physiological temperature of zebra finches: Calder, 1964). Next, 6 µl of the sperm suspension was loaded into a chamber slide (depth 20 µm, Leja®, Netherlands) and sperm motion was recorded for six unique fields of view (5s each for total 30s) at 400x using phase contrast microscopy (CX41, Olympus, Japan) connected to a digital camera (Legria HF G25, Canon, Japan). All samples, chamber slides, and the heated microscope stage (TP-S, Tokai Hit, Shizuoka, Japan) were maintained at a constant temperature of 40ºC throughout. Finally, an aliquot (*c.* 10-20 µl) of the sperm suspension was fixed in 5% buffered formaldehyde solution and used at a later date to quantify sperm morphology (i.e. sperm length and the proportion of morphologically normal sperm).

Videos of sperm motion were analyzed using computer-assisted sperm analysis (CASA; HTM- CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research) with frame rate was set at 50 Hz and 25 frames (i.e. sperm cells were tracked for 0.5 s) and quality control settings following Rowe et al. (2015) and Hurley et al. (Chapter 6). Each analysis was visually examined and cell detection parameters established to exclude non-sperm, contaminating particles: minimum contrast, 80-100; minimum cell size, 8-12 pixels; sperm head elongation (i.e. width/length), < 75%. Additionally, we excluded non-continuous sperm tracks or sperm tracked for less than 10 frames, as well as tracks for which the maximum frame-to-frame movement exceeded the average frame-to-frame movement by 4 SDs for the same track, as such tracks tend to represent tracking errors. Finally, to account for the effects of drift, tracks with an average path velocity (VAP) of <30 µm s^{-1} or a straight line velocity (VSL) of <25 µm s^{-1} were considered immotile. These cut-offs were based on an analysis of dead sperm cells using the same recording and analysis conditions as those used in the current study. All CASA

was done by the same person (MR) and without information about the male's identity.

Following this filtering process, we excluded samples with less than 10 motile sperm tracks from analyses of sperm swimming speed (see Chapter 6 for justification and methods). After this exclusion, the total number of motile sperm tracked for each male ranged from 15 to 458 (median = 84, mean = 105 ± 6.32). For each sperm, we recorded cell straight-line velocity (VSL, i.e. average velocity on a straight line between the start and endpoint of the sperm track), average path velocity (VAP, i.e. average velocity over a smoothed sperm track), and curvilinear velocity (VCL, i.e. velocity over the actual sperm track). We choose to use VCL for statistical analyses because this metric measures the actual path of sperm movement. Finally, we calculated the proportion of motile sperm as the number of motile tracks divided by the total number of cells.

Next, we quantified sperm length and the proportion of sperm with normal morphology by placing an aliquot (*c.* 2 µl) of formaldehyde-fixed sperm on a microscope slide, allowing it to air-dry overnight, and then rinsing it with distilled water before allowing it to air-dry again. We then captured high magnification (320x) digital images of sperm cells using a light microscope (BX53), a digital camera (DP73) and cellSens software (all products of Olympus, Australia). Next, 10 morphologically normal sperm (following: Bennison et al., 2015, Laskemoen et al., 2007) were measured from each individual using ImageJ (v. 2.0.0_rc_43/1.51a: Schindelin et al., 2015) to obtain measurements (±0.001 µm) of the following sperm traits: head length, mid-piece length, flagellum length, and total sperm length. These measurements were also used to calculate the ratio of flagellum length:head length. These measures were taken as head and total length has been found to be important to the success of sperm reaching the ovum (Bennison et al., 2015, Hemmings et al., 2016), and in sperm performance (Humphries et al., 2008).

To assess the proportion of sperm with normal morphology, we examined 100 randomly chosen sperm on each of two replicate slide smears (200 sperm cells examined in total) and scored sperm as having either normal or abnormal morphology (i.e. cells with

macrocephaly; microcephaly; broken heads, midpieces or flagellum; or multiple flagellum).

We then averaged these values, to determine the proportion of normal sperm in the sample

(repeatability of replicate smear $R = 0.90$, $p < 0.001$) (Nakagawa and Schielzeth 2010). All

length measurements and scoring of sperm morphology was done blind to information about

clutch number or success by the same person (LLH). Due to poor fixation of early samples,

data on sperm morphology are taken from phase 3 of the experiment only. Measures of sperm

length have been found to be highly repeatable in zebra finches (Birkhead & Fletcher, 1995)

with low variation (Birkhead et al., 2005), so is unlikely to have changed. However, changes

in normal morphology has not been addressed, but it is potentially susceptible to change (e.g.

environment impact: Chapter 6), which may reflect a sperm's fertilisation ability at the time it

was collected (Hemmings et al., 2016).

Statistical analysis

All statistical analysis was performed using R (version 3.3.3; R Core Team, 2017) and the

'*lme4*' package (Bates et al., 2015). To quantify the effects of experience and success on

sperm swimming speed (VCL: single cell recordings to better represent variation in an

individual than overall average), proportion of motile sperm (logit transformed), egg and yolk

mass, egg volume, and delay to initiating egg laying (in days) we fitted six different linear

mixed models (LMM) for each dependent variable. Fixed effects were breeding experience

(three-level factor: clutch number 1, 2, or 3) and success class (three-level factor: first, later, or

never successful). Models included the interaction between success class and experience to

test potential differences in how experience impacted each success class. We also fitted pair

identify as a random factor to account for repeated measures. Additionally, we fitted a

generalized mixed models (GLMM Poisson family, log link) with the same fixed factors and

random effects as described above, but with the dependent variable being PVL sperm

numbers, and correcting for over dispersion by adding data row number as a random factor.

We first ran full models with the interaction between success class and experience using

likelihood ratio tests using maximum likelihood estimation. If the interaction was non-significant we removed it, but success class and experience were not removed from the model. All final LMM models were fitted with REML (restricted maximum likelihood).

To quantify the effects of experience and success on percent normal sperm (logit transformed) and sperm morphology measures (ln-transformed) we fitted two linear mixed models (LMM). As these measures were only sampled at clutch 3 (due to poor early fixation), this was run with success class as the only fixed factor in both models. As individual morphology measures were used (not the average), male identity was included as a random factor.

In order to investigate hatching success we used two different types of models using GLMM binomial (family, logit). First, we looked at proportion hatching success (logit transformed) overall for the entire study by totaling all of a pair's hatched and unhatched eggs across the experiment, with success class as a fixed factor. Then to determine whether hatching success improved with the number of breeding attempts we isolated data by first, later or never class. For first and never successful pairs we ran the models with clutch number as a fixed factor and pair identity as a random factor. For the pairs in the 'later' group (successful on a breeding attempt other than the first) we added a covariate of success (reared chicks or not) of the clutch to determine if successful chick rearing is what improved the hatching rate.

Lastly, to investigate if the overall number of fledglings produced was impacted by when pairs were first successful (e.g. which breeding attempt first yielded fledgligns) we summed the number of chicks produced by each pair then ran a GLMM (Poisson family, log link) model with first successful breeding attempt as a fixed effect.

When main effects were significant, pairwise comparisons were done by rerunning models and iteratively changing the reference level for the variable of interest. To estimate the significance of these tests: Summary z values are reported with p-value generated using package *lmerTest* (Kuznetsova et al., 2016), and GLMM ANOVA p-values for regression

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coefficients were obtained using the *car* package which reports chi-square (X^2) with the p-value (Fox & Weisberg, 2011).

Finally, to investigate variability we calculated the coefficient of variation (CV) for each response variable. This was done between success classes (i.e. first, later, never) and within class by clutch (for PVL sperm and egg measures), within males by class (for sperm swimming speed and sperm morphology), or between males by class (proportion normal sperm). Differences were found by visually examining the overlap of 95% confidence intervals (CI) calculated using 1000 bootstrap samples and correcting for bias. Figures were constructed using ggplot2 (Wickham, 2009), and modelling assumptions (normality and heterogeneity of variance of residuals) were assessed visually (following Zuur et al., 2009). All tests were two-tailed and considered significant at $\alpha < 0.05$, and all means are shown $\pm$ SD unless otherwise noted.

Results

General breeding success

Of the initial 20 pairs, we collected data for 17 pairs throughout the entire experiment (i.e. the third PVL egg collection). In the remaining 3 pairs, one member died during the study and consequently data from these pairs was excluded from all analyses. Across the four breeding attempts, success varied across the 17 pairs. We therefore categorised birds into three classes, those that were successful in their first attempt to rear chicks (i.e. 'first', n=6), those that were successful later after at least one initial failed attempt (i.e. 'later', n=6: 2 on the second, 3 on the third, 1 on the fourth attempt) and those that were never successful (i.e. 'never', n=5) (figure 1).

Reproductive initiation and output

With experience, all pairs reduced the number of days taken to lay a new clutch from an average of 6 to 3 days ($F_{2, 31.7} = 3.24$, p = 0.05), with no significant difference in this delay

observed between success classes ($F_{2, 14.2} = 1.31$, $p = 0.30$). The overall decline was driven by all pairs reducing average time taken (in days) to clutch initiation from clutch 1 to clutch 3, so that pairs were 58.1% (first successful), 54.5% (later successful), and 31.4% (never successful) faster to start laying eggs.

All pairs hatched a proportion of their eggs during each breeding attempt, therefore any chicks produced by never successful pairs hatched subsequently died (typically with in 2 days of hatch). Across all clutches, pairs that were successful on the first attempt hatched 97.8% of their eggs, while those that succeeded later had a hatch rate of 92.8%. Pairs that were never successful hatched only 88.4% of their eggs, which was significantly different from those that were successful at first clutch ($z = 2.05$, $p = 0.04$), but not later successful pairs ($z = 1.24$, $p = 0.22$). In clutches that resulted in fledglings, only 3 of 179 eggs failed to hatch (all were in a pair's first successful breeding attempt, as second fledgling clutches had a 100% hatch rate). Experience did not improve hatching success in later successful pairs, as those that reared chicks for the first time on the third or fourth breeding attempt did improve hatching success from their first to second clutch attempts from 66.7% to 93.8% ($z = 2.37$, $p = 0.02$). Hatching success did not improve with experience in pairs that were never successful ($X^2{}_3 = 0.60$). Overall, hatching failure could be characterised as follows: 10.5% appeared infertile (no apparent development), 57.9% failed at < 4 days embryonic age, 31.6% failed after 10 days of embryonic age. Of the three failed eggs from clutches that yielded fledglings, 2 failed < 4 days of age and one failed approximately day 11.

Once pairs successfully fledged one set of chicks, their next clutch always yielded successful fledglings (N = 12). Pairs that successfully bred on the first attempt (N=6) on average raised more chicks over their two breeding successes (7.7 ± 2.2 fledglings) than pairs that were successful later (5.2 ± 1.5 fledglings). The pairs that successfully reared chicks in their second breeding attempt (N = 2) raised significantly fewer chicks from those successful on first try (t = -4.17, p = 0.02, 3.5 ± 0.7 fledglings), and pairs were successful on the third attempt (N = 3: 6.0 ± 1.0 fledglings) .

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PVL Sperm

Birds that bred successfully on their first attempt decreased their average total PVL sperm across the experiment ($X^2_2 = 7.46$, p = 0.02), with the decline occurring after rearing chicks (figure 2): average sperm number per egg from PVL clutch 2 to PVL clutch 3 decreased (z = -2.63, p < 0.01). Pairs that successfully bred later also had significantly decreased total PVL sperm over the experiment ($X^2_2 = 41.0$, p < 0.01), again with the decline occurring after rearing chicks (PVL clutch 2 to PVL clutch 3: z = -5.74, p < 0.001). Unsuccessful birds did not show a significant decline in average total sperm on the PVL with experience ($X^2_2 = 1.12$, p = 0.57).

After breeding attempts, average total PVL sperm in clutch 3 of first successful pairs did not differ significantly from those with later success (z = -1.92, p = 0.06) or were never successful (z = 1.22, p = 0.22). Successful pairs that bred later did significantly differ at clutch 3 from those that never fledged chicks (z = 3.02, p < 0.01).

In first success birds, there was no significant change in the variation between clutches with CV declining from 0.87 (*95% CI* = 0.67 to 1.12) in clutch 1 to 0.80 (*95% CI* = 0.64 to 1.00) in clutch 3. Later success birds showed a significantly decreased CV of 1.32 (*95% CI* = 1.06 to 1.69) in PVL clutch 1 to a CV of 0.57 (*95% CI* = 0.40 to 0.81) in clutch 2, but not significantly different CV of 0.84 (*95% CI* = 0.62 to 1.07) at clutch 3. Those pairs that had no success in producing offspring had an initial CV of 1.33 (*95% CI* = 0.10 to 1.80) in clutch 1 decreasing by more than half to 0.49 (*95% CI* = 0.35 to 0.67) in clutch 2, and staying low in the clutch 3 at 0.57 (*95% CI* = 0.46 to 0.70).

Sperm motility and morphology

Sperm swimming speed (VCL) did differ overall across clutches (i.e. with experience) ($F_{2,3999.8} = 10.83$, p < 0.001), but did not differ significantly between success class groups ($F_{2,14.2} = 1.49$, p = 0.26). However, there was a significant interaction of success class and

clutch ($F_{2,3995.2}$ = 4.16, p < 0.01) as both first and later successful males generally showed a decrease in average VCL sperm speed with experience while never successful males had sperm speed increase.

This interaction was created by how each group's sperm swimming speed changed across clutches (figure 3). First successful males did not significantly change average swimming speed across clutches, although it did tend to decline. Later successful males had an average sperm swimming speed that was significantly lower at clutch 2 than either clutch 1 (t = 3.67, p < 0.001) or clutch 3 (t = 3.57, p < 0.001). Later successful males' sperm swimming speed at clutch 2 was significantly slower than first success males (t = 2.32, p = 0.03). Never successful males showed significantly increased VCL after breeding attempts (clutch 1 to clutch 3: t = 3.93, p < 0.001).

Sperm swimming speed variance differed significantly between groups. Males that were successful on their first breeding attempt had a significantly lower coefficient of variance than both other groups (CV = 0.26, *95% CI* = 0.25 to 0.27). Later successful males had significantly higher variance in their sperm swimming speed (CV = 0.37, *95% CI* = 0.36 to 0.38) than both other groups, with never successful males also having high variance in speed (CV = 0.29, *95% CI* = 0.28 to 0.31).

Across clutches, sperm speed in first success males showed a slight increase in variance from clutch 1 CV = 0.26 (*95% CI* = 0.24 to 0.28) to clutch 2 (PVL clutch 2: CV = 0.29, *95% CI* = 0.26 to 0.31), followed by a significant decrease in variance after breeding (clutch 3: CV = 0.23, *95% CI* = 0.21 to 0.25). Later and never successful males showed a non-significant decline in sperm speed CV with experience based on CIs.

We found no overall significant differences in the proportion of motile sperm between success class ($F_{2,13.8}$ = 1.45, p = 0.27) or across clutches ($F_{2,27.5}$ = 0.22, p = 0.80). First successful birds were the only class to show a marginally significant change between clutches, increasing the proportion motile from clutch 2 (46.6 ± 37.4%) to clutch 3 (77.4 ± 6.3%: t = 2.06, p = 0.05), but the sperm in clutch 3 was not significantly more motile than clutch 1(65.4

± 25.1%: t = 0.72, p = 0.48). Later males that were successful demonstrated an increased proportion of motile sperm from clutch 1 (42.5 ± 13.3%) to clutch 3 (53.1 ± 20.4%), while in unsuccessful birds the proportion of motile sperm declined (from 63.0 ± 25.5% to 48.9 ± 42.2%), but neither change was significant (later: t = 0.67, p = 0.51, never: t = -1.21, p = 0.23). However, this meant that males successful on their first attempt had more motile sperm after breeding than those that were never successful (t = -2.08, p = 0.04), but not those that succeeded later (t = -1.72, p = 0.09). There was no difference in variance in sperm motility between groups (ca. CV = 0.38), or with experience in the later and never successful groups. However, males successful on their first attempt showed significantly decreased variance in sperm motility from their first reproductive bout (CV = 0.38, *95% CI* = 0.15 to 0.67) to after breeding (CV = 0.08, *95% CI* = 0.05 to 0.13).

After breeding attempts, when the third clutch was collected for PVL examination, the fixed sperm sample revealed a significant difference between success class types (table 1: $F_{2,14}$ = 8.40, p < 0.01) with unsuccessful males having proportionally less normal sperm compared to males that were successful (table 1): first success (t = 4.08, p = 0.001), later success (t = 2.55, p = 0.02). Between first success pair males there was significantly lower variance in proportion of normal sperm (CV = 0.02, *95% CI* = 0.01 to 0.04) compared to later (CV = 0.14, *95% CI* = 0.10 to 0.22) and never (CV = 0.27, *95% CI* = 0.18, 0.47) successful males. The total sperm length of first success males was on average longer than later or never successful males, but there was no significant difference between groups in any individual sperm measure (table 1): total, head, mid-piece, or flagella-head ratio. Sperm flagella of the males that were successful from the outset were marginally longer than those that were successful eventually (t = -2.09, p = 0.05) and tended to be longer that those not successful at all, but not significantly (t = -1.95, p = 0.07). There was only a significant difference in the variance in head length and flagella length. Sperm head length tended to be less variable in first (CV = 0.07, *95% CI* = 0.06 to 0.09) compared to never successful pairs (CV = 0.11, *95% CI* = 0.09 to

0.15). Flagella length was significantly less variable in sperm from first (CV = 0.23, *95% CI =* 0.20 to 0.28) compared to later successful pairs (CV = 0.33, *95% CI =* 0.30 to 0.39).

Egg measures

We found an increase in egg investment with experience: yolk mass ($F_{2,234.7}$ = 11.22, p < 0.001), egg mass ($F_{2,234.5}$ = 101.9, p < 0.001) and egg volume ($F_{2,234.5}$ = 79.4, p < 0.001) increased with clutch number. Yolk mass did not significantly increase until the third clutch collection (e.g. first attempt success: clutch 1 – 2: t = 0.84, p = 0.40; clutch 2 – 3: t = 4.43, p = 0.001), whereas egg mass and volume increased with each clutch collection. In contrast, there was no effect of timing of success (i.e. first, later, never) on any measure of egg investment: yolk mass ($F_{2,\ 14.1}$ = 0.80, p = 0.47), egg mass ($F_{2,14.0}$ = 0.80, p = 0.47) and egg volume ($F_{2,14.0}$ = 0.58, p = 0.58).

There was no significant difference in the variation in yolk mass with success or experience. First attempt successful pairs had less variation in egg mass (CV = 0.08, *95% CI =* 0.07, 0.09), suggesting more consistent investment, than unsuccessful pairs (CV = 0.12, *95% CI =* 0.10, 0.14). This was also true of egg volume: first (CV = 0.08, *95% CI =* 0.07, 0.09), never (CV = 0.12, *95% CI =* 0.10, 0.14). There was no change with experience within each category of success in egg mass and volume, except variation in egg volume of pairs successful later was lower in the first (CV = 0.05, *95% CI =* 0.04, 0.06) compared to clutch 3 (CV = 0.10, *95% CI =* 0.07, 0.12).

Discussion

We investigated how experience and breeding success in forced-paired inexperienced zebra finches impacted sperm morphology, sperm motility, and the number of sperm that reach the ovum, as well as reproductive output. Our results revealed that breeding success (i.e. success at fledging chicks) is associated with a decrease in the number of sperm found trapped in the egg membrane, perhaps reflecting a better coordination of reproductive physiology in these

pairs, and a closer match to the optimum level of polyspermy (not too many or too few sperm) in this species. In part, this finding may be determined by variation in sperm quality, as we also found significant differences in proportion of normal morphology sperm and marginal differences in sperm length across males with respect to the reproductive success of the pair. As in previous studies, we found that egg mass and volume increased with experience, as delay to initiate clutch laying decreased (Adkins-Regan & Tomaszycki, 2007, Sanchez-Macouzet et al., 2014), as well as an increase in yolk mass. These changes occurred independently of a pair's success in rearing chicks to fledging.

In pairs that bred successfully, we saw a reduction in the number of sperm reaching the ovum when compared to a pair's first egg production. If this phenomenon was due to experience alone it would have meant that the longer a female is reproductively active the better she is at regulating the number sperm reaching the ovum. Again, this is important to ensure fertilisation and embryo development (Hemmings et al., 2016, Hemmings & Birkhead, 2015), and reduce the risk of early development failure from genetic abnormality (Christensen et al., 2005, Fechheimer, 1981, Mizushima et al., 2008, Forstmeier & Ellegren, 2010). However, pairs that were never successful did not display significantly reduced total PVL sperm numbers (figure 2), so this suggests another factor – or an interaction with another factor – is impacting post-copulatory selection. There are a number of factors, such as divesting in amount of sperm produced or put into an ejaculate or a refinement of the female's selection of sperm, both of which could be influenced by sperm morphology.

Sperm morphology traits can be important to fertilisation success under both competitive (i.e. sperm competition) and non-competitive conditions (Simmons & Fitzpatrick, 2012). Work in zebra finch has shown that when sperm morphology types vary in a single ejaculate certain sperm morphological sub-groups can have better success in reaching and fertilizing the egg (Bennison et al., 2015, Hemmings et al., 2016). Thus, it is likely that certain traits increase a sperm's chance for reaching the ovum. Recently in zebra finches, fertilisation success has been linked to the composition of sperm morphology traits, specifically those with

longer total length (Bennison et al., 2015) and longer tails and shorter heads (Hemmings et al., 2016). In our study, after breeding, males in pairs successful on their first breeding attempt on average had longer sperm heads (with significantly less variation), longer flagella and the greatest total length in their ejaculate sperm (table 1). This small difference in sperm morphology could account for the on average higher swimming speed in the first successful males, following the hypothesis that longer sperm swim faster, but this is still debated (Bennison et al., 2015). Interestingly, first and later successful males' sperm swimming speed did decrease in average speed and variation with successful breeding, compared to unsuccessful males. Perhaps this reflects a change or refinement in sperm investment after successful reproductive experience in a pair reduces competition pressures (perceived or actual) (Simmons & Fitzpatrick, 2012, Immler et al., 2010). Conversely, the increase in the swimming speed of those males that were never successful (across the experiment) could reflect an investment change to increase the competitiveness of their sperm.

