1 **Reproductive coordination breeds success: the importance of the partnership in avian sperm**
2 **biology**

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Abstract

Previous experience with a partner can improve reproductive coordination between a pair and increase offspring production. We paired inexperienced zebra finches and investigated how a pairs' experience and their reproductive success together (i.e. whether they were successful or unsuccessful at rearing chicks) related to the number of sperm reaching the ovum, sperm motile performance, and hatching success. In contrast to unsuccessful pairs, successful pairs increased their relative hatching rates over sequential breeding attempts, with pairs hatching 100% of eggs after successfully fledging their previous clutch. Across the study, hatching failure was primarily due to early embryo death. Further, the number of sperm reaching the perivitelline layer (PVL) significantly decreased after fledging chicks in successful pairs, and overall less sperm was found on the PVL in successful pairs compared to unsuccessful pairs. Across breeding attempts, males in successful pairs also exhibited a significant decline in sperm swimming speed, whereas it increased over breeding attempts in unsuccessful pairs. Our results support the idea of an optimal level of supernumerary sperm on the avian egg. However, our data suggest that there are likely to be interactions between the quality of a partnership and male sperm traits that may contribute to fitness in socially monogamous birds and that have been largely neglected to date.

Keywords: perivitelline layer, reproductive success, sperm motility, sperm usage, Zebra Finch

Significance Statement

Breeding experience with a partner can improve physiological and behavioural coordination and the reproductive success of a pair. In the zebra finch, we assessed whether experience and reproductive success altered sperm swimming speed, the number of sperm reaching the ovum, and the number of chicks hatching. These three measures are potentially correlated, given that birds require multiple sperm to fuse with the ovum for fertilisation and development to occur, and the idea that sperm swimming speed impacts a sperm's success at fertilising ova. However, too many or too few sperm can lead to hatching failure, so there will be an optimal number of sperm to ensure fertilisation and healthy development. We found that only pairs that successfully reared chicks increased hatching success in subsequent clutches and decreased the amount of sperm reaching the ovum. Additionally, with more

50 experience, the swimming speed of the top 10% of their sperm decreased. These findings are important
51 because they link partnership quality to sperm biology.

Introduction

Over the past four decades, considerable effort has been directed towards understanding the potential genetic benefits that individuals can gain from their choice of a reproductive partner (Slatyer et al. 2012), as well as how mate choice can lead to sexual conflict between males and females (Arnqvist and Kirkpatrick 2005; Arnqvist and Rowe 2005). Recent work, however, has indicated that the intrinsic variation in the quality of a partner might be somewhat over-stated (e.g. Wang et al. 2017; Griffith 2019). In animals that form pair bonds, social benefits and the quality of the partnership may be important for individual reproductive success (Griffith 2019); perhaps even more so than the additive genetic benefits that can be acquired from the partner. Pair coordination, also referred to as pair synchrony, is an emergent property of the social pair, and describes the quality of the pair in terms of their behaviour and physiological state (e.g. Mariette and Griffith 2015). Importantly, pairs that are better coordinated with respect to reproductive behaviour (Spoon et al. 2006; Bebbington and Hatchwell 2016) and physiological state (Riechert et al. 2014), appear more likely to reproduce successfully.

Pair coordination might also be important for the optimisation of fertilisation and developmental success of embryos, especially in species that lack straightforward extrinsic determinants of reproductive periodicity. Most bird species in the northern hemisphere temperate zone have a breeding phenology and male and female reproductive physiology that is largely set by extrinsic factors such as day length (Hahn et al. 2009). Extrinsic factors may help to ensure that pair reproductive physiology is coordinated, such that the male is producing an appropriate amount of sperm during the female's fertile period and thus sufficient sperm are available at the time of female ovulation. However, coordination of reproductive physiology between the individuals within a pair is likely more challenging for species with flexible or extended breeding periods, where the rapid onset of reproduction may be advantageous. For example, species breeding in the Australian temperate zone were found to typically have breeding seasons that were 2.3 times longer than those breeding in an equivalent area in the European temperate zone (Englert Duursma et al. 2017).