In this study, we did not examine the morphology of the sperm that reached the ovum (i.e. long or short: Hemmings et al. 2016) or the genetics of the birds, which controls sperm morphology and impact sperm success (Kim et al. 2017, Knief et al 2017), so we are unable to speculate if a male's sperm karyotypes impacted their eventual success. However, one set of factors that could impact the number of sperm reaching the egg is the proportion of sperm that has a normal morphology sperm and is motile. We found that males that never had a successful breeding attempt had a lower average of morphologically normal sperm and greater variation between males in that group compared to those that were successful on the first breeding attempt. Unfortunately, we were unable to directly assess the sperm morphology and proportion of normal sperm of the different males in the early part of the experiment due to poor sample fixation at that time. Therefore, we are unable to assess whether the difference between the groups of males at the end of the experiment was a result of the different reproductive history of the males by that point, or whether this poorer quality sperm was the normal state of these birds. The repeatability in zebra finch sperm morphology (Birkhead &

Fletcher, 1995, Birkhead et al., 2005) and sperm quality in chickens (Parker et al., 2002, Parker et al., 2000) suggests that the latter is certainly a possibility. The higher percentage of morphologically normal sperm could explain why first success birds had more motile sperm than never successful birds after successful breeding. It could also explain the differences in the total number of sperm on the PVL in these pairs. Previous work has shown that females appear to compensate when sperm insemination numbers are low by allowing a greater number of sperm to reach the ovum (Hemmings & Birkhead, 2015), but the mechinsim is unclear. Therefore, the never successful pairs' higher average PVL sperm numbers could reflect a similar response to low number of normal sperm. Similar, since only normal sperm can be stored for use in fertilisation (Lake, 1975), perhaps a low number of normal sperm cells in an ejaculate equals a low insemination number, so it is compensated for in the same way. The resulting higher number of sperm could account for why we saw no change in the hatching failure in unsuccessful pairs (Christensen et al., 2005, Fechheimer, 1981). If this is true, then the decrease in sperm numbers in later successful pairs would account for their improved hatching rate, but this could also reflect a change in pair behaviour.

In a previous study looking at the impact of forced-pairing vs. free choice on reproduction, it was found that forced-pairs yielded significantly more dead offspring, not a differential hatching rate (Ihle et al., 2015). Our difference in hatching success between and across our success classes could reflect the interaction of biological process that results in some pairs being very compatible (high rate of hatching from the start), very incompatible (consistently poor hatching), and birds that – given no other mate choice – made it work (improved hatching after first failure). Increased hatching in the later successful birds could stem from changes in sperm usage or improved behavioural compatibility (Mariette & Griffith, 2012). Mariette and Griffith (2012), showed that pairs that were better synchronised while feeding nestlings hatched more eggs (possibly due to better coordination while incubating, or during the fertile period of the female) and therefore fledged more offspring. Future work should explore potential causes and effects of such interactions.

Overall, our study showed that as pairs gain experience together they increased their egg measures (egg mass, yolk mass, and egg volume) and hatching, and a decreased clutch initiation. Our study is the first to demonstrate that the total number of sperm on the outer PVL decreases following successful rearing of chicks to fledging, which coincided with a decrease in hatching failure. Experience alone was not enough to decrease hatching failure or total PVL sperm, as pairs that never successfully reared fledglings showed no significant changes in these measure from their first reproductive efforts. Furthermore, unsuccessful pairs had a lower percentage of normal morphology sperm. However, it is unclear if this contributes to the higher number of sperm on the PVL and early embryo death hatching failure, or is a result of multiple failures. Given that only about 64% of zebra finch females successfully reproduce in an experimental setting (Griffith et al., 2017), our findings suggest that looking at the number of sperm reaching the PVL should be considered when evaluating the impact of experimental manipulation on breeding success and failure especially in forced-pair designs.

Authors' contributions. LLH and SCG conceived and designed the experiment. LLH led the writing of the manuscript, with contributions from other authors. LLH conducted the empirical work. LLH and MR processed samples. LLH conducted the statistical analysis.

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Table 1. Overall mean of sperm quality, performance, and morphology measures for males after breeding attempts, classified by success at rearing chicks to fledge multiple breeding attempts.

Trait	Success			F	p
	First	Later	Never		
	Mean ± SD	Mean ± SD	Mean ± SD		
VCL (µm s⁻¹)	118.32 ± 27.70	109.16 ± 38.70	131.07 ± 35.01	1.18	0.34
Proportion motile	*0.77 ± 0.06* [a]	*0.53 ± 0.20* [ab]	**0.48 ± 0.24** [b]	**4.17**	**0.04**
Normal morphology (%)	**82.40 ± 1.95** [a]	**73.0 ± 10.52** [a]	**55.71 ± 15.57** [b]	**8.40**	**0.004**
Total length (µm)	70.84 ± 5.92	66.24 ± 6.13	65.91 ± 6.12	2.09	0.16
Head length (µm)	11.28 ± 0.84	10.91 ± 1.12	10.61 ± 1.25	1.59	0.24
Midpiece length (µm)	28.17 ± 5.14	31.57 ± 4.56	31.36 ± 4.63	2.09	0.16
Flagellum length (µm)	*31.38 ± 7.49* [a]	*23.76 ± 8.06* [b]	*23.94 ± 6.11* [b]	*2.57*	*0.10*
Flagellum:head	2.78 ± 0.69	2.19 ± 0.76	2.28 ± 0.59	1.92	0.18

bold indicates significance difference p < 0.05, *italics* indicates marginal significant difference p < 0.10, different letters indicate difference between groups.

Figure 1.
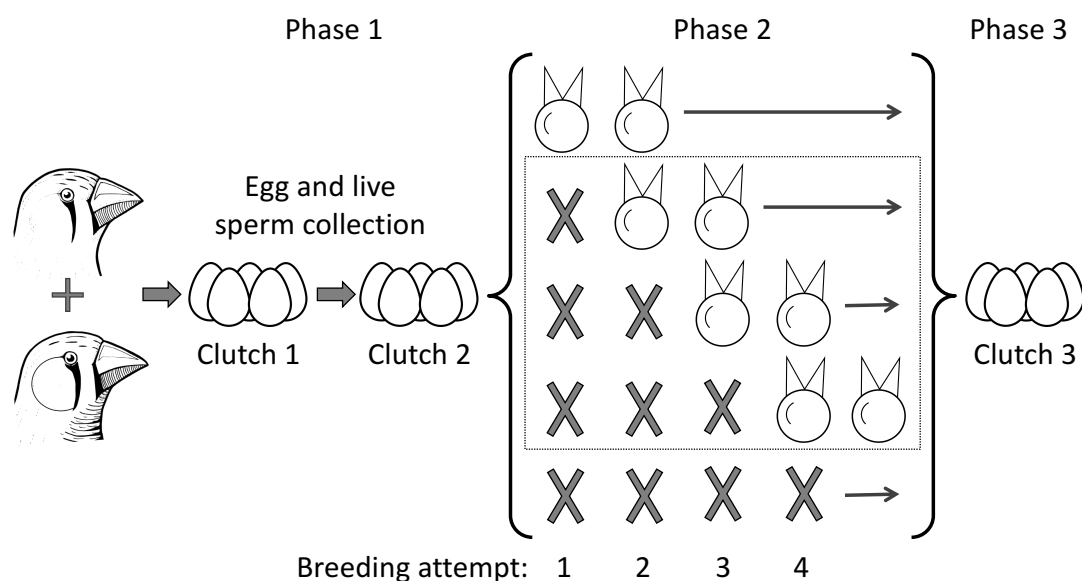
Figure 1. Experimental set up. Pairs were formed using virgin birds. Phase 1: The first two clutches of eggs they produced were collected for PVL analysis. Phase 2: They were then given 4 breeding attempts to produce a clutch of eggs that resulted in fledglings. Pairs were successful (chick head) on the first attempt, later (in box) on a subsequent attempt after failing (X) one or more attempts, or never. If pairs reared a clutch to fledging they were allowed to produce a second, which was always successful. Phase 3: After breeding attempts were completed, a third clutch of eggs was collected for PVL analysis.

Figure 2.
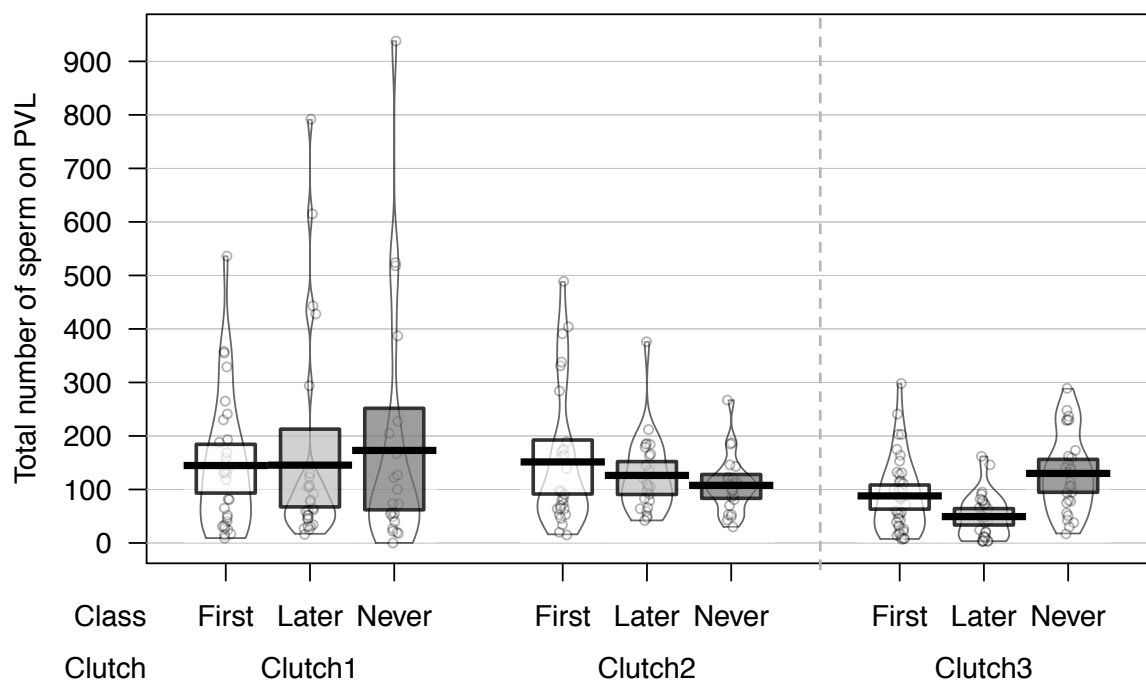
Figure 2. Total number of sperm on outer PVL with experience. Clutch 1 and clutch 2 were first ever produced for pair and individual. Clutch 3 was produced after attempts to rear chicks to fledge (hatched line), which pairs did successfully on the first attempt (white bar), a later attempt (light grey), or never (dark grey). Circles represent raw data points with bean smooth density outline, bar denotes central tendency, box shows 95% high density interval.

Figure 3.
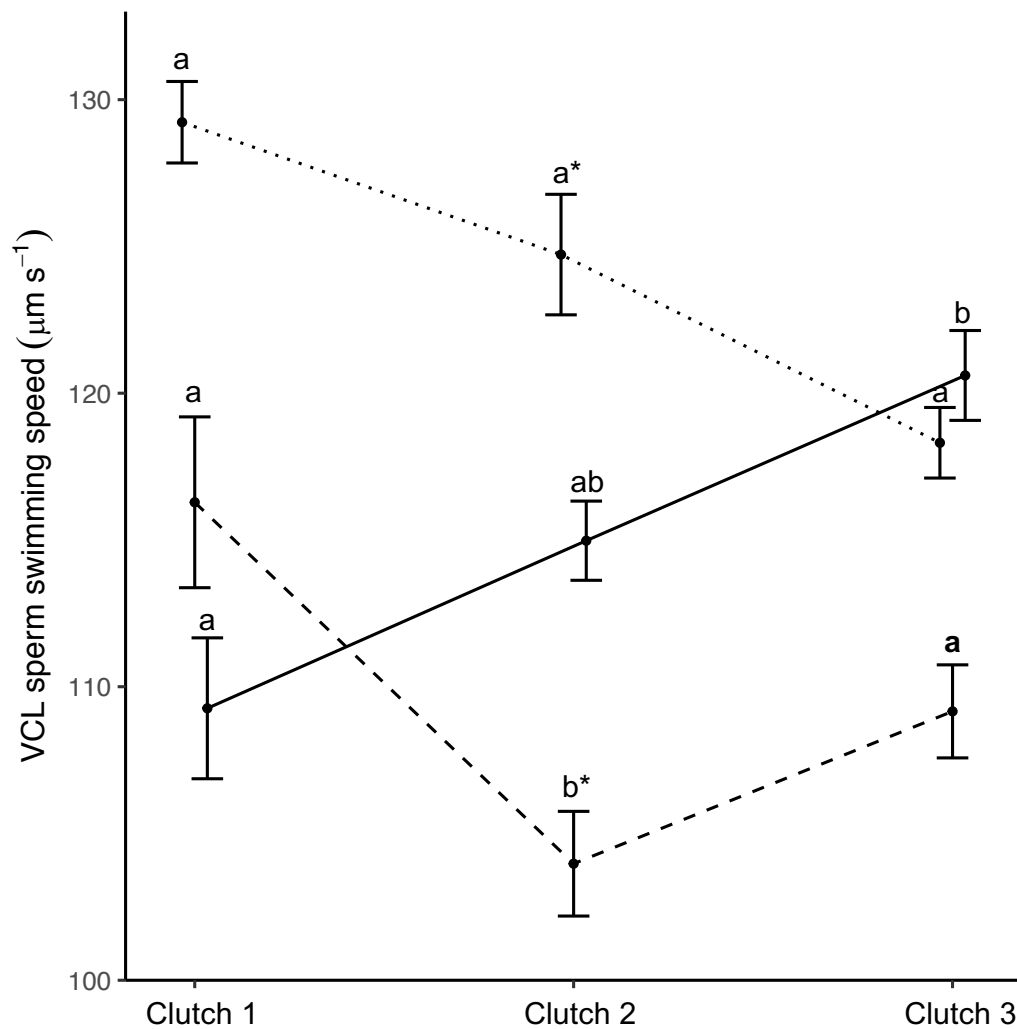
Figure 3. Sperm swimming speed (VCL: μms^{-1}) with experience. Clutch 1 and clutch 2 were first ever produced for pair and individual. Clutch 3 was produced after attempts to rear chicks to fledge, which pairs did successfully on the first attempt (dotted line), a later attempt (dashed line), or never (solid line). Letters denote statistical significance between means within a success class across clutches. Bars with the same letter are not significantly different from one another, while different letters represent significant differences ($p < 0.05$). Asterisks denote between group significant difference at a given clutch ($p < 0.05$). Error bars $\pm$ SE.

Chapter Five

Variation in reproductive tract development and sperm performance across a single reproductive cycle in zebra finch

Chapter Five Vignette

In the previous chapter I have shown how experience and breeding success with a partner can alter the ejaculate-female and sperm-egg interactions. To examine how male ejaculate related to sperm reaching the ovum I collected sperm from males on the day the third egg in a clutch was laid. I did this to standardise the sample time point, but since zebra finch females can store sperm for 10+ days (Birkhead et al. 1989) sperm used in fertilising the eggs collected could have come from a much earlier copulation, and therefore ejaculate type. This raised the question of how sperm performance and morphology in a male's ejaculate may change over time. Most of the species in which investigations of sperm competition (both within and between males) and sperm performance have been conducted are from the northern hemisphere where breeding is more seasonal and predictable than in most Australian species (e.g. Cramer et al., 2014; Laskemoen et al., 2013). Additionally, studies with zebra finch sperm have been done on captive birds under controlled conditions (e.g. Birkhead 2010; Hemmings et al. 2016). Therefore, in this chapter I strive to describe the change in gonadal development in both male and female zebra finches across a single reproductive cycle, and how assessment of sperm performance in the ejaculate and peripheral testosterone can differ depending on when in the cycle the sample is taken. The goal is to provide a guide for interpreting ejaculate and hormone samples taken from a population where the reproductive state of the individual is unknown.

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Research Article

Variation in reproductive tract development and sperm performance across a single reproductive cycle in zebra finch

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Abstract

Many Australian bird species demonstrate prolonged and variable periods of reproduction across the year, which is in stark contrast to birds in the northern temperate zones that have predictable and often short springtime breeding. The majority of studies looking at reproductive phenology in birds – including change in reproductive organs, hormones, and sperm production – have focused on northern hemisphere temperate species. Work that has been done on Australian species often occurs in controlled environments or by assigning reproductive state (e.g. breeding or non-breeding) to an individual based on observations of the population as a whole. Here we describe changes in reproductive morphology and physiology in the zebra finch, *Taeniopygia guttata*, in females and males at time points across a single breeding cycle. We found a dramatic change in female reproductive tract following egg laying. Testes of paired males were largest and heaviest during pre-breeding and gradually declined across the cycle so that they were significantly smaller by mid incubation and lighter when chicks fledged. In contrast, males that were unpaired only had smaller and lighter testes than paired males before incubation. Post-hatch males had slower sperm than incubating males, and less motile sperm than nesting, incubating and unpaired males. Testosterone varied significantly across the cycle, potentially influencing sperm performance, but not in direct relation to testes size. Therefore, we conclude that it is important to know the reproductive state of opportunistic breeders when taking samples or measurements related to reproduction, and suggest that researchers need to think beyond the classic northern temperate avian reproductive model when interpreting data gathered from bird species outside the northern temperate zone, that multi-brood, or are flexible in their breeding response.

Introduction

The zebra finch, *Taeniopygia guttata*, is often put forth as the iconic opportunistic breeding bird, depicted as a species that breeds less seasonally and responds primarily to non-photic cues to stimulate reproduction (Hahn et al., 1997; Hahn et al., 2008; Zann, 1996). This is a relatively northern temperate zone hemisphere-centric view of bird species, in that most species in these regions breed on a relatively rigid seasonal schedule dictated primarily by photoperiod producing one or at two clutches in that season (figure 1A) (Charmantier et al., 2008; Hahn et al., 1997). These temperate species also often show a predictable pattern of dramatic recrudescence and regression of their reproductive tract at the beginning and end of the breeding season (Davis and Davis, 1953; Jenkins et al., 2007), which is often viewed as an energy conservation measure for flight (Deviche et al., 2011; Johnson, 2011). A few species show some flexibility to their reproduction, but this is more the exception than the rule and often relates to variation in timing of full development and regression of the gonads (Hahn et al., 2009).

A recent analysis of breeding records shows that breeding in most Australian species is variable and opportunistic across the year and in different biomes, with many species breeding year round (Duursma et al., 2017). The breeding periods for these species are typically more than twice as long as those seen in avian species in the northern hemisphere temperate zone (Duursma et al., 2017). This means that many of these species show a less generalised population synchrony to their reproductive timing (figure 1B), with spikes in reproductive activity varying across the country and even within a population. For example, in the zebra finch, whilst breeding occurs on a very flexible and protracted time scale, there still tends to be a rough seasonality to when the majority of the population breeds or does not breed (Zann, 1996; Zann et al., 1995). These breeding periods shift depending on the unpredictability of rainfall and range of temperatures in the region of Australia where the birds are studied (Duursma et al., 2017; Zann, 1996; Zann et al., 1995). Similarly, the number of pairs breeding during any given period in a region can shift across the year (Zann, 1996), with some level of

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breeding activity when average ambient temperatures are as low as 2.2ºC (Zann, 1996), and up to and in excess of 36ºC (Griffith et al., 2016). Flexible breeding such as this would require development of the gonads to be much more constant or flexible than that seen in northern temperate breeders. A major challenge for research is that in such opportunistic populations, the level of synchrony across breeding pairs is relatively low, and different individuals are at various stages of reproduction. Even in a population in which breeding is actively occurring, a reasonable proportion of adults might be in a non-breeding phase.

The limited study of gonad size in wild zebra finches suggests that reproductive organ size also differs between birds in temperate environments and those in unpredictable arid environments (a large portion of the zebra finches' range), with males and females in the latter maintaining a more activated reproductive system in what was inferred to be a non-breeding period (Perfito et al., 2007). One study examining domestic females shows that oviduct regression occurs even as the last egg is being laid (Williams and Ames, 2004). Additional studies on wild caught and captive birds have revealed some effects of dehydration, humidity and the state of local vegetation on testes (Priedkalns et al., 1984; Sossinka, 1974; Vleck and Priedkalns, 1985), and ovary size (Sossinka, 1974). However, spermatogenesis did not appear to be greatly impacted by these same parameters (Priedkalns et al., 1984; Sossinka, 1974; Vleck and Priedkalns, 1985) unless the testes significantly regressed (Priedkalns et al., 1984).

Most sperm morphology and competition studies have been conducted in the context of the highly predictable and synchronised northern hemisphere temperate birds (e.g. Calhim et al., 2009; Cramer et al., 2014; Laskemoen et al., 2007; Laskemoen et al., 2013; Lifjeld et al., 2012). Therefore, when wild populations have been sampled, the breeding stage (from pre- to active to post- breeding) of the individual and the population as a whole is relatively equivalent. This means that relatively clear comparisons can be made both between and within populations and individuals, and within and between years with respect to sperm measures (i.e. variability in morphology, quality, quantity, etc). In the laboratory, studies of sperm

competition in the zebra finch can circumvent this potential problem, by comparing individuals at a defined point in the breeding cycle (Bennison et al., 2015), but it is not always clear they have (Birkhead and Fletcher, 1995; Mossman et al., 2009). In the wild, however, the most comprehensive study of reproductive physiology to date, addressing the change in wild zebra finch gonad size – and thus spermatic activity – inferred reproductive state (early, late or non-breeding) from general nest activity and the presence and age of juveniles in the broader local population (Perfito et al., 2011; Perfito et al., 2007). However, as discussed above, the reproductive state of the general populous of opportunistic breeding birds may not reflect an individual's breeding state, and the specific breeding stage differs greatly between individuals. Observations could be further confounded by the fact that zebra finches have a short juvenile refractory period, and are normally considered sexually mature at ~70 days of age (with most males producing mature sperm) (Sossinka, 1970). This means that birds can breed the same year that they are born, which is uncommon in seasonal breeders with prolonged juvenile refractory periods.

Here we characterize gonadal changes in both male and female zebra finches across a single reproductive cycle to describe how they alter reproductive tract investment as energy demands shift across the cycle. Our findings provide important insight into the reproductive flexibility of this well studied species, and help to identify important components of physiological and morphological variation in birds that have more protracted reproductive periods.

Materials and methods

36 pairs of zebra finches, all approximately 18-24 months of age (bred at Macquarie University under similar conditions), were formed prior to the start of the experiment by forced pairing, and had laid at least one clutch of eggs together. From February to May 2015 pairs were moved into one of 12 outdoor aviaries (0.95 x 1.9 x 1.8 m) that physically, but not

visually or vocally separated them from other pairs. Pairs were randomly assigned to one of

six time points (n=6 per time point), so that each time point was represented across the

duration of the experiment. The six time points were spread across the breeding cycle so

samples were taken at pre-breeding, nest building, third day of egg laying, mid-incubation, six

days post hatch, and fledging (day after first fledgling was noted out of nest). We chose to not

sample at first egg laying and hatching days to ensure pairs were committed to the

reproductive stage (i.e. laying a full clutch and rearing chicks). A seventh time point of

unpaired males was formed (n = 6) by visually and acoustically separating them from females

in groups of three per aviary for 6 weeks before sampling in June 2016. All birds were given a

fortnight to acclimatise to the aviaries with no nest box or nesting material before the start of

the experiment, at which time these were provided (with exception of pre-breeding and

unpaired males). All procedures were conducted according to relevant national and

international guidelines and were approved by the Macquarie University Animal Ethics

Committee (Animal Research Authority 2013/29).

The day birds reached their pre-assigned time point, sperm samples were taken from

males vial cloacal massage (Wolfson, 1952), which were then recorded and later analysed to

assess the proportion of motile sperm and sperm swimming speed (VCL) (Supplemental 1)

(Chapter 6; Rowe et al., 2015). The following day the pairs were caught and bled in < 5

minutes of disturbance for hormone analysis, with resulting samples processed at Deakin

University (Supplemental 1: only male data presented) (methods as per Crino et al., 2017).

Samples were taken on separate days to insure quality of sperm and blood samples. It is

required to take blood shortly after capture to prevent influence of handling stress of results

and as euthanasia was an end point ~150μl of blood was collected. Therefore collecting sperm

the day before guaranteed a non-stressed sample was collected. Following blood collection,

males where then euthanized using deep anaesthesia with Isoflurane until breathing stopped.

In quick succession the brain was removed, the testes were dissected out into separate pre-

weighed weigh boats where they were measured and weighed before fixation for later

histology (brain and testis histology data not presented here). Females were similarly treated, taking the diameter (2 times 90 degrees apart) of largest ovarian follicle measurements and dissecting out and weighing the oviduct (cut off at cloacal juncture). If a developing ovum was present in the oviduct it was removed before weighing.

All measures were compared using linear mixed models (*lme4* package, R v. 3.3.3 (R Core Team, 2017)) , oviduct weight (ln transformed), follicle volume, proportion motile sperm (logit transformed), or sperm swimming speed (VCL) as the explanatory variable and sampling time point as fixed effect. Following Pearson's correlation to check for outliers, testes mass (ln transformed) and volume models included the interaction between time point and side as fixed effects and male identity as a random effect. Additionally, we used Pearson's correlation tests to look at relationship between testosterone and VCL, proportion motile sperm, and testes mass and volume. Lastly, we tested for change in the proportion of asymmetry between left and right testes in volume and mass (both proportions logit transformed) with time point as a fixed effect. Post hoc testing was done by iteratively changing reference levels of time point. Scaled mass index (SMI: calculated using tarsus length and body mass: Peig and Green, 2009) was included as a covariate in all models to correct for size differences, but there was no significant difference in SMI in males ($F_{6,35}$ = 0.81, p = 0.57) or females ($F_{5,30}$ = 0.84, p = 0.53). Variability between time points was determined by calculating coefficient of variation (CV) for each response variable and then visually examined for overlap of confidence intervals (CI: calculated using 1000 bootstrap samples and corrected for bias). Figures were constructed using *ggplot2* (Wickham, 2009), and modelling assumptions (normality and heterogeneity of variance of residuals) were assessed visually (following Zuur et al., 2009). All tests were two-tailed and considered significant at α < 0.05, with significance estimate via *lmerTest* (reported as t-value) (Kuznetsova et al., 2016). Data presented are mean ± standard deviation unless otherwise noted.

Results

Testes mass and volume were positively correlated, (r (82) = 0.96, p < 0.001: Figure S1), and there was no significant difference in proportion of testis asymmetry in volume ($F_{6,32}$ = 0.94, p = 0.48) or mass ($F_{6,33}$ = 0.44, p = 0.84) across the study.

Males had a significant difference in mass between left and right testes at all time points ($F_{1,33.0}$, p < 0.001). Left testis mass peaked at pre-breeding (Figure 2A: 31.7 ± 5.8 mg), and significantly decreased by fledging (20.9 ± 4.6 mg) (t = -2.39, p = 0.02). Right testis mass peaked during egg laying (Figure S2: 21.8 ± 7.4 mg), and significantly declined by fledging (14.7 ± 6.1 mg) (t = -2.33, p = 0.02). Unpaired males had significantly smaller left testes (Figure 2A: 18.5 ± 8.0 mg) than: pre-breeding (t = -3.22, p < 0.01), nesting (t = -3.08, p < 0.01), egg laying (t = -3.10, p < 0.01), and incubation (t = -2.08, p = 0.04) and smaller right testes (Figure S2: 13.0 ± 5.7 mg) at the same time point: pre-breeding (t = -3.28, p < 0.01), nesting (t = -2.60, p = 0.01), egg laying (t = -2.64, p = 0.01), and mid-incubation (t = -2.14, p = 0.04). When chicks fledged, males had the highest variance in left testis mass (CV = 0.43, *95% CI* = 0.21 to 0.72) and right testis mass (CV = 0.44, *95% CI* = 0.27 to 0.72), but not significantly more than any other group except of the right testis in pre-breeding males which was less variable than all other time points (CV = 0.08, *95% CI* = 0.06 to 0.13).

The left testis volume was always significantly larger than the right (Figure S3: $F_{1,35}$ = 92.8, p < 0.001). The left testis volume decreased from pre-breeding (30.1 ± 4.8 mm^3) to fledgling (19.9 ± 5.1 mm^3) (t = -3.29, p < 0.01). The right testis volume peaked at nesting (20.6 ± 6.5 mm^3), and significantly decreased by fledging (13.6 ± 5.2 mm^3: t = -2.35, p = 0.02). Unpaired males had left testes that were significantly smaller in volume (17.1 ± 5.8 mm^3) than males in: pre-breeding (t = -4.01, p < 0.001), nesting (t = -2.91, p < 0.01), and egg laying (t = -3.21, p < 0.01). The right testes of unpaired males were also smaller (11.6 ± 2.7 mm^3) and showed significant differences from: pre-breeding (t = -1.40, p = 0.02), nesting (t = -2.84, p < 0.01), and egg laying (t = -3.36, p = 0.02). There was no difference in coefficient of

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variance between the volumes of testes on either side, except again right testis volume was less variable than all other time points (CV = 0.08, *95% CI* = 0.05 to 0.12).

Male testosterone (Figure 2B) was significantly higher during nesting compared to unpaired (t = -3.34, p = 0.002), pre-breeding (t = -2.08, p = 0.04), incubation (t = -2.76, p = 0.01), and post-hatch (t = -2.72, p = 0.01). There was significantly higher variation (ca. CV = 1.40, *95% CI* = 0.97 to 2.58) during laying and fledging than all other groups (ca. CV = 0.49, *95% CI* = 0.30 to 0.79). There was no correlation between testosterone and any measure tested: sperm swimming speed (r (35) = 0.10, p = 0.57), proportion motile sperm (r (37) = 0.10, p = 0.53), and testes mass r (37) = 0.13, p = 0.46).

The proportion of motile sperm recorded did not differ overall by time point (Figure 1C: $F_{6,34}$ = 2.05, p = 0.08). However, post-hatching males showed the lowest proportion motility (0.32 ± 0.29), which was a significant decline from nesting (t = -2.23, p = 0.03) and incubating males (t = -2.74, p < 0.01). Unpaired males had the highest average proportion of motile sperm (0.76 ± 0.17), but only significantly more so that post-hatch (t = 3.03, p < 0.01). Post-hatch period males had the highest variability in motility (CV = 0.93, *95% CI* = 0.45 to 1.49), followed by fledging males (CV = 0.70, *95% CI* = 0.37 to 1.11). Both of these groups had significantly more motile sperm than in males during the egg laying period (CV = 0.24, *95% CI* = 0.16 to 0.36), and unpaired males who had the least variability in motility (CV = 0.22, *95% CI* = 0.12 to 0.35).

There was a significant effect of scale mass index on sperm swimming speed (VCL) ($F_{1,26}$ = 7.69, p = 0.01), but this did not interact with sampling time point ($F_{6,26}$ = 1.28, p = 0.30). Similarly, sperm swimming speed (VCL) did not differ across the breeding cycle, except for a decline from incubation to post-hatch (Figure 2D: t = -2.19, p < 0.04). This was driven by a moderate increase in speed during incubation (106.3 ± 24.1 μm s^{-1}) followed by a moderate decrease in post-hatch speed (86.0 ± 13.5 μm s^{-1}) compared to all other groups (ca. 96.0 ± 14.9 μm s^{-1}). During egg laying, males had the lowest coefficient of variance at 0.07 (*95% CI* = 0.03 to 0.12), this was significantly lower than incubation (CV = 0.22, *95% CI* =

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0.16 to 0.34) and marginally so from fledging (CV = 0.22, *95% CI* = 0.12 to 0.38) and unpaired males (CV = 0.14, *95% CI* = 0.11 to 0.21) (Table S1).

In females, follicle volume (figure 3A) showed a dramatic decline between egg laying and mid-incubation. Follicular volume peaked during laying (73.5 ± 63.4 mm^3) declining at mid-incubation (2.0 ± 0.7 mm^3) (t = -2.40, p = 0.02. The coefficient of variance (Table S1) was highest at the fledging sample at 1.84 (*95% CI* = 1.23 to 3.75), which was significantly greater than in females at mid-incubation (CV = 0.31, *95% CI* = 0.22 to 0.47) and post-hatch (CV = 0.42, *95% CI* = 0.20 to 0.74). Pre-breeding females also had a high coefficient of variance at 1.21 (*95% CI* = 0.58 to 2.01), which was significantly higher during incubation.

Oviduct mass (figure 3B) also showed a dramatic decline between egg laying and mid-incubation. Oviduct mass similarly peaked during lay (0.42 ± 0.13 g) declining at mid-incubation (0.06 ± 0.03 g) (t = -5.54, p < 0.001). There was no significant variation in oviduct mass across the reproductive cycle, but it was highest in the pre-breeding stage (CV = 0.71, *95% CI* = 0.35 to 1.19) and lowest during egg laying (CV = 0.31, *95% CI* = 0.15 to 0.56).

Discussion

In the short predictable breeding season of northern hemisphere temperate breeding birds, we expect to see a correlation between increased testes size, testosterone levels, and sperm production (Dawson et al., 2001; Deviche et al., 2011; Ramenofsky, 2011). Contrary to this, our results suggest that zebra finch reproductive activation and deactivation is variable across a single reproductive cycle, with the male and female responses varying in the degree of change. We found no evidence of a significant change in the proportion of asymmetry between left and right testes across the breeding cycle as hypothesised by Møller (1994), but different peaks in maximum size (with right lagging behind left) suggest differential activation of each testis (Birkhead et al., 1998). However, as expected, the left testis was always larger,

and given that laparotomy based studies typically only measure it (Hurley et al., 2008; Perfito et al., 2007), we will primarily discuss changes in the left testis.

Unpaired males had significantly smaller and lighter testes than paired males in early stages of reproduction (pre-breeding, nesting, and egg laying), showing an increase of 41.6% in mass and 43.1% volume of the left testis at pre-breeding. This is half the ~80% change from non-breeding to breeding zebra finch males sampled in a predictable environment, but closer to the ~50% change of the unpredictable environment males (presuming same maximum recrudescence, as there was no breeding measure in the unpredictable environment birds) (Perfito et al., 2007). In our birds, once breeding pairs finished egg laying there was a gradual decline in both measures, to a point that did not significantly differ from unpaired males. Specifically, across the breeding cycle left testis mass and volume peaked during pre-breeding, and both declined about a 34% by fledging (figure 2A and S3A). However, volume was already significantly 23.7% smaller by mid-incubation (figure S3A). These findings suggest that, if testis volume measures alone are taken in a cross section of males in an asynchronous breeding populations there would be difficulty in clearly delineating between non-breeding birds and those in the latter part of a reproductive cycle.

Change in testes mass and volume did not appear to directly impact sperm function, except during the post-hatching period when there was a significant decline in proportion motile and sperm swimming speed. The fact that viable sperm was always produced is not surprising given that testes that greater than 3 mm in length are considered spermatogenic (shortest length measured was 3.39 m in a unpaired male) (Priedkalns et al., 1984). This implies that something else is influencing sperm performance. This could be due to change in production or the length time (i.e. age) of the sperm being stored at a given time point due to changes in copulation rates. Both of these measures could be influenced by the changing levels of testosterone, with a change in production impacting testis mass and volume.

Testosterone in northern hemisphere seasonal breeding birds is correlated with changes in testes development and regression (Denk and Kempenaers, 2005; Hurley et al., 2008;

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Wingfield et al., 1990), even in a flexibly breeding species (i.e. can slightly adapt timing of reproduction to optimize survivial of young: Deviche et al., 2006). Further, the current belief is that variation in testosterone levels during the reproductive cycle differs in single and double brooded species and with the amount of competition and parental care (Wingfield et al., 1990). These changes in testosterone often coincide with an increase in production and maturation of sperm (Jones and Lin, 1993). However, the link between peripheral testosterone levels and sperm quality is unclear. Treatment with exogenous testosterone appears to have no effect (fowl: Cecil and Bakst, 1986) or limited/reducing effect on ejaculate volume (song bird: Kast et al., 1998). The latter was potentially due to increases in copulatory behaviour (Kast et al., 1998), so if the inverse occurs it could explain the disuse effect on sperm performance. One study in ducks showed that increased plasma testosterone correlated with both total number and proportion of sperm with normal morphology (Penfold et al., 2000), which could explain the differences we saw in sperm performance, especially the proportion of motile sperm.

In our birds, peripheral testosterone did not directly relate to testes size, as testosterone was low in pre-breeding birds while they had testes as large and heavy as when they have high testosterone during nesting (figure 3A and B). However, there is a prolonged drop in testosterone from after egg laying to post-hatching, which perhaps explains the decrease in sperm performance seen during the post-hatching period. Further, if a decrease in testosterone leads to a decline in spermatogenesis it could explain the observed smaller testes mass and volume seen by the time the current brood fledges. Therefore, the high variation in testosterone levels at egg laying and fledging periods (figure 3B), likely represents differential levels of activation and inactivation of sperm production/maturation by a male at these time points. However, we still need to investigate what is occurring on a spermatogenic and structural level in the testes at these time points to see how/if changes in peripheral testosterone relate to these changes.

Across a single reproductive cycle, females showed a dramatic reduction in their follicular and oviduct development once egg laying was complete, similar to that found previously (Williams and Ames, 2004). Variation in both follicular and oviduct development was high during pre-breeding stage, with follicular volume varying the most during the fledging period. The deactivation of the female zebra finch reproductive tract after laying eggs is reminiscent of the regression of these tissues in northern hemisphere temperate zone birds (Dawson, 2008; Hurley et al., 2008; Jacobs and Wingfield, 2000; Ramenofsky, 2011). However, the level of regression takes them down to the more primed state of photosensitive birds, not a fully regressed state (Dawson, 2008; Dawson et al., 2001; Ramenofsky, 2011). This could conserve energy (Nilsson and Råberg, 2001; Vézina and Williams, 2002) but allow the females to maintain a state of readiness. This semi-regressed state would allow females to quickly develop their system again once their current brood is independent. Females starting to transition back to a pre-breeding state was evident in the high level of variation in follicular development at the time their current brood fledged. The oviduct development is sensitive to change in circulating hormones (Yu and Marquardt, 1973), so it is likely some key hormones are cycling. Further work needs to be done to relate changes in follicular size and oviduct weight to changes in hormones.

In summary, our examination of reproductive development across a single reproductive cycle in zebra finches revealed a dramatic change in female reproductive tract, and a less pronounced but significant change in male testes size and mass. Having a mate increased male testes size and mass in early reproduction (pre-breeding, nesting and egg laying), but did not change sperm performance. Testes mass and volume decreased during incubation, post-hatching and fledging periods, so they did not differ from unpaired males. Post-hatch males had slower sperm than incubating males, and less motile sperm than nesting, incubating and unpaired males. Males showed pronounced shift in testosterone levels across the reproductive cycle, with significant variation during egg laying and after fledging current brood of chicks. Taken together, these findings suggest that it is important to know what

reproductive state a zebra finch is in when taking samples or measurements related to reproduction. This is especially true when sampling a wild or free breeding captive population where, given that wild zebra finch produce multiple clutches in succession and across the year (figure 1B: Zann, 1996), its current breeding stage may be unknown. Given the extent of opportunistically breeding birds in Australian beyond the zebra finch (Duursma et al., 2017), our findings suggest that researchers need to think outside the framework that has been generated by work on northern hemisphere temperate zone avian species. Birds such as the zebra finch, that are multi-brooded and highly flexible in their breeding response can exhibit significant amounts of reproductive plasticity that is capable of effecting variation in sperm motility and performance. Future work should consider how the morphology sperm change over the reproductive cycle. Total sperm length has been shown to be highly repeatable (Birkhead and Fletcher, 1995) with low variation (Birkhead et al., 2005) in zebra finch. However, given that social context has been shown to change in another estrildid finch (Immler et al., 2010), a closer look should be taken at all morphological measures as it is possible that morphology (at least the amount of variation) may change across reproductive context.

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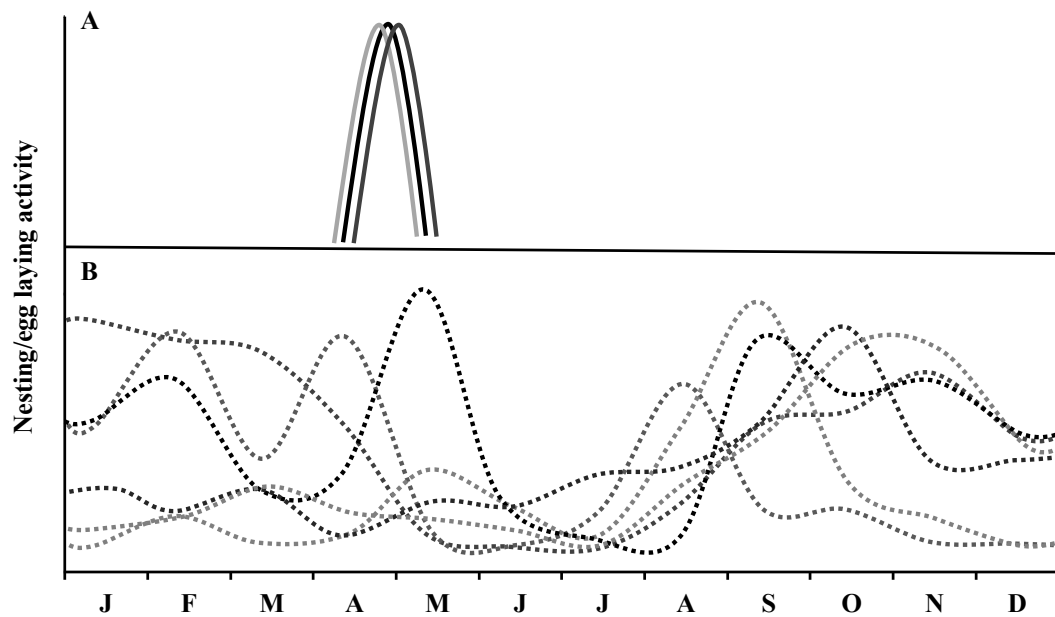
Figure 1.

Figure 1. Breeding activity in A) northern temperate species representative, one population of great tits, *Parus major*, reflecting shift in mean egg laying date over 47 years (adapted from data in Charmantier et al. (2008) figure 1A), B) Australian representative species: six populations of zebra finch around Australia reflecting nesting percentage over one to six years (adapted from Zann (1996) figure 7.1).

Figure 2.
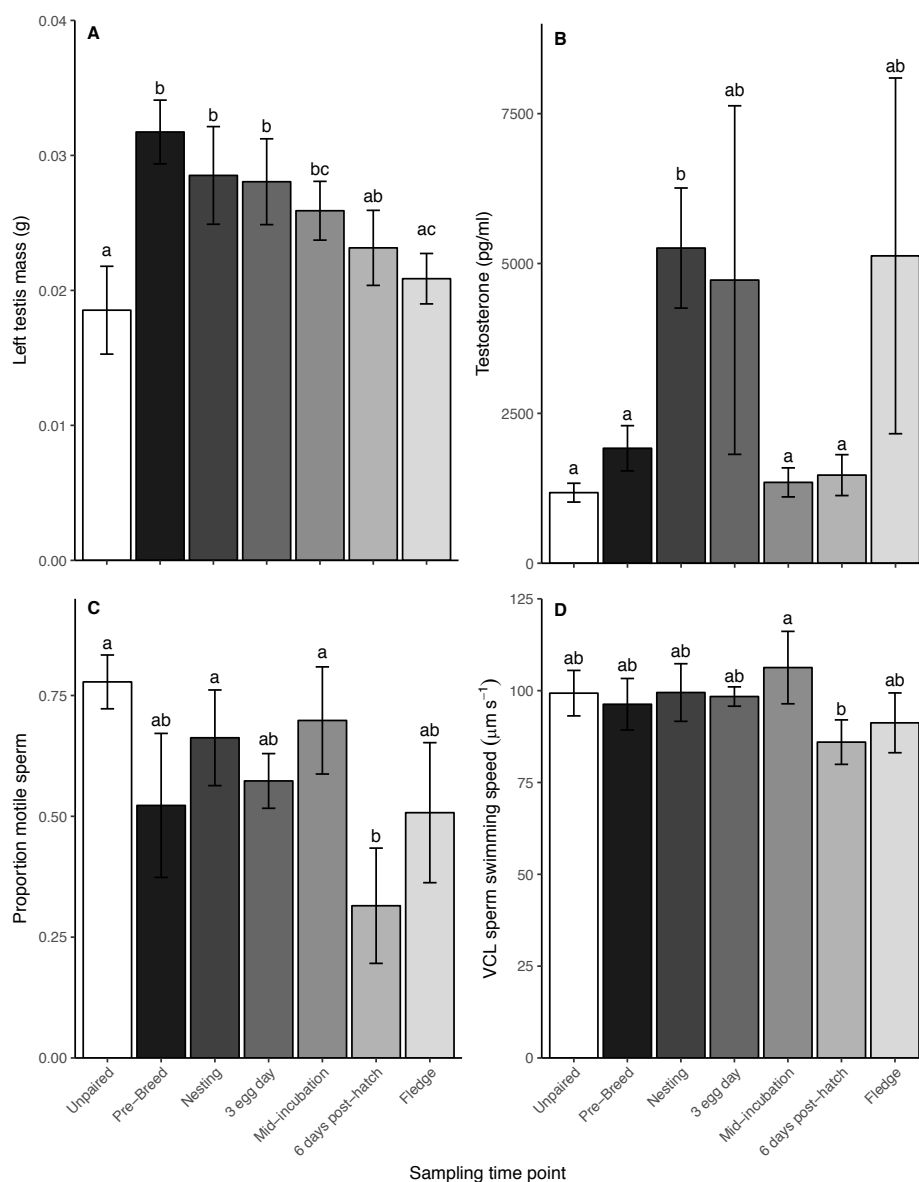
Figure 2. Change in male across a single breeding cycle. A) left testes volume (mm^3), B) testosterone (pg/ml), C) proportion motile sperm, D) sperm swimming speed in VCL (μms^{-1}). Letters denote statistical significance between means at given time points, columns with the same letter are not significantly different from one another, columns with different letters are significant different ($p < 0.05$). Error bars represent ± SE.