One of the most well-recognised flexibly breeding birds is the Australian zebra finch, *Taeniopygia guttata*, which typically breeds across eight months of the year (Englert Duursma et al. 2017). A recent analysis of data from captive populations of zebra finch from 21 institutions worldwide found that 36% of all females fail to reproduce successfully when given the opportunity in a captive

setting (Griffith et al. 2017). A significant driver of this reproductive failure is the number of eggs that fail to hatch (just 42% hatched in one study of 1,670 eggs; and just 88% of 3,213 females overall managed to hatch one or more chicks (Griffith et al. 2017). In a study of wild birds, the level of hatching failure was also considerable; 21% of all clutches failed completely, with only 84% of eggs successfully hatching in clutches where at least one egg hatched (Griffith et al. 2008). These numbers are similar to those seen across species where a significant determinant of variation in reproductive success is the failure to successfully hatch all of the eggs that are laid, with a study of 99 species finding an average hatching rate of 89% (Spottiswoode and Møller 2004). As such, egg failure is widespread and accounts for a significant reduction in of potential reproductive output. There are a number of factors that could contribute to hatching failure, as well as the subsequent survival of any nestlings that do hatch. In the wild, behavioural coordination between a male and female (i.e. nest visit synchrony) is correlated with the number of nestlings in the nest just prior to fledgling; a pattern with variance largely driven by the percentage of eggs that hatch successfully (77% of all eggs hatched: Mariette and Griffith 2012). Several studies have also experimentally revealed the benefit of prior breeding experience with the same partner, and demonstrate that experience may help with physiological and behavioural coordination (Adkins-Regan and Tomaszewski 2007; Crino et al. 2017). Conversely, experimental disruption of a pair bond can have a negative impact on chicks (e.g. increased stress response: Crino et al. 2017), and force-paired birds, in contrast to those free to choose partners, have a higher level of hatchling mortality (Ihle et al. 2015).

Another relatively unexplored factor that may contribute to hatching failure in socially monogamous birds is the inability of males to produce and transfer an appropriate quantity of functionally competent sperm in their ejaculate due to poor coordination or compatibility between partners. Birds are obligately polyspermatric, requiring more than the one sperm to penetrate the ovum for successful embryonic development (Snook et al. 2011). However, when either too many or too few sperm reach the site of fertilisation (Christensen et al. 2005; Forstmeier and Ellegren 2010; Hemmings and Birkhead 2015), early embryonic death can lead to hatching failure. The quality of sperm transferred by males can also impact the reproductive success of pairs. For example, fertilisation by post-meiotic aged sperm can slow embryonic development, increase embryonic mortality, and reduce post hatch condition of nestlings (Dharmarajan 1950; White et al. 2008).

The number of sperm reaching the ovum in birds likely reflects an interaction between ejaculate sperm number and quality and female usage of sperm (i.e. sperm storage and transport in oviduct) (Hasson and Stone 2009). The number of sperm found on the perivitelline layer (PVL) of avian eggs (i.e. those sperm reaching the ovum) differs significantly across species, generally scaling with both body and egg mass (Birkhead et al. 1994). From an evolutionary perspective, this pattern may reflect a species-specific optimum number of sperm present on the PVL that ensures fertilisation and successful embryonic development (Hasson and Stone 2009). However, even within-species, the number of sperm found on the PVL varies considerably both across pairs and across eggs within a clutch (Johnsen et al. 2012; Hurley et al. 2017). The factors underlying this variation and the potential impact it may have on reproductive success are currently unclear, though at least one study suggests that male age may be a contributing factor (Girndt et al. 2019). The optimal balance between too many and too few sperm at the egg may therefore contribute to hatching and fledging success in birds. Achieving the optimal number of sperm on the egg membrane may be something that is best achieved by a well coordinated pair.

Huxley (1914) recognized that courtship displays in birds primarily function to enhance physiological coordination between the male and female. Here, we extend the idea of physiological pair coordination to the gamete level, and suggest that gametic coordination may help ensure fertilisation and developmental success of embryos. Using zebra finches, we examined sperm motile performance, and the number of sperm reaching the surface of the ovum (i.e. the number trapped in the ovum's outer PVL) in breeding pairs that were either successful or unsuccessful at rearing fledglings. We also tested for a change in sperm motile performance and the number of sperm reaching the PVL across a number of sequential breeding attempts in order to investigate potential effects of increase in breeding experience and breeding success on sperm quality. We accounted for sperm characteristics because studies have demonstrated that the social environment or a male's social status can affect sperm traits (Cornwallis and Birkhead 2007; Pizzari et al. 2007; Immler et al. 2010), and in socially monogamous species, pair coordination likely constitutes an important component of the social environment (Griffith 2019).