Figure 3.
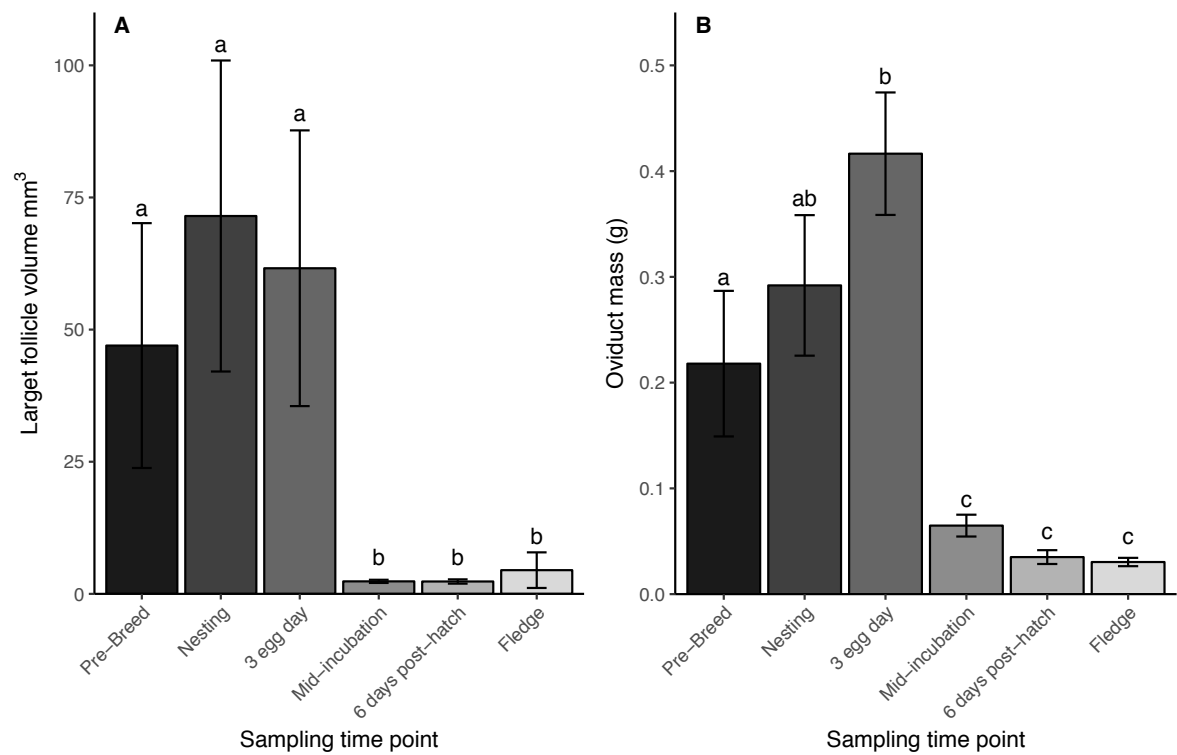
Figure 3. Change in female reproductive tract across a single breeding cycle. A) volume of largest follicle (mm^3), B) ovarian mass (g). Letters denote statistical significance between means at given time points, columns with the same letter are not significantly different from one another, columns with different letters are significant different (p < 0.05). Error bars represent ± SE.

Supplemental 1

Variation in reproductive tract development and sperm performance across a single reproductive cycle in zebra finch

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Methods Supplemental

Sperm sampling

Sperm samples were obtained from males by cloacal massage (Wolfson, 1952), and recorded to calculate proportion motile and sperm swimming speed, and fixed for later quantification of quality and morphology (see Chapter 6 for method). Briefly, sperm were collected and diluted in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd) pre-heated to 40ºC (the approximate physiological temperature of zebra finches: Calder, 1964). Next, 6 μl of the sperm suspension was loaded into a chamber slide (depth 20 μm, Leja®, Netherlands) and sperm motion was recorded for six unique fields of view (5s each for total 30s) at 400x (CX41, Olympus, Japan) using a digital camera (Legria HF G25, Canon, Japan). All samples, chamber slides, and the heated microscope stage (TP-S, Tokai Hit, Shizuoka, Japan) were maintained at a constant temperature of 40ºC throughout.

Sperm motility analysis

Videos of sperm motion were analyzed at a later date using computer-assisted sperm analysis (CASA; HTM- CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research) under the same criterion detailed in Rowe et al. (2015) and Hurley et al. (Chapter 6). In short, the frame rate was set at 50 frames/s, and sperm were tracked for 0.5 s in each field of view, including only sperm tracked for more than 10 frames, with drift corrected based on analysis of a dead sperm recording. Analysis output was visually inspected to remove cases where two sperm

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crossed paths and CASA tracking switched mid-track and to remove debris (i.e. non-sperm particles, such as red blood cells and faecal matter) that was incorrectly identified as a sperm. All processing was done by the same person (MR) and without information about the male's identity. The total number of sperm, number of motile sperm, sperm cells' straight-line velocity (VSL: average velocity on a straight line between the start and endpoint of the sperm track), average path velocity (VAP: average velocity over a smoothed sperm track), and curvilinear velocity (VCL: velocity over the actual sperm track) were recorded.

The total number of motile sperm tracked for each male ranged from 11 to 912 (median = 88.5, mean = 162.4 ± 179.7). We quantified sperm swimming speed as curvilinear velocity (VCL: using the average of all motile sperm per bird for analysis) and the proportion of motile sperm (i.e. number of motile sperm tracks divided by the total number of cells). For VCL, samples with less than 10 motile sperm tracks were excluded from analyses of sperm swimming speed, as ten motile cells represented a minimum threshold at which sample VCL values approximately reached a mean asymptote. This criteria was applied to ensure that sperm speed was adequately estimated, and was determined using a means accumulation approach (see Chapter for methods). The average of all an individual's single cell recordings were used in the analysis.

Blood sampling and processing

On the day of euthanasia, males and females were netted in the aviary and a 75ul blood sample was taken in < 5 min (data for only males are presented here). This was done by pricking the alar vein with a 27G needle and collecting welling blood into 75ul heparinised microhermatocrit capillary tubes. Samples put into 0.5 μl eppendorf tubes on ice until spun (< 1 hr) at 7000 rpm for 10 min to separate plasma. Isolated plasma was stored at -20 ºC until assayed.

Testosterone levels were quantified with Enzyme Immunoassay (EIA) kits (Cat No. ADI 901-065, Enzo Life Sciences). We used an adjusted protocol to assay the raw plasma samples using half the volume of all the reagents supplied with the EIA kits (methods as per Crino et al., 2017). An external standard of 500 pg/ml was run on every plate and used to calculate inter-plate variation. All samples and standards were run in triplicate. Plates were read on a FLUOstar Omega microplate reader at 405 nm corrected at 570 nm. Levels of testosterone were determined from a five-point standard curve ranging from 2,000 to 7.81 pg/ml. Intra- and inter-plate variation was 3.49 and 19.24% respectively. All assays were run blind to information about clutch number or success by the same person (OLC).

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Supplemental 2

Variation in reproductive tract development and sperm performance across a single reproductive cycle in zebra finch

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Table and Figures supplement

Table S1. Proportion motile sperm and sperm swimming speed (VCL) coefficient of variance (CV) with lower and upper 95% confidence intervals (CI). Letters denote significantly different CIs between groups rows, where the same letter are not significantly different from one another, and rows with different letters had significantly different CIs.

Stage	Proportion motile				Sperm swimming speed (VCL)			
	CV	CI_{low}	CI_{up}	sig	CV	CI_{low}	CI_{up}	sig
Pre-breed	0.70	0.10	1.33	ab	0.16	0.06	0.34	ab
Nesting	0.37	0.08	0.72	ab	0.19	0.11	0.33	ab
Laying	0.24	0.16	0.36	a	0.06	0.03	0.12	a
Incubation	0.39	0.14	0.72	ab	0.23	0.16	0.34	b
Post-hatch	0.93	0.45	1.49	b	0.16	0.09	0.28	ab
Fledging	0.70	0.37	1.11	b	0.22	0.12	0.38	b
Unpaired	0.22	0.12	0.35	a	0.14	0.10	0.21	ab

Figure S1.
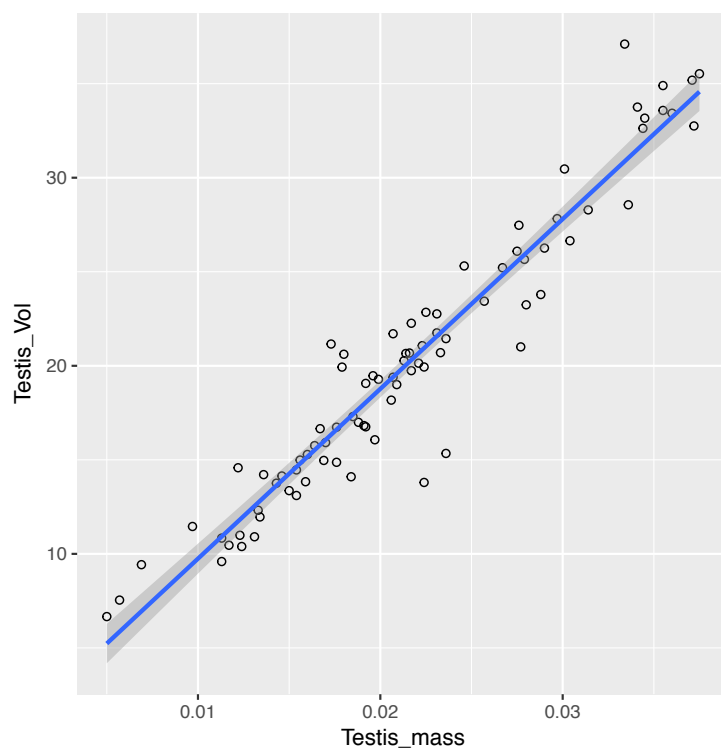
Figure S1. Correlation between testis mass and volume (both left and right side), with regression line and 95% confidence interval (shaded region).

Figure S2.
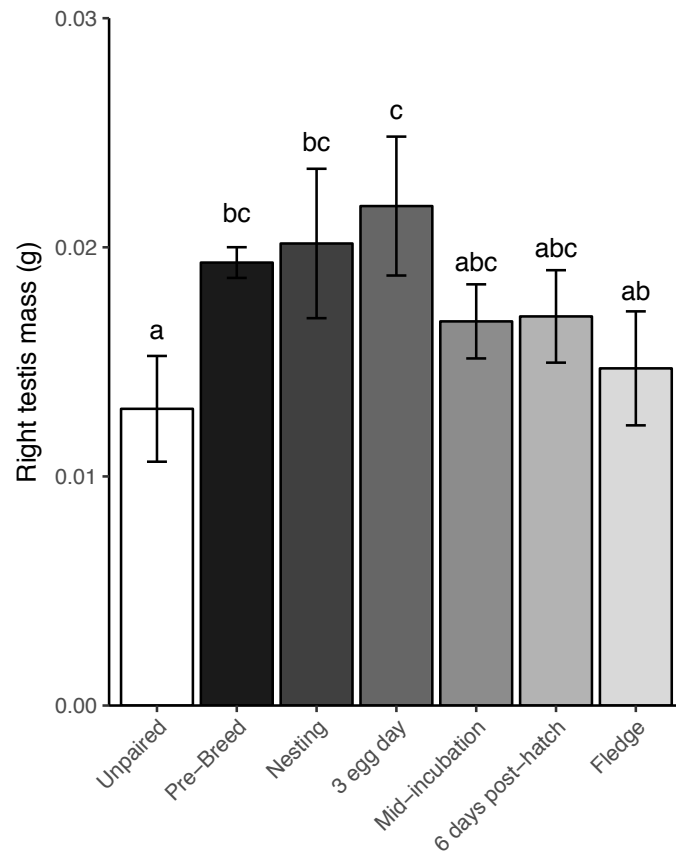
Figure S2. Change in right testis mass across reproductive cycle. Letters denote statistical significance between means at given time points. Columns with the same letter are not significantly different from one another, columns with different letters are significantly different ($p < 0.05$).
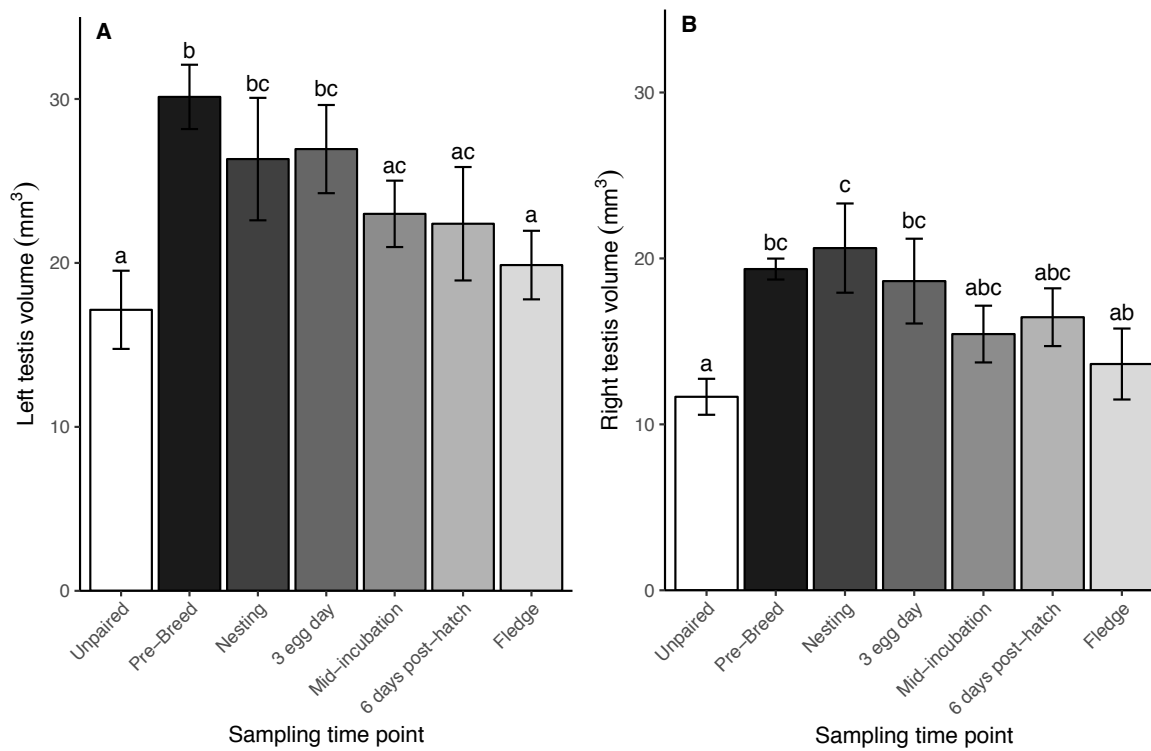
Figure S3. Change in testis volume over time in A) left testis and B) right testis. Letters denote statistical significance between means at given time points, columns with the same letter are not significantly different from one another, therefore, columns with different letters are significantly different ($p < 0.05$).

Chapter Six

Experimental heatwaves negatively impact sperm quality in the zebra finch

Chapter Six Vignette

In previous chapters I described my examinations of the zebra finch and the normal variation in the number of sperm that reach the ovum, how sperm morphology and performance differ with experience and success, and how sperm performance changes across a reproductive cycle. The variation between pairs in sperm numbers reaching the ovum revealed in the research presented in Chapter 2 and 4 is likely to do with differential sperm performance based mainly on genetic-based morphological traits of the sperm (Simmons and Fitzpatrick 2012, Pizzari and Parker 2009). The changes seen in sperm performance across the breeding cycle likely reflects a change in the internal environment in the male (e.g. change in production and length of time the sperm has been stored) (Reinhardt et al. 2015). How changes in external environmental factors impact sperm performance (i.e. sperm ecology) has largely been ignored (Reinhardt et al. 2015). Work concerning temperature and avian reproduction has mainly focused on the shift in reproductive timing in northern hemisphere birds (Visser et al 2009), but it has been seen that temperature impacts embryonic development in zebra finches (Griffith et al. 2016). In this chapter, I investigate the impact of ecologically relevant elevated temperatures on cloacal temperature and sperm morphology and motility in zebra finch, highlighting the potential role of temperature in determining male fertility in birds. Understanding how high temperatures impact on sperm performance, and ultimately fertility, is vital given the increased frequency of heatwaves in our warming world.

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Experimental heatwaves negatively impact sperm quality in the zebra finch

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Abstract

For sexually reproducing species, functionally competent sperm are critical to reproduction. While high atmospheric temperatures are known to influence the timing of breeding, incubation, and reproductive success in birds, the effect of temperature on sperm quality remains largely unexplored. Here, we experimentally investigated the impact of ecologically relevant elevated temperatures on cloacal temperature and sperm morphology and motility in zebra finches *Taeniopygia guttata*. We periodically sampled males exposed to 30 or 40°C temperatures daily for 14 consecutive days. Following a 12-day (23ºC) recovery period, birds were exposed to heat a second time, but under the alternate treatment (i.e. birds initially exposed to 40ºC, were now exposed to 30ºC). Elevated ambient temperatures led to a reduction in sperm quality and an increase in cloacal temperature; though these effects were most notable under 40ºC conditions and were influenced by the duration of heat exposure and prior exposure to high temperature. Our findings highlight the potential role of temperature in determining male fertility in birds, and perhaps also in constraining the timing of avian breeding. Given the increased frequency of heatwaves in a warming world, our results suggest the need for further work on climatic influences on sperm quality and male fertility.

Keywords: avian reproduction, climate change, heatwave, male fertility, sperm swimming speed, sperm morphology

Introduction

Normal sperm function is critical to reproductive success as only functionally competent sperm are capable of fertilising an egg. Over the past few decades, understanding how post-copulatory sexual selection (i.e. sperm competition) shapes sperm morphology and performance has been a key focus of studies in a wide range of taxa. Indeed, it is now widely accepted that sperm competition can drive evolutionary changes in sperm size, morphology, swimming speed, metabolic performance, viability, and longevity [1, 2]. In contrast, the impact of external environmental effects on sperm function has received relatively little attention in the evolutionary and ecological literature. This is surprising given the considerable body of evidence suggesting that a range of environmental factors (e.g. temperature, diet, UV radiation) may impact sperm quality in both external and internal fertilising species [reviewed in 3].

Given warming global temperatures and an increased frequency of heat waves [4], it may be particularly important to understand the consequences of temperature variation for sperm function and quality. Environmental temperature variation affects organisms across all life stages, and is known to influence animal physiology, behaviour, and global distribution. In birds, ambient temperature influences breeding phenology (e.g. [5]), incubation behaviour (e.g. [6]), and reproductive success [7]. In some taxa, temperature has also been shown to influence sperm function and fertilizing ability. For example, in mammals, exposure to high ambient temperatures leads to decreased sperm motile performance and increased levels of morphological defects [8, 9]. Heat stress has also been linked to male infertility in poultry [10, 11]. The effect of high ambient temperatures on avian sperm quality, however, has rarely been considered (see [10-12] for exceptions in domestic poultry).

The zebra finch *Taeniopygia guttata* has served as a model system for the study of avian sperm biology and sperm competition [13]. In the wild, zebra finch are opportunistic breeders, capable of breeding year round given suitable environmental conditions [14]. Furthermore, breeding activity is known to occur in average ambient temperatures as low as

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2.2ºC [14] and up to, and in excess of 36ºC [15]. However, for all birds in the Australian arid zone, including the zebra finch, breeding activity appears to be somewhat suppressed during the hot summer months [16]. At one site, even in late spring, during a period of active breeding, the ambient temperature within nest chambers was regularly found to be over 40°C and even exceeded 50°C [15]. Thus adult male zebra finches are, on occasion, naturally exposed to high ambient temperatures that may negatively affect sperm quality. Here, using domesticated zebra finches, we experimentally investigated the impact of elevated ambient temperatures on avian sperm function. Furthermore, we examined how the duration of heat exposure and prior exposure to elevated temperatures might affect potential temperature related changes in sperm function. Finally, we assessed whether sperm function was restored when birds were returned to milder ambient temperature conditions.

Methods

(a) Experimental design

In 2016, 20 male zebra finches from a captive population at Macquarie University (Sydney, Australia) were housed indoors in single-sex cages (dimensions 0.7 × 0.5 × 1.3 m, 5 males/cage) under baseline climate-controlled conditions (23ºC, ~50% humidity, 12L:12D cycle) with *ad libitum* food and water. All birds were sexually mature (15-18 months of age), hatched under the same conditions, and were previously maintained under identical housing conditions in outdoor aviaries. Birds were randomly selected from a single similar-aged cohort, which lead to inclusion of some siblings (controlled for in the analyses). Throughout the experiment males were kept in visual and vocal contact with females; this was achieved by placing a cage housing five female zebra finches immediately adjacent to the male cages, such that there were two cages of five males each separated by a cage of females.

Following a three-week acclimation period at baseline conditions, males were randomly allocated to one of two heat treatment groups: (1) 40ºC, and (2) 30ºC (~50% humidity for both treatments). We choose these temperatures as they are representative of (1)

relatively normal maximum daily temperatures experienced during active breeding periods (30ºC), and (2) extreme heat conditions experienced intermittently during active breeding periods (40ºC). We choose not to exceed 40ºC temperature treatments in order to minimise the likelihood of birds dying, as death has been shown to occur if sustained body temperatures reach 45-46ºC [14]. Immediately prior to the treatment (i.e. 0700-0800 on day 1), we collected sperm (see below) and measured cloacal temperature by gently inserting an internal probe thermometer (QM1601, Digitech, TechBrands, Australia) into the cloaca, and measured tarsus length and body mass. Birds were then exposed to the heat treatment (30 or 40ºC) for an 8-h period each day for 14 consecutive days. Outside of these periods, birds were maintained under baseline conditions. To avoid temperature shock, birds in the 40ºC treatment were acclimatised by initially being held at 30ºC for a 30-m period, after which time the temperature was increased to 40ºC. We collected sperm and measured cloacal temperature and body mass throughout the experiment on days 3, 7, 11, and 14; in these instances all samples were collected after birds had been exposed to experimental temperatures for several hours (i.e. 1400-1600). Following sample collection on day 14, birds were returned to baseline conditions for 12 days (i.e. day 14-26), and sperm collected and cloacal temperature measured on day 26. On that day we repeated the experiment, exposing birds to a second heat exposure period. During this second period, sample collection and environmental conditions were identical to those described above, but this time birds were exposed to the alternate temperature treatment (i.e. birds that experienced 40ºC in the first exposure, were subject to conditions of 30ºC in the second exposure period). The one exception to this is that we also collected samples on day 21, 7 days after birds were returned to baseline conditions, to more closely examine how quickly sperm quality recovered from temperature-dependent damage.

(b) Sperm quality analyses

Sperm samples were obtained by cloacal massage [17], and sperm swimming speed was quantified immediately using standard methods (e.g. [18]). Briefly, fresh sperm were collected

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and immediately diluted in a small volume (*c.* 50 μl) of pre-heated (40°C) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd). Following this dilution, we then immediately loaded 6 μl of the diluted sperm sample into a pre-heated slide chamber (depth 20 μm, Leja®, Netherlands) and sperm videos were captured at 400x magnification using a phase contrast scope (CX41, Olympus, Japan) fitted with a heated stage plate (TP-S, Tokai Hit, Shizuoka, Japan) and connected to a digital camera (Legria HF G25, Canon, Japan). The media, heat stage plate, and counting chambers were all maintained at 40°C (the approximate physiological temperature of zebra finches; [19]). For each sample, we recorded six unique fields of view for 5 s, for a total recording time of 30 s.

Videos were analysed at a later date using computer-assisted sperm analysis (CASA; Sperm Class Analyzer ® 5.4.0.0, SCA Motility, Microptic, Barcelona, Spain). The frame rate was set at 50 frames/s, and sperm were tracked for 0.5 s in each field of view. To control for the effects of drift, sperm cells with an average path velocity (VAP) of <30 μm s^{-1} or a straight line velocity (VSL) of <25 μm s^{-1} were considered immotile. These values were obtained from an analysis of dead sperm using the same recording and analysis conditions as those used in the current study. In addition, sperm tracked for less than 10 frames were excluded and we set the following cell detection parameters: minimum cell size of 10 μm^2. We also visually inspected each analysis, and in a few cases where two sperm crossed paths and CASA switched sperm mid-track, the affected tracks were deleted. Similarly, when a sperm track was interrupted, and thus two non-independent tracks were recorded, the earlier track was deleted (the remaining track was still required to fit the above criteria). Finally, this visual inspection also allowed us to manually delete any debris (i.e. non-sperm particles, such as red blood cells and faecal matter) that was incorrectly identified as a sperm so it was not included in the analysis (elongated/cylindrical zebra finch sperm heads are readily distinguishable from circular non-sperm particles). These analyses were performed blindly with respect to treatment group (all by CSM).

Using only sperm tracks that passed these criteria, we quantified sperm swimming speed as curvilinear velocity (VCL; see Rowe et al. 2015 for justification for using VCL) and the proportion of motile sperm (i.e. number of motile sperm tracks divided by the total number of cells). For VCL, samples with less than 10 motile sperm tracks were excluded from all analyses of sperm swimming speed (see supplementary material for justification of this cut-off). We calculated the mean VCL for each sample for statistical analysis. Finally, a small aliquot of the sperm suspension was fixed in 5% buffered formaldehyde solution and used at a later date to quantify the proportion of morphologically normal sperm. Specifically, one person (LLH) quantified the proportion of sperm with normal morphology (i.e. no obvious damage to the entire sperm cell or visible morphological abnormalities) for each sample. All scoring of sperm morphology was done blind to experimental treatment (see supplementary material S1 for full details of sperm morphology assays).

(c) Statistical analysis

Statistical analyses were run using R (version 3.3.2, R Core Team 2016), and all proportion data was normalized by logit transformation. We tested for differences between the two treatment groups at the start of the experiment (day 1), using a two-sample t-test, in the following traits: body mass, body condition (i.e. the residuals from the regression of body mass on tarsus length, both log-transformed), cloacal temperature, sperm swimming speed (VCL), and both the proportion of motile sperm and normal sperm. We investigated how the effect of temperature was influenced by both the duration of exposure (i.e. number of days in heat treatment) and previous exposure to elevated ambient temperatures using linear mixed models (LMMs). For these analyses, the change in trait value (i.e. value at day$_x$ – value at day 1, where x is day 3, 7, 11, or 14) was the dependent variable. Thus, our models considered the effect of temperature at 30ºC and 40ºC relative to trait values measured under baseline conditions (i.e. 23ºC) for the following traits: cloacal temperature, VCL, proportion motile sperm and proportion normal sperm. In these models, temperature (30ºC vs. 40ºC),

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experimental exposure period (1 vs. 2), and experimental day (hereafter referred to as exp-day; i.e. day 3, 7, 11, 14), together with their three-way interaction (and all constituent pairwise interactions), and body condition were included as fixed effects. Male identity was included as a random effect in all models. We also ran models that included cage number and mother's identity (to control for the potential impact of genetic background given the inclusion of four sets of siblings, accounting for 9 birds in total) as random effects, and compared these models to those with the single random effect (male identity) with likelihood ratio tests using maximum likelihood estimation. In all instances, these additional random effects did not significantly improve the models, and thus were not included in the final analysis. Finally, we assessed whether or not cloacal temperature and sperm quality recovered from the potential effects of heat exposure when birds were returned to baseline conditions (23ºC) using LMMs. For these models, we included exp-day (1 vs. 26), exposure period (1 vs. 2), and heat treatment (30ºC vs. 40ºC), together with their three-way interaction (and all constituent pairwise interactions), as fixed effects, and male identity as a random effect.

For all LMMs, non-significant interaction terms were removed in a backwards-stepwise fashion, beginning with the highest order interaction [20], and models compared using likelihood ratio tests. For post-hoc testing, we re-ran models and iteratively changed the reference level for variables that were in significant interactions or that had significant main effects. All final models were fitted with REML, and we summarised result using an ANOVA with Type III sum of squares for presentation in the main text. All LMMs were run using the package 'lme4 ' [21], graphs were constructed using ggplot2 [22], and modelling assumptions (normality and heterogeneity of variance of residuals) were assessed visually (following [20]). All tests were two-tailed and considered significant at $\alpha < 0.05$.

Results

No significant differences in body mass or condition were detected between the treatment groups before the experiment began (mass: $t_{18} = -0.59$, $p = 0.56$; body condition: $t_{18} = -0.61$, p

= 0.55). Similarly, groups did not differ with respect to sperm quality traits (sperm swimming speed: $t_{17} = 1.01$, $p = 0.33$; proportion motile sperm: $t_{18} = 0.92$, $p = 0.37$; proportion normal sperm: $t_{18} = 1.65$, $p = 0.12$) or cloacal temperature ($t_{18} = 0.78$, $p = 0.44$).

Experimental ambient temperatures strongly affected the proportion of normal sperm in a sample (table 1; figure 1). Patterns of change in the proportion of sperm with normal morphology differed between the two treatment groups, resulting in a significant temperature by exp-day interaction (table 1). During the first exposure period, 40°C birds showed a significant decline in the proportion of normal sperm with increasing duration of heat exposure ($t_{134.0} = -9.97$, $p < 0.0001$). There was also a significant, negative relationship between the proportion of normal sperm and exp-day in birds exposed to 30°C ($t_{131.0} = -2.81$, $p = 0.006$; table S2, figure S3); however this was immediately preceded by a slight increase in the proportion of normal sperm on day 3 relative to pre-experimental levels (mean ± SE; 0.90±0.01 vs. 0.84±0.02; $t_9 = -1.92$, $p = 0.09$) and even at their lowest levels (i.e. day 14) the proportion of normal sperm did not differ from values obtained under baseline conditions (mean ± SE; 0.82±0.02 vs. 0.84±0.02; $t_9 = 0.92$, $p = 0.38$). Moreover, the impact of heat was significantly greater in the 40°C group (i.e. the relationship was significantly more negative; table S2, S3). At the end of the first exposure period and following the 12-day recovery period, the proportion of normal sperm in a sample for birds exposed to 30°C did not differ significantly from values collected prior to the experiment ($t_{54.7} = -1.14$, $p = 0.26$). In contrast, birds in the 40°C group showed a significantly lower proportion of normal sperm in samples relative to those samples collected prior to the experiment ($t_{53.1} = -3.58$, $p = 0.0008$), despite the 12-day recovery period. During the second exposure period, the treatment groups again showed significantly different responses to heat exposure (table S4, S5). Specifically, while birds held at 40°C showed a significantly, negative relationship between change in proportion normal sperm and exp-day ($t_{131.0} = -4.79$, $p < 0.0001$), birds held at 30°C exhibited a positive relationship between these variables ($t_{133.5} = 2.27$, $p = 0.03$). In addition, there was a significant interaction between exp-day and exposure period (table 1), showing that the

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response to heat exposure was significantly stronger during the first exposure period (figure 1; table S2-5). At the end of the second exposure period, birds held at 40°C appeared to recover from heat treatment as the proportion of normal sperm in samples returned to baseline levels following the 12-day recovery period ($t_{54.7}$ = -1.11, p = 0.27). Similarly, in the 30°C birds there was no significant difference between the proportion of normal sperm in a sample collected at the beginning of the second exposure period and again following the 12-day recovery period ($t_{53.1}$ = 1.28, p = 0.21), however in this case the proportion of normal sperm remained significantly lower relative to pre-experimental levels (paired t-test: t_9 = 3.64, p = 0.005). Finally, body condition was negatively associated with the change in the proportion of normal sperm (table 1).

Change in sperm swimming speed (VCL) was significantly affected by heat treatment ($F_{1,99.5}$ = 5.85, p = 0.017) and exposure period ($F_{1,99.1}$ = 20.92, p < 0.0001). However, these effects were primarily related to the differential response of birds exposed to 30 vs. 40°C. Specifically, in the first exposure period and relative to samples collected under baseline (23°C) conditions, birds exposed to 40°C conditions showed a non-significant tendency towards a reduction in swimming speed (figure S4), whereas birds at 30°C tended to show an increase in sperm speed (figure S4) resulting in a significant difference between the groups in terms of the change in sperm swimming speed (Tables S7, S8, figure S4). Interestingly, following the 12-day recovery period, birds in both groups showed an increase in sperm swimming speed relative to pre-experimental levels; though while this increase was significant in the 30°C (t_{11} = -2.57, p = 0.03), it was not significant for 40°C birds ($t_{10.8}$ = -0.48, p = 0.64). For both treatment groups, the decline in sperm swimming speed was significantly greater in the second heat exposure period relative to the first exposure period (table S9, S10, figure S4). Moreover, during the second exposure period, the intercept for the 40°C birds differed significantly from 0 ($t_{86.6}$ = -3.16, p = 0.002); suggesting that in birds exposed to 40°C conditions, sperm swimming speed decreased significantly compared to sperm samples collected under baseline (23°C) conditions prior to the second heat exposure (table S10).

However, following the 12-day recovery period at 23°C, all birds showed recovery of sperm swimming speed to baseline levels (i.e. no difference between day 1 and 26 in second exposure period: 30°C: $t_{10.7}$ = -0.15, p = 0.89; 40°C: $t_{12.1}$ = -0.12, p = 0.91). The change in sperm swimming speed was not influenced by the duration of exposure (i.e. exp-day number; $F_{1,88.4}$ = 2.15, p = 0.15) or body condition ($F_{1,19.9}$ = 2.15, p = 0.52) and all interaction terms were non-significant and removed from the model (table S6).

The change in the proportion of motile sperm in a sample was also significantly affected by temperature ($F_{1,130.1}$ = 7.95, p = 0.006). More specifically, birds in the two treatment groups showed a significantly different initial response to elevated temperatures; birds exposed to 30°C temperatures showed a slight tendency towards an increase in the proportion of motile sperm, whereas birds in the 40°C treatment showed a tendency towards a slight decrease in the proportion of motile sperm (table S12-15, figure S5). However, neither group differed significantly from samples collected under baseline (23°C) conditions in either the first (table S12-S13) or second (table S14-S15) exposure period. The change in the proportion of motile sperm was not affected by exp-day number ($F_{1,121.9}$ = 1.06, p = 0.31), exposure period ($F_{1,126.2}$ = 3.21, p = 0.08) or body condition ($F_{1,28.0}$ = 0.01, p = 0.92). Finally, all interaction terms were non-significant and removed from the model (table S11).

Finally, high ambient temperature also affected male cloacal temperature (table 2, figure 2). In the first exposure period, birds in both treatment groups (30°C and 40°C) showed an increase in cloacal temperature, though this increase was significantly greater in the 40°C group relative to birds at 30°C ($t_{32.5}$ = 4.55, p < 0.0001, table S17, S18). In the second exposure period, birds in both the 30°C and 40°C treatment groups again showed an initial rise in cloacal temperature, though in this instance there was no significant difference between the groups in this response (figure 2; table S19, S20). Change in cloacal temperature varied significantly with exp-day (table S17-S20; figure S6), though the pattern of change over exp-day differed by exposure period resulting in a significant exposure period by exp-day interaction (table 2). More specifically, during the first exposure period, the change in cloacal

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temperature increased significantly over exp-day for both 30ºC and 40ºC treatment groups (table S17, S18), indicating a continuous rise in cloacal temperature with continued heat exposure (figure 2). Moreover, cloacal temperatures did not return to pre-experimental levels at the end of the first exposure period, but instead remained elevated even though birds were held at 23ºC for 12 days (30ºC: $t_{55} = 8.11$, $p < 0.0001$; 40ºC: $t_{55} = 4.24$, $p < 0.0001$). During the second exposure period, the change in cloacal temperature was significantly, negatively associated with exp-day (table S19, S20; figure S6). Thus, after an initial increase in cloacal temperature upon secondary heat exposure, cloacal temperature for all birds began to stabilise and decrease towards values obtained under 23ºC conditions immediately prior to the second heat period (figure 2). Notably, however, cloacal temperature of the birds exposed to 30ºC in the second exposure period again remained elevated relative to baseline conditions following a 12-day recovery period at 23ºC ($t_{55} = 3.32$, $p = 0.002$). Moreover, while cloacal temperatures of birds exposed to 40ºC in the second exposure period returned to levels at the beginning of this period ($t_{55} = -0.55$, $p = 0.58$), cloacal temperature following the 12-day recovery period was significantly greater than that recorded prior to the experiment (paired t-test: $t_9 = -3.84$, $p = 0.004$).

Discussion

We found that elevated ambient temperatures resulted in a decrease in sperm quality and an increase in cloacal temperature in male zebra finches. Most notably, when birds were exposed to 40ºC temperatures, we observed a strong decline in the proportion of sperm with normal morphology within a sample, and these proportions continued to decline with continued exposure to 40ºC conditions. Moreover, birds exposed to 40ºC during the first exposure period followed by 30ºC during the second exposure period, showed incomplete recovery from heat exposure, as the proportion of sperm with normal morphology was significantly lower at the end of the experiment relative to pre-experimental levels, even after a 12-day recovery period at 23ºC. We also found that prior exposure to elevated temperatures (i.e. if birds were exposed

to 30ºC conditions during the first exposure period) somewhat mitigated the negative impact of 40ºC temperatures on the proportion of normal sperm. Finally, 30 and 40ºC temperatures also lead to a *c*. 2ºC rise in cloacal temperature, which is consistent with studies in poultry and other passerine species [e.g. 10, 23]. In this study, however, we noted that the increase in cloacal temperature persisted even 12 days after the last heat exposure, which may at least partially explain the lasting effects of temperature on sperm morphology.

We observed a decline in the proportion of sperm with normal morphology across the duration of each heat exposure treatment. During the first seven days of heat treatment, the proportion of normal sperm was reduced to a similar level in both exposure periods, whereas further decline in the proportion of normal sperm on day 11 and 14 was considerably stronger for birds subjected to 40ºC temperatures during the first heat exposure. In birds, spermatogenesis is divided into three major phases: 1) the spermatogonial stage, involving mitotic cell division, 2) the spermatocyte stage, involving cell division via meiosis, and 3) the spermatid stage, involving differentiation to produce mature spermatozoa [24]. In Japanese quail, the duration of spermatogenesis has been estimated at 12.77 days, with each spermatogenic phase lasting *c*. 4-4.5 days [25]. In other non-passerine species, such as the domestic fowl and Barbary drake, the duration from the onset of meiosis to spermiation (phases 2-3) has been estimated at 11-12 days [24]. Sperm are then transported along the ductus deferens to the seminal glomera, a process that takes approximately 1 day [24, 26]. Studies of passerines are generally lacking; though one study of the yellow-throated sparrow *Gymnoris xanthocollis*, suggests that spermatogenesis may occur more rapidly in passerines relative to non-passerine taxa [27]. This may offer some clue as to the nature of sperm damage in our study. One explanation for the patterns of sperm damage observed in this study is that sperm stored in the seminal glomera are damaged by our treatment, with the continued decline in the proportion of morphologically normal sperm with prolonged heat exposure resulting from the accumulation of damaged sperm. However, an alternate explanation is that sperm damage may, at least in part, result from temperature effects on developing sperm cells during

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spermatogenesis. In mammals, the primary spermatocytes (especially pachytene and diplotene) of phase 2 and early spermatids of phase 3 appear particularly susceptible to heat stress [28]. Thus it is possible that the observed reduction in the proportion of normal sperm early in the exposure period (e.g. exp-day 3) reflects damage to sperm populations in the seminal glomera and ductus deferens, while the further decline in sperm quality observed as the treatment continued reflects the cumulative effects of damage to these same sperm populations combined with damage to developing sperm cells. We found that the proportion of sperm with normal morphology was at its lowest at experimental days 11 and 14, and suggest this may reflect damage inflicted on these cells whilst undergoing meiosis (e.g. pachytene and diplotene spermatocytes in phase 2) and spermatid development (spermatids in phase 3). Such a pattern fits well with the 11-12 days taken for sperm to transition from primary spermatocytes to mature spermatids (see above) with an additional day for sperm to be transported along the ductus deferens.

Interestingly, the fact that we did not observe as strong a decline in the proportion of normal sperm in birds exposed to 40ºC temperatures during the second exposure period suggests that these birds may have become acclimated to higher ambient temperatures, via prior exposure to 30ºC conditions, and that this may allow them to minimise temperature-induced defects to spermatogonia, spermatocytes, and developing spermatids. All organisms respond to elevated ambient temperatures by inducing heat shock proteins (HSPs). HSPs function as molecular chaperones to mitigate heat-induced damage by binding to proteins and preventing protein denaturation and incorrect folding [26]. Acute heat stress has been shown to increase the expression levels of genes belonging to the heat-shock protein family in testis tissue, attenuating the heat-induced damage to sperm [27]. In chickens *Gallus gallus domesticus* high environmental temperatures elevated expression of several HSPs (e.g. HSP25, HSPA2: [28, 29]). In the current study, we observed a sustained increased in male cloacal temperature. While we have no information on testis gene expression in our study, we suggest it is plausible that this rise in cloacal temperature lead to the upregulation of genes

related to the heat shock response, which would explain the diminished response to elevated temperatures we observed during the second exposure period.

In contrast to sperm morphology, we found that the motile performance of sperm (i.e. sperm swimming speed and the proportion of motile sperm) was relatively unaffected by high ambient temperatures. Although we did observe an initial decline in sperm swimming speed under 40ºC conditions during the second exposure period, both within-male (across exp-day) and between-male variability was high and no clear effects of temperature were apparent, which was also the case for the proportion of motile sperm in a sample. Sperm motile performance appears to show relatively low within-male repeatability in passerine birds [30, 31]. Moreover, in domestic fowl, sperm swimming speed is influenced by seminal fluid proteins [32] and the presence of extracellular ions, e.g. calcium and sodium [33]. While it is not known how quickly components of seminal fluid may change in birds, plasticity in seminal fluid production and composition has been reported in rodents [34] and *Drosophila* [35], and in fowl sperm swimming speed is capable of rapid change (i.e. within days) in response to shifts in social competitiveness [36]. As such, sperm motile performance may be a relatively plastic trait, which may be buffered from the negative effects of temperature via rapid changes in the chemical and protein milieu of seminal fluid.

Reductions in sperm quality (e.g. sperm motility, viability) due to elevated body temperatures have been linked to infertility in poultry [10, 37] and a range of mammalian species [38]. Our findings suggest high ambient temperatures may result in a limited supply of functional sperm, and thus may also impact male fertility in passerine birds. In birds, only morphologically normal sperm appear to be able to enter the sperm storage tubules (SSTs) of females [39], and there also appears to be a minimum number of sperm required at the site of fertilization to ensure successful embryonic development [40]. Thus, it is plausible that natural selection may act on males to protect sperm from the detrimental effects of elevated temperatures, particularly when these effects are not transient such as those we observed when the proportion of normal sperm failed to return to pre-experimental levels. In a general sense,

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our results might help to explain the recent finding of constrained avian breeding activity across the hotter parts of Australia, during the hottest parts of the year [16], a finding that has yet to be explored across other hot regions of the world. More importantly, functional infertility may be an important selective pressure in systems where females mate with multiple males. Under conditions of sperm competition, males that are best able to mitigate temperature-induced sperm damage are likely to be superior competitors for the fertilization of ova during hot conditions. Selection may therefore drive changes in gene expression and sequence evolution of proteins linked to spermatogenesis (e.g. HSPs), the composition and plasticity of seminal fluid, or male behaviours (e.g. shade seeking or other thermoregulatory behaviours) in response to increasing global temperatures and frequency of heatwaves. Male functional infertility may also generate selection on females to seek extra-pair copulations to avoid the potential costs of infertile social mates [41, 42]. Thus, elevated temperatures may have profound ecological and evolutionary consequences for the reproductive biology and behaviour of birds.

An important next step will be to determine if the negative effects of temperatures extend to additional passerines species. We found an effect of 40ºC, but not 30ºC, conditions in this study, and indeed globally numerous species will be regularly exposed to such high temperatures [7]. However, it remains to be determined if sperm function in temperate species or species breeding at high latitudes are similarly affected by increases in maximum temperatures. The effect of elevated temperature on female processes also warrants investigation; for example, whether sperm are prone to temperature-induced damage whilst residing in female SSTs, and the implications of this for female reproductive success. We suggest that temperature-induced or temperature-associated reductions in sperm quality may be an important biological consequence of the anthropocene as global temperatures rise and the frequency of extreme heat events increase.

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Table 1.

Reduced LMM examining the change in the proportion of sperm with normal morphology
with heat treatment, exposure period, experimental day (exp-day), and male body condition as
fixed factors (see supplementary material S3 for full models).

Predictor	F_{df}	p
Temperature	$F_{1,132.7} = 2.44$	0.12
Exposure period	$F_{1,132.6} = 5.53$	0.02
Experimental day	$F_{1,131.0} = 44.44$	< 0.0001
Body condition	$F_{1,34.6} = 5.78$	0.02
Temp*Exp-day	$F_{1,133.2} = 38.18$	< 0.0001
Temp*Exposure period	$F_{1,17.5} = 6.31$	0.02
Exposure period*Exp-day	$F_{1,132.9} = 18.97$	< 0.0001

Table 2.

Reduced LMM examining the change in male cloacal temperature with heat treatment,
exposure period, experimental day (exp-day), and male body condition as fixed factors (see
supplementary material S3 for full models).