Specifically, the social context of a partnership may reflect the level of coordination and communication within pairs, and may also change as the pair becomes more experienced. Moreover, coordination may affect sperm production to ensure sufficient numbers of quality sperm being

produced by the male during the fertile period of the female and minimise post-meiotic sperm aging of sperm stores prior to copulation, as well as the way that the female reproductive tract interacts with sperm. We therefore hypothesised that experience and social context (i.e. breeding success) impacts the number of sperm reaching the PVL and sperm motile performance traits. However, given the novelty of the questions that we are addressing, the lack of theoretical work in this area, and the largely observational nature of the study, we refrain from making exact predictions about the directions of relationships between the social context and either sperm motile performance or the number of sperm on the PVL. In order to measure these and best detect any variation, we have used a structured and controlled approach in our study design. Our approach provides the first observational examination of the variation in PVL sperm and sperm performance traits present across pairs as they gain experience, with the only clear expectation being that successful or experienced pairs are likely to be different in these measures than unsuccessful or inexperienced pairs.

Methods

General procedures and study design

Twenty pairs of virgin domestic zebra finches, between 12 -18 months of age, were force-paired (i.e. given a genetically unrelated partner randomly selected from across the given age range) and each pair was placed in one of 20 outdoor aviaries (4.1 x 1.85 x 2.24 m: bred and housed at Macquarie University) fitted with 4 nest boxes and nesting material. We force-paired birds to maximise the among-pair variance in the level of coordination, given that if pairs were allowed to choose each other or had spent time together prior to the start of the work such pairs would be likely to already exhibit some level of coordination (Spoon et al. 2006; Schuett et al. 2011). Birds were maintained under these conditions and provided with food and water *ad libitum*, as well as a daily supplement (green pea mix and hard-boiled egg) for the entire duration of the breeding trials (October 2014 - April 2015). The work consisted of three phases: 1) egg collection and sperm sampling, 2) breeding and chick production, and 3) collection of third clutch and sperm sampling (Fig. 1). Pairs were allowed to mate and nest freely across the experiment in order to best reflect natural reproduction patterns.

During Phase 1, we collected the first two clutches of eggs (comprised of a minimum of 3 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 the third egg was laid/collected (see below for details of

sperm sampling). During Phase 2, pairs were allowed up to four attempts to incubate a clutch and rear the brood to fledging. When pairs successfully fledged a brood of at least one chick, they were allowed to rear a second brood before moving to Phase 3 to examine whether hatching and fledgling success increased with experience. Therefore, successful breeders laid anywhere between 2 and 5 clutches of eggs in Phase 2. Pairs that were unsuccessful in either hatching eggs or rearing chicks across the four breeding attempts were automatically moved to Phase 3 after the failure of their fourth attempt. Finally, during phase 3, we collected the next laid clutch of eggs (hereafter referred to as clutch 3) for the PVL sperm analysis and again collected a sperm sample on the day the third egg of this clutch was collected/laid.

During Phase 1 and 3, nest boxes were checked daily for evidence of nest building or egg laying until complete clutches were produced. Eggs were collected on the day they were laid and replaced with a dummy egg. Dummy eggs were left in the nest for five days after the final egg of the clutch was laid and then removed with the nest to promote the production of a new clutch. During Phase 2, nest boxes were checked daily until a complete clutch was laid, after which the nest box was left alone until the day prior to expected hatching (~12 d after 4 egg laid: Zann and Rossetto 1991), when it was again checked daily until all eggs had hatched. Unhatched eggs were removed 5 days after expected hatch date. Eggs that failed to hatch were dissected and scored for fertility and embryo age at time of developmental failure (scoring based on: Birkhead et al. 2008; Murray et al. 2013) to assess the cause of failure. Finally, fledglings were removed from their natal aviary when the youngest was at least 32 days of age. The number of eggs, hatchlings and fledglings were recorded to assess the success of reproductive attempts.