Predictor	F_{df}	p
Temperature	$F_{1,136.0} = 19.323$	< 0.0001
Exposure period	$F_{1,136.8} = 0.004$	0.95
Experimental day	$F_{1,136.0} = 0.082$	0.78
Body condition	$F_{1,25.8} = 0.013$	0.91
Temp*Exposure period	$F_{1,17.3} = 6.931$	0.017
Exposure period*Exp-day	$F_{1,137.3} = 11.291$	0.001

Figure 1.
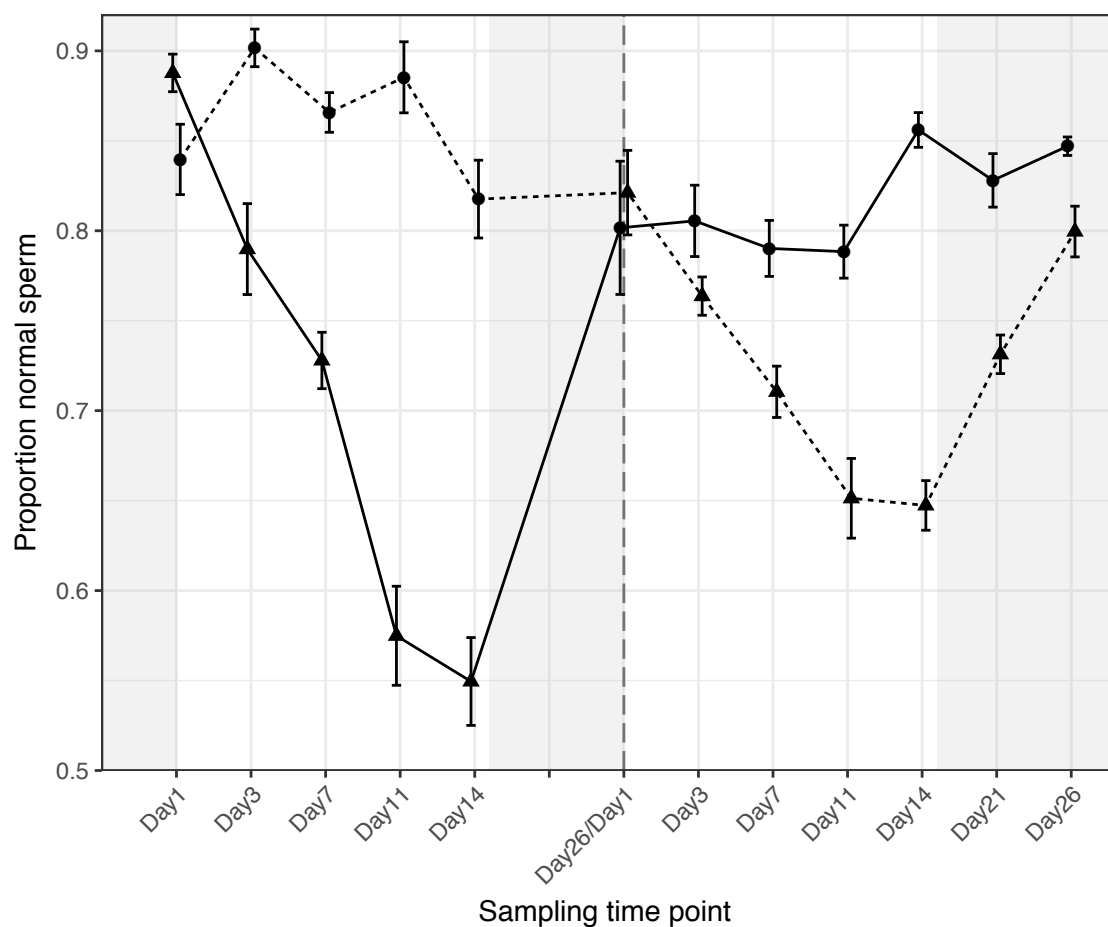
Figure 1. Proportion of sperm in a sample with normal morphology. Mean ± SE of proportion of normal sperm on given experimental day (exp-day) over two experimental heat exposure periods (separated by vertical hatched line). Spacing between time points is related to sampling on exp-day, not continuous time. Males exposed first to 40ºC in part 1 of experiment then to 30ºC in part 2 (solid line), or 30ºC then 40ºC (dashed line). Temperature during experimental heat exposure: 30ºC (circles) or 40ºC (triangles). During experimental treatment birds were exposed to heat for 8 hours a day (white background) and returned to 23ºC at other times. During acclimation and recovery periods birds were kept continuously at 23ºC (shaded background).

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Figure 2.

Figure 2. Male cloacal temperature. Mean ± SE of cloacal temperature on given experimental day over two experimental heat exposure periods (separated by vertical hatched line). Males exposed first to 40°C in part 1 of experiment then to 30°C in part 2 (solid line), or 30°C then 40°C (dashed line). Temperature during experimental heat exposure: 30°C (circles) or 40°C (triangles). During experimental treatment birds were exposed to heat for 8 hours a day (white background) and returned to 23°C at other times. During acclimation and recovery periods birds were kept continuously at 23°C (shaded background).

Data supplement

Files in this Data Supplement:

Supplementary Material S1: Supplementary information on sperm quality analyses. Figures S1, S2.

Supplementary Material S2: Expanded statistical output. Tables S1 – S21, Figures S3 – S6.

~~Supplementary Material S3: Supporting data~~ – NOT PROVIDED IN THESIS

~~Supplementary Material S4: R code~~ – NOT PROVIDED IN THESIS

Experimental heatwaves negatively impact sperm quality in the zebra finch

Laura L. Hurley, Callum S. McDiarmid, Christopher R. Friesen, Simon C. Griffith, and

Melissah Rowe

Sperm Quality Analyses

Sperm motile performance

For VCL, we excluded samples with < 10 motile sperm tracks from all analyses of sperm

swimming speed. This criteria was applied to ensure that sperm speed was adequately

estimated, and was determined using a means accumulation approach. To do this, single cell

VCL recordings from16 birds at baseline 23ºC were randomly sampled 5 times each at 2

sperm intervals (2, 4, 6, …30), taking the standard deviation and mean of each sample.

Accumulation of means was done by dividing these values to calculate a coefficient of

variation (CV). Ten motile cells represented a threshold at which sample VCL values

approximately reached an asymptote (Figure S1). Using only those samples that passed our

criteria, the total number of motile sperm that were tracked for each male ranged from 11 to

353 (mean = 107.2 ± 4.7 s.e.).

For the proportion of motile sperm, we set our exclusion criteria as those samples with

fewer than 5 cells (either motile or immotile) tracked by CASA. All samples had more than 5

cells identified in CASA, however, thus no samples were excluded in analysis of the

proportion of motile sperm. For calculations of proportion of motile sperm, the total number

of sperm that were tracked for each male ranged from 6 to 497 (mean = 179.2 ± 6.0 s.e).
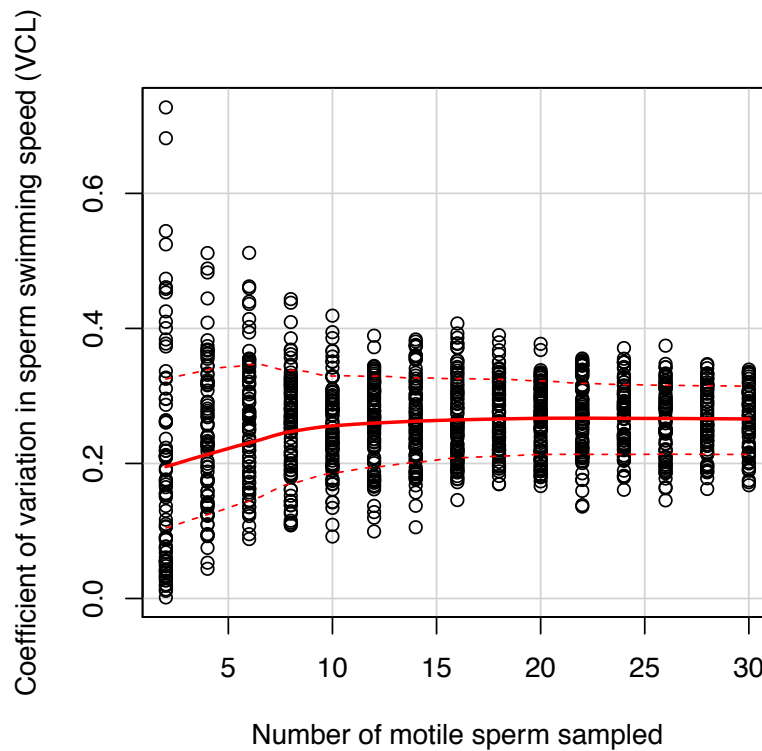
Figure S1. Coefficient of variation (CV) of sperm swimming speed (VCL) in relation to the number of motile sperm used to calculate it. Swimming speed of individual sperm cells were taken from 16 males from whom > 54 sperm cells were tracked. For those males, we randomly chose sperm swimming speed values for 2, 4, 6, 8, 10, ……30 (i.e. samples collected at intervals of 2, between 2 and 30) individual sperm cells and calculated the CV in sperm swimming speed for each of these subsamples. This process was repeated five times for each male, resulting in five estimates of CVswimming speed at each of the 15 intervals.

Sperm morphology: proportion of sperm with normal morphology

Immediately after assessment of sperm swimming speed, we fixed *c.*20 μL of the diluted sperm sample in 5% buffered formaldehyde solution to permit the examination of sperm morphology. Specifically, one person (LH) quantified the proportion of sperm with normal morphology (i.e. no obvious damage to the entire sperm cell or visible morphological abnormalities) for each sample. Sperm abnormalities included cells with large heads (macrocephaly), small heads (microcephaly) or broken heads, cells with broken or unattached

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midpieces, and cells with multiple flagellum, broken flagellum, or bent flagellum (see Figure S2 for examples). In each case, we assessed 100 randomly chosen sperm on each of two replicate slide smears (200 sperm cells examined in total) and scored sperm as having either normal or abnormal morphology. We then calculated the proportion of normal sperm per slide and averaged these values to determine the proportion of normal sperm in sample (repeatability of replicate smear $R = 0.93$, $p < 0.005$; Nakagawa and Schielzeth, 2010).
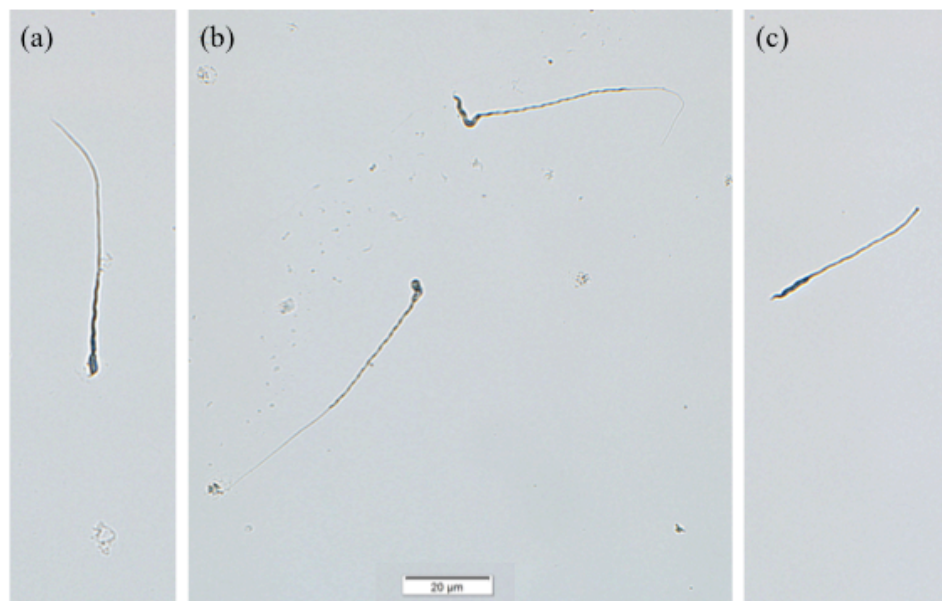
Figure S2. Sperm abnormalities (a) microcephaly head, (b) macrocephaly head (left, deflated by fixation) and bent head (right), (c) broken flagellum. Imaging at 320x, scale bar insert in b, provides scale for all images.

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Experimental heatwaves negatively impact sperm quality in the zebra finch

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Proportion of Sperm with Normal Morphology

Table S1. Results of the full LMM (summarised with Type III sum of squares, ANOVA) examining the change in the proportion of sperm with normal morphology in relation to temperature (30 vs. 40ºC), experimental exposure period (1st vs. 2nd), and experimental day (hereafter referred to as exp-day; i.e. day 3, 7, 11, 14), together with their three-way interaction (and all constituent pairwise interactions), and body condition.

Predictor	F_{df}	p
Temperature:	$F_{1,131.8} = 2.44$	0.12
Exposure period:	$F_{1,1231.5} = 5.50$	0.02
Exp-day	$F_{1,130.0} = 44.02$	<0.0001
Body condition	$F_{1,33.4} = 5.57$	0.02
Temp*Part	$F_{1,55.3} = 2.90$	0.09
Temp*Exp-day	$F_{1,132.4} = 37.80$	<0.0001
Exposure period*Exp-day	$F_{1,131.8} = 18.86$	<0.0001
Temp*Exposure period*Exp-day	$F_{1,132.9} = 0.03$	0.86

Table S2. Reduced model results relating change in the proportion of sperm with normal morphology to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period, temperature by exp-day, and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 1). There was a significant effect of including male identity to the models, with it accounting for only 11.8% of variance in the final model regardless of leveling.

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	0.09 ± 0.03	$t_{82.3} = 3.10$	<0.01
Temperature: 40ºC	-0.11 ± 0.04	$t_{64.1} = -2.89$	<0.01
Exposure period: 2nd	-0.13 ± 0.04	$t_{63.8} = -3.44$	0.001
Exp-day	-0.01 ± 0.002	$t_{131.0} = -2.81$	<0.01
Body condition	-0.32 ± 0.13	$t_{34.6} = -2.40$	0.02
Temp*Exposure Period	0.13 ± 0.05	$t_{17.5} = 2.51$	0.02
Temp*Exp-day	-0.02 ± 0.002	$t_{133.2} = -6.18$	<0.0001
Exposure period*Exp-day	0.01 ± 0.002	$t_{132.9} = 4.36$	<0.001

Table S3. Reduced model results relating change in the proportion of sperm with normal morphology to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period, temperature by exp-day, and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 40°C, exposure period 1).

Predictor	Estimate $\pm$ SE	t_{df}	p
(intercept)	-0.02 $\pm$ 0.03	$t_{81.8} = -0.62$	0.54
Temperature: 30°C	0.11 $\pm$ 0.04	$t_{64.1} = 2.89$	<0.01
Exposure period: 2nd	0.002 $\pm$ 0.04	$t_{65.1} = 0.05$	0.96
Exp-day	-0.02 $\pm$ 0.002	$t_{134.0} = -9.97$	<0.0001
Body condition	-0.32 $\pm$ 0.13	$t_{34.6} = -2.40$	0.02
Temp*Exposure Period	-0.13 $\pm$ 0.05	$t_{17.5} = -2.51$	0.02
Temp*Exp-day	0.02 $\pm$ 0.002	$t_{133.2} = 6.18$	<0.0001
Exposure period*Exp-day	0.01 $\pm$ 0.002	$t_{132.9} = 4.36$	<0.001

Table S4. Reduced model results relating change in the proportion of sperm with normal morphology to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period, temperature by exp-day, and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	-0.04 ± 0.03	$t_{82.1}$ = -1.35	0.18
Temperature: 40ºC	0.02 ± 0.04	$t_{65.1}$ = 0.61	0.54
Exposure period: 1st	0.13 ± 0.04	$t_{63.8}$ = 3.44	0.001
Exp-day	0.01 ± 0.002	$t_{133.5}$ = 2.27	0.02
Body condition	-0.32 ± 0.13	$t_{34.6}$ = -2.40	0.02
Temp*Exposure Period	-0.13 ± 0.05	$t_{17.5}$ = -2.51	0.02
Temp*Exp-day	-0.02 ± 0.002	$t_{133.2}$ = -6.18	<0.0001
Exposure period*Exp-day	-0.01 ± 0.002	$t_{132.9}$ = -4.36	<0.001

Table S5. Reduced model results relating change in the proportion of sperm with normal morphology to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period, temperature by exp-day, and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 40°C, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	-0.02 ± 0.03	$t_{87.8}$ = -0.53	0.60
Temperature: 30°C	-0.02 ± 0.04	$t_{65.1}$ = -0.61	0.54
Exposure period: 1st	-0.002 ± 0.04	$t_{65.1}$ = -0.05	0.96
Exp-day	-0.01 ± 0.002	$t_{131.0}$ = -4.79	<0.0001
Body condition	-0.32 ± 0.13	$t_{34.6}$ = -2.40	0.02
Temp*Exposure Period	0.13 ± 0.05	$t_{17.5}$ = 2.51	0.02
Temp*Exp-day	0.02 ± 0.002	$t_{133.2}$= 6.18	<0.0001
Exposure period*Exp-day	-0.01 ± 0.002	$t_{132.9}$= -4.36	<0.001
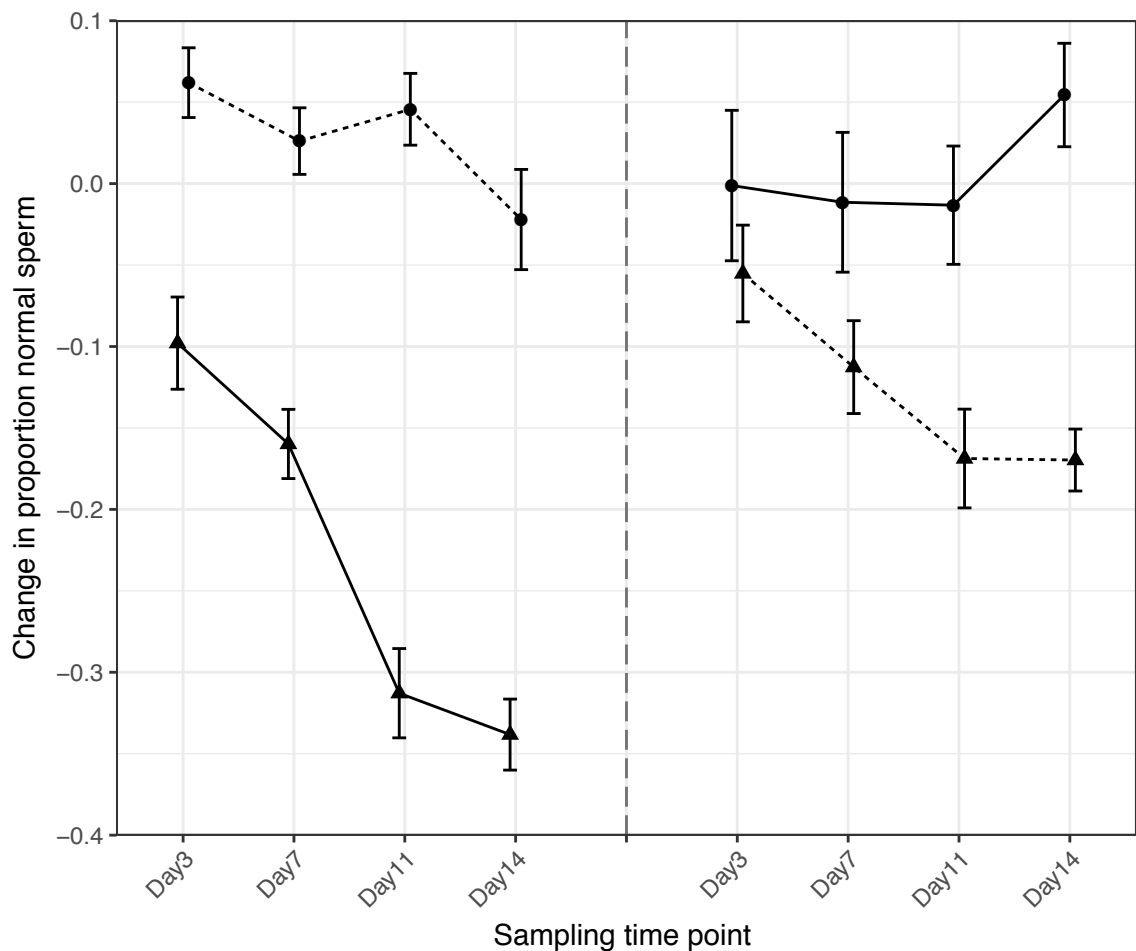
Figure S3. Patterns of change in the proportion of sperm with normal morphology over time during the two heat exposure periods. Values show the change in normal morphology (mean ± SE) relative to measurements taken on day 1 of each exposure period (i.e. change = value at day_x − value at day 1, where x is day 3, 7, 11, or 14). The two experimental heat exposure periods are separated by vertical hatched line. Solid line represent males exposed to 40ºC (triangles) in first experiment period then to 30ºC (circles) in second, while dashed line with represent males exposed to 30ºC in first exposure period then to 40ºC in second exposure period.

Sperm Swimming Speed (VCL)

Table S6. Results of the full LMM (summarised with Type III sum of squares, ANOVA) examining the change in sperm swimming speed in relation to temperature (30 vs. 40ºC), experimental exposure period (1st vs. 2nd), and experimental day (hereafter referred to as exp-day; i.e. day 3, 7, 11, 14), together with their three-way interaction (and all constituent pairwise interactions), and body condition.

Predictor	F_{df}	p
Temperature	$F_{1,94.3} = 3.06$	0.08
Exposure period	$F_{1,94.0} = 8.56$	<0.01
Exp-day	$F_{1,91.6} = 2.83$	0.10
Body condition	$F_{1,20.4} = 0.47$	0.50
Temp*Part	$F_{1,86.7} = 0.06$	0.81
Temp*Exp-day	$F_{1,93.0} = 0.52$	0.47
Exposure period*Exp-day	$F_{1,91.7} = 0.75$	0.39
Temp*Exposure period*Exp-day	$F_{1,93.5} = 0.46$	0.50

Table S7. Reduced model results model relating change sperm swimming speed (VCL) to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 1). There was a significant effect of including male identity to the models, with it accounting for 17.0% of variance in the final model regardless of leveling.

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	4.21 ± 2.25	$t_{85.2} = 1.87$	0.06
Temperature: 40ºC	-3.87 ± 1.60	$t_{99.5} = -2.42$	0.017
Exposure period: 2nd	-7.29 ± 1.59	$t_{99.1} = -4.57$	<0.0001
Exp-day	-0.26 ± 0.18	$t_{88.4} = -1.47$	0.15
Body condition	8.53 ± 13.12	$t_{19.9} = 0.65$	0.52

Table S8. Reduced model results relating change sperm swimming speed (VCL) to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 40ºC, exposure period 1).

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	0.34 ± 2.32	$t_{90.2} = 0.15$	0.88
Temperature: 30ºC	3.87 ± 1.60	$t_{99.5} = 2.42$	0.017
Exposure period: 2nd	-7.29 ± 1.59	$t_{99.1} = -4.57$	<0.0001
Exp-day	-0.26 ± 0.18	$t_{88.4} = -1.47$	0.15
Body condition	8.53 ± 13.12	$t_{19.9} = 0.65$	0.52

Table S9. Reduced model results relating change sperm swimming speed (VCL) to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	-3.08 ± 2.36	$t_{88.7} = -1.31$	0.19
Temperature: 40ºC	-3.87 ± 1.60	$t_{99.5} = -2.42$	0.017
Exposure period: 1st	7.29 ± 1.59	$t_{99.1} = 4.57$	<0.0001
Exp-day	-0.26 ± 0.18	$t_{88.4} = -1.47$	0.15
Body condition	8.53 ± 13.12	$t_{19.9} = 0.65$	0.52

Table S10. Reduced model results relating change sperm swimming speed (VCL) to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 40ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	-6.95 ± 2.20	$t_{86.6} = -3.16$	0.002
Temperature: 30ºC	3.87 ± 1.60	$t_{99.5} = 2.42$	0.017
Exposure period: 1st	7.29 ± 1.59	$t_{99.1} = 4.57$	<0.0001
Exp-day	-0.26 ± 0.18	$t_{88.4} = -1.47$	0.15
Body condition	8.53 ± 13.12	$t_{19.9} = 0.65$	0.52
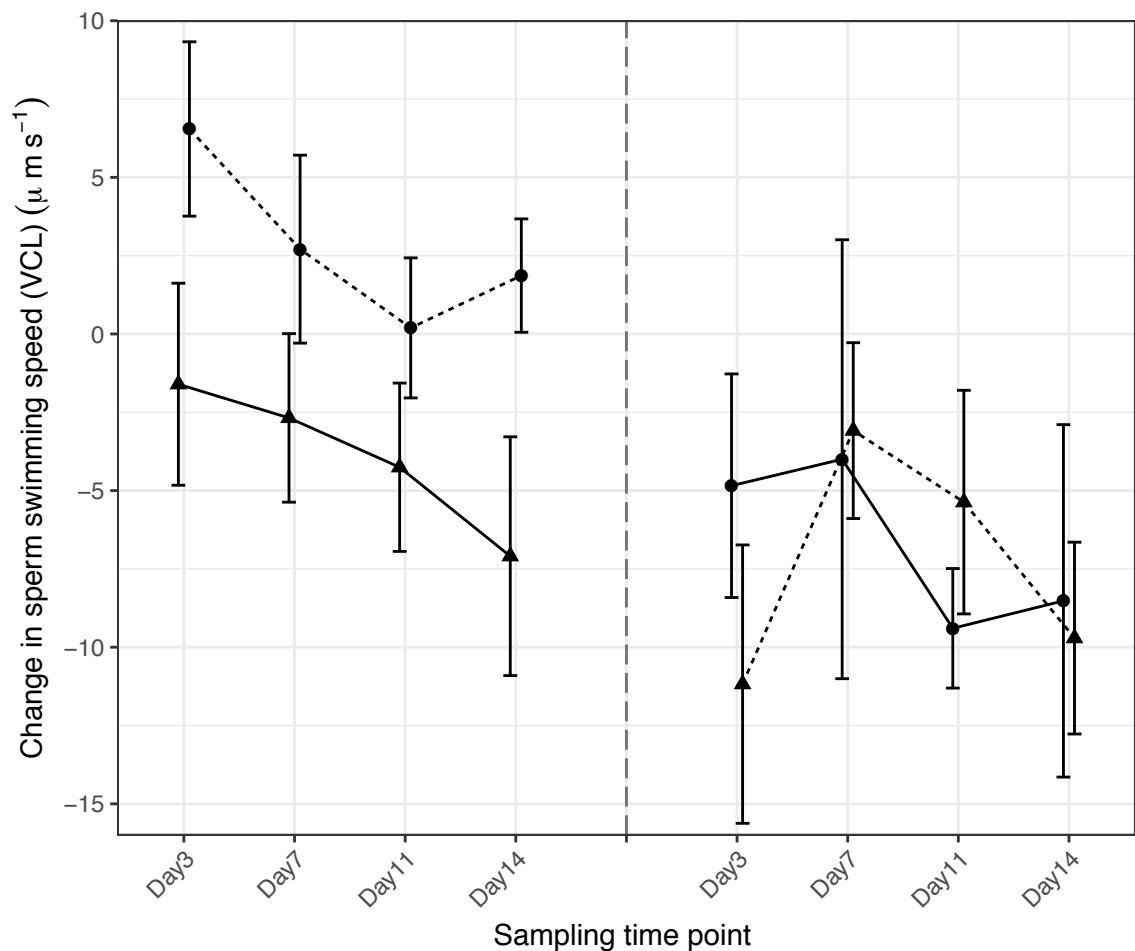
Figure S4. Patterns of change in sperm swimming speed (VCL) over time during the two heat exposure periods. Values show the change in sperm swimming speed (VCL) (mean ± SE) relative to measurements taken on day 1 of each exposure period (i.e. change = value at day$_x$ – value at day 1, where x is day 3, 7, 11, or 14). The two experimental heat exposure periods are separated by vertical hatched line. Solid line represent males exposed to 40ºC (triangles) in first experiment period then to 30ºC (circles) in second, while dashed line with represent males exposed to 30ºC in first exposure period then to 40ºC in second exposure period.

Proportion of Motile Sperm

Table S11. Results of the full LMM (summarised with Type III sum of squares, ANOVA) examining the change in the proportion of motile sperm in a sample in relation to temperature (30 vs. 40°C), experimental exposure period (1st vs. 2nd), and experimental day (hereafter referred to as exp-day; i.e. day 3, 7, 11, 14), together with their three-way interaction (and all constituent pairwise interactions), and body condition.

Predictor	F_{df}	p
Temperature	$F_{1,130.2} = 0.35$	0.56
Exposure period	$F_{1,129.5} = 0.29$	0.59
Exp-day	$F_{1,127.0} = 0.96$	0.33
Body condition	$F_{1,30.6} = 0.02$	0.89
Temp*Part	$F_{1,72.2} = 0.05$	0.81
Temp*Exp-day	$F_{1,129.4} = 0.54$	0.46
Exposure period*Exp-day	$F_{1,128.5} = 0.05$	0.83
Temp*Exposure period*Exp-day	$F_{1,129.3} = 0.37$	0.37

Post-hoc comparisons via re-levelling

Table S12. Reduced model results relating change in the proportion of motile sperm in a sample to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 1). There was a significant effect of including male identity to the models, with it accounting for 28.8% of variance in the final model regardless of leveling.

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	0.11 ± 0.08	$t_{84.0} = 1.34$	0.18
Temperature: 40ºC	-0.14 ± 0.05	$t_{130.1} = -2.82$	<0.01
Exposure period: 2nd	-0.08 ± 0.05	$t_{130.5} = -1.61$	0.11
Exp-day	-0.01 ± 0.01	$t_{124.2} = -1.00$	0.32
Body condition	-0.08 ± 0.51	$t_{31.2} = -0.16$	0.88

Table S13 Reduced model results relating change in the proportion of motile sperm in a sample to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 40ºC, exposure period 1).

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	-0.03 ± 0.08	$t_{82.2} = -0.02$	0.68
Temperature: 30ºC	0.14 ± 0.05	$t_{130.1} = 2.82$	<0.01
Exposure period: 2nd	-0.08 ± 0.05	$t_{130.5} = -1.61$	0.11
Exp-day	-0.01 ± 0.01	$t_{124.2} = -1.00$	0.32
Body condition	-0.08 ± 0.51	$t_{31.2} = -0.16$	0.88

Table S14. Reduced model results relating change in the proportion of motile sperm in a sample to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	0.03 ± 0.08	$t_{86.2} = -0.34$	0.73
Temperature: 40ºC	-0.14 ± 0.05	$t_{130.1} = -2.82$	<0.01
Exposure period: 1st	0.08 ± 0.05	$t_{130.5} = 1.61$	0.11
Exp-day	-0.01 ± 0.01	$t_{124.2} = -1.00$	0.32
Body condition	-0.08 ± 0.51	$t_{31.2} = -0.16$	0.88

Table S15 Reduced model results relating change in the proportion of motile sperm in a sample to temperature, experimental exposure period, exp-day, and body condition. Parameter estimates are contrasts relative to the reference category (temperature 40ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	*p*
(intercept)	-0.11 ± 0.08	$t_{82.4} = -1.43$	0.16
Temperature: 30ºC	0.14 ± 0.05	$t_{130.1} = 2.82$	<0.01
Exposure period: 1st	0.08 ± 0.05	$t_{130.5} = 1.61$	0.11
Exp-day	-0.01 ± 0.01	$t_{124.2} = -1.00$	0.32
Body condition	-0.08 ± 0.51	$t_{31.2} = -0.16$	0.88
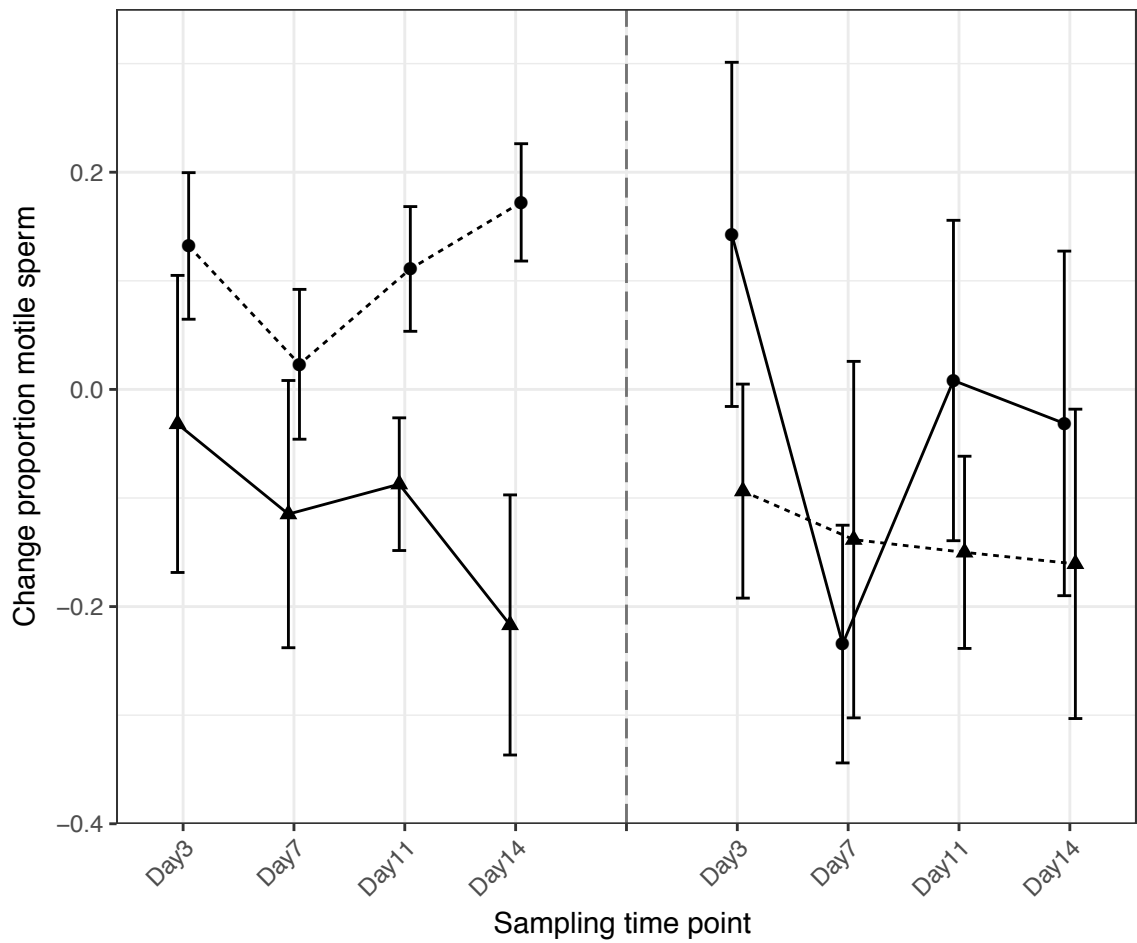
Figure S5. Patterns of change in the proportion of motile sperm in a sample over time during the two heat exposure periods. Values show the change in proportion of motile sperm (mean ± SE) relative to measurements taken on day 1 of each exposure period (i.e. change = value at day_x − value at day 1, where x is day 3, 7, 11, or 14). The two experimental heat exposure periods are separated by vertical hatched line. Solid line represent males exposed to 40ºC (triangles) in first experiment period then to 30ºC (circles) in second, while dashed line with represent males exposed to 30ºC in first exposure period then to 40ºC in second exposure period.

Cloacal Temperature

Table S16. Results of the full LMM (summarised with Type III sum of squares, ANOVA) examining the change in male cloacal temperature in relation to temperature (30 vs. 40°C), experimental exposure period (1st vs. 2nd), and experimental day (hereafter referred to as exp-day; i.e. day 3, 7, 11, 14), together with their three-way interaction (and all constituent pairwise interactions), and body condition.

Predictor	F_{df}	p
Temperature	$F_{1,135.1} = 7.47$	<0.001
Exposure period	$F_{1,134.9} = 0.003$	0.95
Exp-day	$F_{1,134.0} = 0.08$	0.78
Body condition	$F_{1,24.9} = 0.01$	0.92
Temp*Exposure period	$F_{1,90.5} = 5.37$	0.02
Temp*Exp-day	$F_{1,135.6} = 0.90$	0.34
Exposure period*Exp-day	$F_{1,135.4} = 11.3$	0.001
Temp*Exposure period*Exp-day	$F_{1,136.0} = 0.83$	0.36

Table S17. Results of the reduced model relating change in cloacal temperature to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 1). There was a significant effect of including male identity to the models, with it accounting for only 11.1% of variance in the final model regardless of leveling.

Predictor	Estimate ± SE	t_{df}	p
(intercept)	0.54 ± 0.38	$t_{104.7} = 1.41$	0.16
Temperature: 40ºC	1.64 ± 0.36	$t_{32.5} = 4.55$	<0.0001
Exposure period: 2nd	0.84 ± 0.54	$t_{105.0} = 1.55$	0.13
Exp-day	0.08 ± 0.03	$t_{136.8} = 2.58$	0.01
Body condition	-0.19 ± 1.70	$t_{25.8} = -0.11$	0.91
Temp*Exposure Period	-1.62 ± 0.62	$t_{17.3} = -2.63$	0.02
Exposure period*Exp-day	-0.15 ± 0.05	$t_{25.8} = -3.36$	0.001

Table S18. Reduced model results relating change in cloacal temperature to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 40ºC, exposure period 1).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	2.19 ± 0.38	$t_{105.6} = 5.72$	<0.0001
Temperature: 30ºC	-1.64 ± 0.36	$t_{32.5} = -4.55$	<0.0001
Exposure period: 2nd	-0.78 ± 0.54	$t_{105.8} = -1.44$	0.15
Exp-day	0.08 ± 0.03	$t_{136.8} = 2.58$	0.01
Body condition	-0.19 ± 1.70	$t_{25.8} = -0.11$	0.91
Temp*Exposure Period	1.62 ± 0.62	$t_{17.3} = 2.63$	0.02
Exposure period*Exp-day	-0.15 ± 0.05	$t_{25.8} = -3.36$	0.001

Table S19. Reduced model results relating change in cloacal temperature to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 30ºC, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	1.38 ± 0.38	$t_{105.9} = 3.61$	<0.001
Temperature: 40ºC	0.03 ± 0.36	$t_{32.5} = 0.07$	0.94
Exposure period: 1st	-0.84 ± 0.54	$t_{105.0} = -1.55$	0.13
Exp-day	-0.07 ± 0.03	$t_{136.5} = -2.18$	0.03
Body condition	-0.19 ± 1.70	$t_{25.8} = -0.11$	0.91
Temp*Exposure Period	1.62 ± 0.62	$t_{17.3} = 2.63$	0.02
Exposure period*Exp-day	0.15 ± 0.05	$t_{25.8} = 3.36$	0.001

Table S20. Reduced model results relating change in cloacal temperature to temperature, experimental exposure period, exp-day, body condition, and the 2-way interactions: temperature by exposure period and exposure period by exp-day. Parameter estimates are contrasts relative to the reference category (temperature 40°C, exposure period 2).

Predictor	Estimate ± SE	t_{df}	p
(intercept)	1.40 ± 0.38	$t_{105.9} = 3.61$	<0.001
Temperature: 30°C	-0.03 ± 0.36	$t_{32.5} = -0.7$	0.94
Exposure period: 1st	0.78 ± 0.54	$t_{105.8} = 1.44$	0.13
Exp-day	-0.07 ± 0.03	$t_{136.5} = -2.18$	0.03
Body condition	-0.19 ± 1.70	$t_{25.8} = -0.11$	0.91
Temp*Exposure Period	-1.62 ± 0.62	$t_{17.3} = -2.63$	0.02
Exposure period*Exp-day	0.15 ± 0.05	$t_{25.8} = 3.36$	0.001
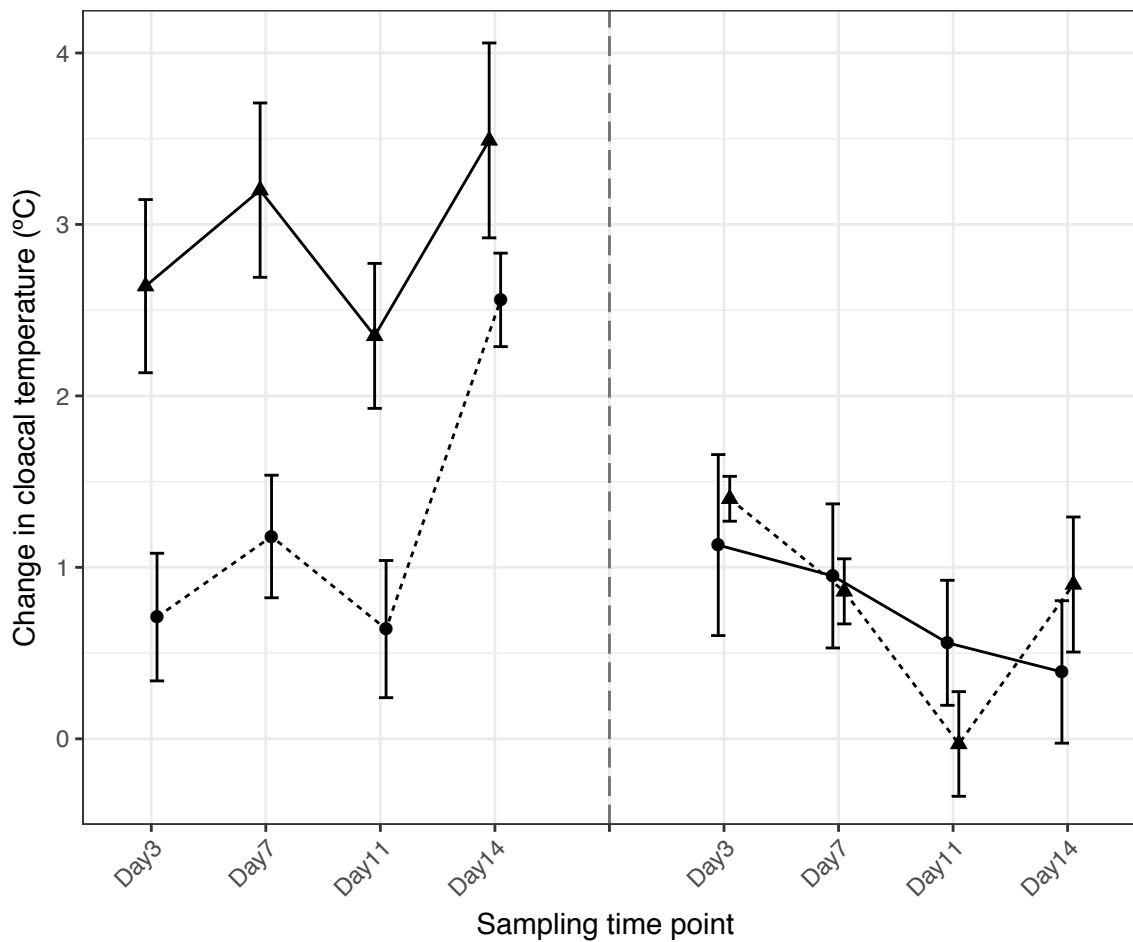
Figure S6. Patterns of change in cloacal temperature over time during the two heat exposure periods. Values show the change in cloacal temperature (mean ± SE) relative to measurements taken on day 1 of each exposure period (i.e. change = value at day_x − value at day 1, where x is day 3, 7, 11, or 14). The two experimental heat exposure periods are separated by vertical hatched line. Solid line represent males exposed to 40ºC (triangles) in first experiment period then to 30ºC (circles) in second, while dashed line with represent males exposed to 30ºC in first exposure period then to 40ºC in second exposure period.

Overall response variable variance

Table S21. Percentage of final model variance attributed to random effect of male identity (Male ID).

Response Variable	% Male ID variance
Cloacal Temperature	11.1
Sperm swimming speed (VCL)	17.0
Prop Motile	28.8
Prop Normal	11.8

Chapter Seven

General Discussion

Understanding the role polyspermy plays in successful fertilization and development of avian embryos starts with understanding the variation in the number of sperm that reach the ovum in different species. It is clear that part of this difference in perivitelline layer (PVL) sperm numbers is due to the body and ovum size of a species (Birkhead et al. 1994), which likely reflects the fact that the site of fertilisation, the germinal disk, is a very small target on a relatively large yolky egg. It has long been understood that sperm that do not fuse with the inner perivitelline layer (PVL) are trapped by the outer PVL (Bobr et al. 1964, Bakst and Howarth 1977). The PVL was later recognised as a tool for investigating the number of sperm reaching the ovum (Wishart 1987), but little work has utilised its capacity to characterise polyspermy. Current studies are mostly descriptive, limited to detailed study in a handful of species (Brillard and Bakst 1990, Birkhead et al. 1993, Wishart and Staines 1999, Lifjeld et al. 2000, Johnsen et al. 2012), or a limited number of samples per species (Birkhead et al. 1994). These studies have shown a correlation between the number of sperm in the inner and outer PVL (Birkhead and Fletcher 1994, Birkhead et al. 1994), and that sperm number in the PVL vary between species, and between eggs with lay order (Birkhead et al. 1994, Lifjeld et al. 2000, Johnsen et al. 2012). A small number of studies have shown that the number of sperm that reach the PVL can tell us more about the causes of hatching success and failure (Christensen et al. 2005, Birkhead et al. 2008, Hemmings and Birkhead 2015), the outcome of post-copulatory pre-zygotic selection (Bennison et al. 2015, Hemmings et al. 2016), and how environmental stressors can alter fertility (McDaniel et al. 1995, McDaniel et al. 1996).

In this thesis, I have used the performance and morphology of sperm in ejaculates, and the number of sperm reaching the PVL to investigate how plasticity in post-copulatory performance impacts reproduction in Australian estrildid finches. For the most part, I focused on these two aspects of sperm plasticity separately, investigating how different contexts (i.e. species, behaviour, the environment) influences sperm variation. I will first discuss the main findings of my studies, and then discuss how it can inform future research on what factors impact reproductive success in Australian birds and beyond.

Contextual differences in post-copulatory performance

The simplest interpretation of my findings about the number of sperm that reach the PVL, is that it is highly variable within and between species. While this has been shown over broader contexts (Birkhead et al. 1994, Lifjeld et al. 2000, Johnsen et al. 2012); I showed that, even in closely related species, there are differences in pattern and consistency of change in PVL sperm numbers within and between clutches in the same pairs. This was further emphasised when I turned my focus more specifically on how post-copulatory constraint differed in long-tailed and zebra finch in different contexts.

When long-tailed finches were in matched subspecies colour pairs, their sperm numbers on the PVL were high, and consistent between clutches produced by a pair. Pairs of mixed colours, especially with a hybrid individual, typically led to a dramatic decline in sperm numbers. Although future work is needed to understand how this influences reproductive success, differences in sperm number provides a potential mechanism for why the hybridisation zone of the two subspecies remains narrow (Griffith and Hooper 2017).

On the other hand, the number of sperm that reached the ovum decreased in zebra finch that had successfully reared chicks to fledge. Pairs that were never successful consistently had high numbers of sperm trapped on the PVL, and a higher rate of hatching failure than pairs that were successful in fledging chicks. Therefore, my findings suggest that high sperm numbers on the PVL in long-tailed finch are more likely to indicate a well matched – and therefore successful – pair, while the opposite is true in zebra finch. This highlights the need to better understand the role of sperm competition and polyspermy on reproductive success in different species.

Contextual differences in sperm performance and morphology

Previous work done on the regulation of reproduction in opportunistic breeders has examined changes in the zebra finch gonads in several contexts: under various photoperiods (Bentley et al. 2000), dehydration levels (Prior et al. 2013), and seasonal predictability

(Perfito et al. 2007). These studies have treated the zebra finch as an anomaly in breeding phenotype, but recent work has shown that the zebra finch is not an anomaly, but a true model for opportunistic breeding (Duursma et al. 2017). My work showed that male zebra finches show plasticity in testes size, testosterone levels, and sperm performance across a single reproductive cycle. This plasticity is outside of what would be predicted by the short seasonal temperate breeding birds from the northern hemisphere, even those that breed on a more flexible schedule (Dawson 2008, Hahn et al. 2009, Ramenofsky 2011). Due to these changes care should be taken when sampling ejaculate for use in studies looking at sperm performance in opportunistic breeders, as different reproductive states could yield contrasting results.

In zebra finches, I also showed that the proportion of normal morphology sperm in an ejaculate is a predictor of a pair's reproductive success, as pairs with more abnormal sperm had higher rates of hatching failure and failed to rear chicks. This suggests that abnormal sperm morphology might be one reason why only 64% of captive zebra finch females successfully breed (Griffith et al. 2017). Therefore, my finding that high ambient temperatures increase the amount of abnormal sperm in a male's ejaculate is a potential explanation for the decrease in observations of reproduction (e.g. egg laying) as temperatures increase (Duursma et al. 2017). Previously, it has been shown that increased ambient temperature impacts fertility in chickens (McDaniel et al. 1995, McDaniel et al. 1996). However, my findings suggest that in the heat adapted zebra finch, it is heatwaves that have the greatest impact on sperm, not normal seasonal temperature increases.

Future directions

In this thesis, I present the Australian estrildid finches, primarily the zebra and long-tailed finch, as models for studying plasticity in sperm performance and post-copulatory performance. Further, I have demonstrated that using sperm trapped on the perivitelline layer to is a valuable tool for this research. There are several different ways that research can progress from the ground work I have laid:

1) Chapter 2 showed that the optimal range (not too many or too few) for sperm reaching the ovum differ in Gouldian, long-tailed, and zebra finches, despite similar body and ovum size. Future research should investigate if differential requirement of polyspermy in related species is due to different levels of sperm competition within or between males in the population (Kleven et al. 2009), and compare the requirement and tolerance for polyspermy in these species (Hemmings and Birkhead 2015). Such studies would lead to a better understanding of the role of polyspermy in birds.

2) In Chapter 3, I showed that long-tailed finch is a perfect model for future work investigating the role sperm phenotype plays in post-copulatory preformance in speciation and hybridization. Given that they readily breed in captivity, more controlled work can be conducted with them than in wild models, such as the *Ficedula* flycatchers (Veen et al. 2001, Veen et al. 2009).

3) To date, most research on the post-copulatory success of sperm has focused on the success of normal sperm with given morphological characteristics (Bennison et al. 2015, Hemmings et al. 2016) without considering the overall proportion of normal sperm morphology in the ejaculate. The finding in Chapter 4, that poor levels of normal sperm morphology impacts reproductive success, suggests that future work should investigate why lower numbers of normal morphological sperm leads to higher numbers of sperm reaching the ovum than when there is less abnormal sperm. The mechanisms could be the same that compensates for low insemination numbers (Hemmings and Birkhead 2015). Additionally, future studies should consider how resulting poorer success rates impacts divorce rate and extra pair copulations in species with high pair fidelity, such as the zebra finch, and how genetic influences on sperm traits can impact success (Kim et al. 2017, Knief et al 2017).

4) Chapter 5 showed that the zebra finch physiology across a reproductive cycle is dynamic, but most research on zebra finches has primarily investigated what various external cues stimulate them to breed (Zann et al. 1995, Hahn et al. 2008). Future

research should investigate how opportunistic breeders' cycling activation of their reproductive organs helps to conserve energy, and that regulation of reproduction is perhaps due more to inhibitory cues than stimulatory ones. Further, Chapter 5 suggests that such work should look at changes on a finer reproductive time scale than using the more general categories pre-breeding (i.e. sensitive to be stimulated to breeding, but not stimulated), breeding, and non-breeding states used in northern temperate research (Perfito et al. 2007, Perfito et al. 2011).

5) Chapter 6 presented the zebra finch as an ideal model to understand the impact of heat on fertility in a species that regularly breeds at high temperatures, but my findings suggest that such research also needs to be investigated in species that do not, as they may show differential adaptation and response to temperature changes. Given the rise in global temperature, and increased rate of extreme weather events such as heatwaves, future research needs to address the impact of temperature on reproductive success due to sperm function. So far, most work focusing on the impact of climate change has explored the shift of timing of reproduction in northern temperate birds, but has ignored the impact on fertility (Charmantier et al. 2008, Visser et al. 2009, Naef-Daenzer et al. 2012).

This thesis emphasises the need for future research to work more towards understanding how context matters in sperm competition and post-copulatory performance of sperm and influences the resulting fertilisation and developmental success of the embryo. The definition of context in these studies needs to broaden beyond genetically inherited traits, to consider life history, breeding phenology, social interactions, and environmental (internal and external) influences. In other words, the more consideration should be given to the ecology of sperm (Reinhardt et al. 2015). Research is clearly moving in this direction, as there is recent work looking at how sperm performance is impacted by heterospecific vaginal fluid (Cramer et al. 2014, Cramer et al. 2016), seminal fluid composition (Froman et al. 2006) and proteomics

(Borziak et al. 2016), and social context (Immler et al. 2010). However, to date only a few studies have addressed how different contexts, for example low insemination numbers (Hemmings and Birkhead 2015) and heat stress (McDaniel et al. 1995, McDaniel et al. 1996), impact successful fertilization and early development. More work is needed to understand how not only genetics, but the ecology of sperm ultimately alters reproductive success.

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Griffith, S. C., O. L. Crino, S. C. Andrew, et al. 2017. Variation in Reproductive Success Across Captive Populations: Methodological Differences, Potential Biases and Opportunities. Ethology **123**:1-29.

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Appendix I: List of Conference Presentations

Conference presentations made during my PhD candidature at Macquarie University.

Hurley, L.L., Akinfiresoye, L., Kalejaiye, O., Tizabi, Y. 2014. Antidepressant effects of resveratrol in an animal model of depression. International Congress of Neuroendocrinology, Sydney, Australia (poster)

Hurley, L.L., Crino, O.L., Buchanan, K.L., Rowe, M., Griffith, S.C. 2015. Post-copulatory constraints to hybridization across subspecies of long-tailed finch. Behaviour. Cairns, Australia. (talk)

Hurley, L.L., Fanson, K.V., Buchanan, K.L., Rowe, M., Griffith, SC. 2016. Variation in polyspermy in three Australian estrildid finches. Australasian society for the study of animal behaviour. Katoomba, Australia. (talk)

Hurley, L.L., McDiarmid, C.S., Rowe, M., Griffith, SC. 2017. The heat is on: Decrease in avian sperm functionality at high ambient temperatures. Society for integrative and comparative biology. New Orleans, USA. (talk)

Appendix II: Related work published during candidature

These publications partially inspired the work conducted in Chapter 4. My main contributions to these papers: 1) Crino et al., I collected part of the data used in the paper. 2) Griffith et al., I was part of the initial group (Griffith, Crino, Andrew, Bolton, Mainwaring, McCowan, Trompf) that conceptualized, did background research, organized data, and wrote the first draft of the manuscript. 3) Kim et al. I collected sperm and blood for the Australian birds (wild, wild captive, domestic) analysed in the study. 4) I bred and collected data from the laboratory birds used in the study.

Crino, O.L., Buchanan, K.L., Fanson, B.G., **Hurley, L.L**., Smiley, K.O., Griffith, S.C., 2017. Divorce in the socially monogamous zebra finch: Hormonal mechanisms and reproductive consequences. Horm Behav 87, 155-163.

Griffith, S.C., Crino, O.L., Andrew, S.C., Nomano, F.Y., Adkins-Regan, E., Alonso-Alvarez, C., Bailey, I.E., Bittner, S.S., Bolton, P.E., Boner, W., Boogert, N., Boucaud, I.C.A., Briga, M., Buchanan, K.L., Caspers, B.A., Cichon, M., Clayton, D.F., Deregnaucourt, S., Forstmeier, W., Guillette, L.M., Hartley, I.R., Healy, S.D., Hill, D.L., Holveck, M.J., **Hurley, L.L**., Ihle, M., Krause, E.T., Mainwaring, M.C., Marasco, V., Mariette, M.M., Martin-Wintle, M.S., McCowan, L.S.C., McMahon, M., Monaghan, P., Nager, R.G., Naguib, M., Nord, A., Potvin, D.A., Prior, N.H., Riebel, K., Romero-Haro, A.A., Royle, N.J., Rutkowska, J., Schuett, W., Swaddle, J.P., Tobler, M., Trompf, L., Varian-Ramos, C.W., Vignal, C., Villain, A.S., Williams, T.D., 2017. Variation in Reproductive Success Across Captive Populations: Methodological Differences, Potential Biases and Opportunities. Ethology 123, 1-29.

Kim KW, Bennison C, Hemmings N, Brookes L, **Hurley LL**, Griffith SC, Burke T, Birkhead TR, Slate J. 2017. A sex-linked supergene controls sperm morphology and swimming speed in a songbird. Nature Ecology & Evolution. 1, 1168-76.

Andrew SC, **Hurley LL**, Mariette MM, and SC Griffith. 2017 Higher temperatures during development reduce body size in the zebra finch in the lab and in the wild. *Accepted J Evol Bio. 28 Aug 2017*.

Appendix III: Unrelated work published during candidature

These publications were part of research I conducted at Howard University (Washington DC USA) as a Research Associate before I started my tenure at Macquarie University. I preformed the live animal research and wrote the first author publication. Quall et al. research was conducted by an undergraduate student as an honours project, I rewrote and prepared the paper for publication. Tizabi et al. I contributed to doing background research and some writing of the paper.

Qualls, Z., Brown, D., Ramlochansingh, C., **Hurley, L.L.** and Tizabi, Y., 2014. Protective effects of curcumin against rotenone and salsolinol-induced toxicity: implications for Parkinson's disease. *Neurotoxicity research*, *25*(1), pp.81-89.

Hurley, L.L., Akinfiresoye, L., Kalejaiye, O. and Tizabi, Y., 2014. Antidepressant effects of resveratrol in an animal model of depression. *Behavioural brain research*, *268*, pp.1-7.

Tizabi, Y., **Hurley, L.L.,** Qualls, Z. and Akinfiresoye, L., 2014. Relevance of the anti-inflammatory properties of curcumin in neurodegenerative diseases and depression. *Molecules*, *19*(12), pp.20864-20879.

Appendix IV: Animal Ethics Approvals

Macquarie University Approvals:

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2013/028-3   **Date of Expiry:** 28 February 2014

Full Approval Duration: *30 September 2013 to 28 February 2014 (5 Months)*
This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator: **Associate Investigators:**
Dr Simon Griffith   Andrea Crino   0406 398 817
Biological Sciences   Mark Mainwaring   (02) 9850 1302
Macquarie University, NSW 2109   Kate Buchanan   0429 398 460
simon.griffith@mq.edu.au   Lori Hurley   0419 978 077
0425 746 674

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
Animal Welfare Officer - 9850 7758 / 0439 497 383, *or* **Manager, Fauna Park** - 9850 4109 / 0425 213 420

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **The effect of pair disruption on corticosterone physiology, prolactin, and male parental investment in two estrildid finch species**

Purpose: 4 - Research: Human or Animal Biology

Aims: **To study the effects of pair disruption on parental behavior and reproductive success by examining the specific hormonal mechanism (prolactin and corticosterone) that may explain changes in physiology and behavior following pair disruption in Zebra finches and long tailed finches**

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex/Weight/Age	Total
18	Zebra finch (*Taeniopygia guttata*)	Male / 12g / adult	40
18	Zebra finch (*Taeniopygia guttata*)	Female / 12g / adult	40
18	Long-tailed finch (*Poephila acuticauda*)	Male / 12g / adult	40
18	Long-tailed finch (*Poephila acuticauda*)	Female / 12g / adult	40
18	Zebra finch (*Taeniopygia guttata*)	Any / 17g / juvenile	80 broods × 5 offspring = 400
18	Long-tailed finch (*Poephila acuticauda*)	Any / 17g / juvenile	80 broods × 5 offspring = 400
		TOTAL	960

Location of research:

Location	Full street address
Fauna Park	209 Culloden Road, Marsfield, NSW 2122

Amendments approved by the AEC since initial approval:
1. *Amendment #1: Addition of Laura Hurley as Postgraduate Student **subject to the Research Assistant attending an Animal Ethics Course*** (Executive Approved 7 November 2013). To be ratified at the AEC meeting 5 December 2013.

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

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Professor Mark Connor (Chair, Animal Ethics Committee)   **Approval Date:** 11 November 2013

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY (ARA)

MACQUARIE University

AEC Reference No.: 2013/029-8　　　　　　　　**Date of Expiry:** 31 July 2015

Full Approval Duration: *20 September 2013 to 31 July 2015 (22 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Dr Simon Griffith
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Associate Investigators:
Kate L Buchanan　　0429 398 460
Mark Mainwaring　　(02) 9850 1302
Andrea Crino　　　　0406 398 817
Larissa Trompf　　　0419 371 403

Post-doctoral Fellow
Camille Duval　　　0475 209 328
Postgraduate Student:
Laura (Lori) Hurley　　0419 978 077
Volunteer Research Assistant :
Tiare Broadhead　　0431 570 967
Renee Borg　　　　0468 622 372

> **In case of emergency, please contact:**
> *the Principal Investigator / Associate Investigator named above*
> **Animal Welfare Officer** - 9850 7758 / 0439 497 383, *or* **Manager, Fauna Park** -
> 9850 4109 / 0425 213 420

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:
Title of the project: Physiological coordination of reproduction in a socially monogamous bird
Purpose: 4 - Research: Human or Animal Biology
Aims: To understand how male and female zebra finches are able to coordinate their physiology and how optimize their different reproductive attempts
Surgical Procedures category: 3 - Minor Conscious Intervention
All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.
 Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex	Weight	Age	Already Approved	New Request	New total	Supplier/ Source
18	Zebra finch (*Taeniopygia guttata*)	Both	12g	Adult	1080	112	1192	Current stock
					0	72	72	
18	Zebra finch (*Taeniopygia guttata*)	Both	17g	Juvenile	1080	840	1920	Current stock
18	Long Tailed Finch	Both	12g	Adult	0	80	80	Current stock
18	Long Tailed Finch	Both	12g	Juvenile	0	480	480	Current stock
							3744	

Location of research:

Location	Full street address
Fauna Park	209 Culloden Road, Marsfield, NSW 2122

Amendments approved by the AEC since initial approval:
1. **Amendment #1**: *Addition of Laura Hurley as Postgraduate Student* **subject to the Research Assistant attending an Animal Ethics Course** *(Executive Approved 7 November 2013, AEC ratified 5 December 2013).*
2. **Amendment #2:** *Addition of Camille Duval as Post-doctoral fellow* **subject to attending an Animal Ethics Course** *(Executive approved 28 May 2014, AEC ratified 19 June 2014)*
3. **Amendment # 3.1 -** *Amend current procedure, addition of additional animals, addition of 3 techniques. End point of animals remains the same as original AR. (Approved by AEC meeting on 14 August 2014)A*
4. **Amendment # 3.2 -** *Addition of animal type and number. (Approved by AEC meeting on 14 August 2014)*
5. **Amendment # 3.3 -** *Addition of number of animals, add a "more intensive" data collecting regime to compare data collected by the concurrently run already approve "less intensive" population in the Large Old Aviaries, already approved less intensive regime. Birds to be added and procedures outlined. Same animal endpoint as original ARA. (Approved by AEC meeting on 14 August 2014)*
6. **Amendment # 3.4 -** *Addition of new temperature manipulation study. (Approved by AEC meeting on 14 August 2014)*
7. **Amendment # 3.5 -** *Birds to be added, new procedures outlined, additional housing detailed. Change in animal endpoint (Approved by AEC meeting on 14 August 2014)*
8. **Amendment #4**: *Addition of Larissa Trompf as Associate Investigator (Approved by AEC meeting on 14 August 2014)*
9. **Amendment #5**: *Addition of Tiare Broadhead as Volunteer Research Assistant(Approved by Exec, to be ratified by AEC 19 March 2015)*
10. **Amendment #6**: *Addition of Renee Borg as Volunteer Research Assistant (Approved by Exec, to be ratified by AEC 19 March 2015)*

Conditions of Approval:
1. **Amendment #1:** *Laura (Lori) Hurley to attend the next WWRAW (Working with Research Animals Workshop) on 25 July 2014.*
2. **Amendment #2**: *Dr Camille Duval to attend the next WWRAW on 25 July 2014 in order to be familiar with NSW legislation and expectations.*
3. **Amendment 5 & 6,** *Tiare Broadhead and Renee Borg to be supervised by Principal Investigator only.*

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence

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Professor Mark Connor (Chair, Animal Ethics Committee)　　　　　　　**Approval Date:** 03 March 2015

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/025   **Date of Expiry:** 15 July 2015

Full Approval Duration: 16 July 2014 to 15 July 2017 (36 Months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Dr Simon Griffith
Biological Sciences
Macquarie University, NSW 2109
0425 746 674
Simon.Griffith@mq.edu.au

Associate Investigators:
Laura Hurley   0419 978 077

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Animal Welfare Officer: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Longitudinal breeding plasticity in wild Zebra Finches (Taeniopygia guttata)**

Purpose: **4 - Research: Human or Animal Biology**

Aims: **To clearly define longitudinal physiological and reproductive changes in wild zebra finches to better understand how they respond to short and long term cues (e.g. changes in weather, food availability, reproductive state of partner) to coordinate reproduction**

Surgical Procedures category: **1 - Observation Involving Minor Interference**

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
20 – Native Wild	**Zebra Finch (Taeniopygia guttata)**	**Adult, 10-17 grams**	**300**	**wild**
		TOTAL	**300**	

Location of research:

Location	Full street address
UNSW Arid Zone Research Station	Fowlers Gap, via Broken Hill NSW 2880

Amendments approved by the AEC since initial approval: N/A
Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

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Professor Mark Connor (Chair, Animal Ethics Committee)   **Approval Date: 19 June 2014**

MACQUARIE University — ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2015/028-4   **Date of Expiry:** 11 September 2017

Full Approval Duration: 13 November 2015 to 11 September 2017

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Associate Investigators:
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Other People Participating:
Emilie Vergauwe   0424 389 923
Callum McDiarmid   0435 609 535

> **In case of emergency, please contact:**
> *the Principal Investigator / Associate Investigator named above*
> **Animal Welfare Officer** - 9850 7758 / 0439 497 383, *or* **Manager, Fauna Park** - 9850 4109 / 0425 213 420

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Sociality, dominance and personality in the zebra finch

Purpose: 4 - Research: Human or Animal Biology

Aims: **to identify the exploration, activity and social tendencies of a population of birds by measuring:**
1. **Individual sociality**
2. **Dominance**
3. **Individual movement**

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex	Total	Supplier/Source
18 - Native Captive	Zebra Finch (*Taeniopygia Guttata*)	Adult/Any	210	Current Laboratory Population
			210	

Location of research:

Location	Full street address
Fauna Park	209 Culloden Road, Marsfield, NSW

Amendments approved by the AEC since initial approval:
1. *Amendment #1 – Amend technique/procedure and location of research. (Executive approved, Ratified by AEC 17 March 2016).*
2. *Amendment #2 - Addition of Emilie Vergauwe as Volunteer (Executive approved, Ratified by AEC 17 March 2016).*
3. *Amendment #3 - Addition of Callum McDiarmid as Assistant (Executive approved, Ratified by AEC 18 August 2016).*
4. *Amendment #4 - Add Laura Hurley as Associate Investigator (Executive approved, ratified by AEC 20/10/2016).*
5. *Amendment #5(a) - Extend protocol by 12 months (from 11/09/2016 to 11/09/2017) (Executive approved, ratified by AEC 20/10/2016).*
6. *Amendment #5(b) - Additional two procedures (duration of heat trials & collection of sperm) (Executive approved, ratified by AEC 20/10/2016).*

Conditions of Approval:
1. *Amendment #1 - Approved subject to the following:*
   - *Birds must be acclimated at 30°C for at least 30 minutes prior to being placed in 40°C*
   - *At any point if the birds are not coping with the temperature change, the experiment is to stop and be reviewed*

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Assoc. Professor Jennifer Cornish (Chair, Animal Ethics Committee)   **Approval Date: 20 October 2016**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)