PVL sperm analysis

Eggs were collected and weighed ($\pm 0.001\text{g}$) on the day that they were laid ($N = 282$). All eggs were dipped in mineral oil and maintained at 4°C until the entire clutch was collected, after which they were frozen and stored at -20°C until processing. Eggs were cut open with fine scissors; yolk was weighed ($\pm 0.001\text{g}$) after washing away albumin and blotting on filter paper ($n = 282$). Fresh egg and yolk mass were used as measures of egg investment. The PVL was then extracted, stained with Hoechst 33342 fluorescent dye (Sigma, $5\text{ }\mu\text{g/ml}$), and examined for sperm (following Hurley et al. 2017) using a fluorescence microscope (Leica DM5000B) with a BP 340–380 excitation filter and a LP 425

suppression filter. The total number of sperm present on the PVL membrane ($N = 282$) was quantified blind to breeding success of pair and randomising clutch processing order (all by LLH: see electronic supplementary material S1 for full details).

Sperm sampling and motile performance

Fresh sperm samples were obtained by cloacal massage, and were immediately used to assess sperm motile performance (i.e. swimming speed and proportion of motile sperm) (following Hurley et al. 2018). Briefly, sperm were immediately diluted in pre-heated (40°C) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd.), loaded into a chamber slide (depth $20\text{ }\mu\text{m}$, Leja[®], Netherlands) on a 40°C heated microscope stage (TP-S, Tokai Hit, Shizuoka, Japan), and sperm motion recorded using phase contrast microscopy (CX41, Olympus, Japan) connected to a digital video camera (Legria HF G25, Canon, Japan). Videos of sperm motion were later analyzed using computer-assisted sperm analysis (CASA; HTM- CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research), with outputs visually examined and filtered (following Rowe et al. 2015). All CASA was done by the same person (MR) and blind to both male identity and the status of the pair (see electronic supplementary material S1 for full details of sperm sampling and analysis). Following this filtering process, we excluded samples with fewer than 10 motile sperm tracks from analyses of sperm swimming speed, based on cumulative variance analysis of single cell data (see Hurley et al. 2018). After this exclusion, the total number of motile sperm tracked for each male ranged from 15 to 458 (median = 96, mean $\pm$ SD = 121.3 ± 15.8). From these data, we obtained the proportion of motile sperm in our recording and two measures of sperm swimming speed: 1) the speed of all motile sperm tracked per sample, and 2) the speed of the fastest 10% of the individual's sperm population. We considered the fastest 10% of sperm following the logic adopted in a number of recent papers (Mossman et al. 2009; Bennison et al. 2016) that this subpopulation may include sperm that are more likely to rapidly traverse the vagina and thus have a greater chance of reaching the sperm storage tubules and thus becoming part of the fertilizing set of sperm, as well as avoiding the potentially detrimental effects of vaginal immunological responses against sperm cells. Logically, selection through fertilisation acts, at least in part, on the fastest fraction of sperm produced by a male, not necessarily on the average motility of all sperm, and limiting analysis to just these most functional sperm can provide the best repeated measure of an individual that is relevant to selection.

Statistical analysis

For these analyses we created two classification types based on success in rearing chicks to fledge in Phase 2 of the work. Specifically, pairs that successfully fledged at least one chick during any of the four breeding attempts were classified as ‘successful’, while pairs that hatched eggs but failed to fledge chicks, despite being given four chances to do so, were classified as ‘unsuccessful’ (Fig. 1). We used fledging success as a criterion because it has been shown that too many or too few sperm on the egg can lead to embryonic developmental failure (e.g. Christensen et al. 2005; Mizushima et al. 2008; Forstmeier and Ellegren 2010; Hemmings and Birkhead 2015), and that sperm quality (i.e. post-meiotic aged sperm) can slow embryonic development and reduce post hatch condition (Dharmarajan 1950; White et al. 2008). Therefore, this approach provides a more holistic view of sperm/egg coordination, than hatching success alone. Due to the underlying rationale of our study in using reproductive success to classify individuals into the two groups – successful and unsuccessful – reproductive success is used a fixed effect throughout, rather than as a response variable.

All statistical analyses were performed using R (version 3.5.3: R Core Team 2019). All modelling assumptions (normality and heterogeneity of variance of residuals) were assessed visually (following Zuur et al. 2009), overdispersion was assessed in all models (Harrison 2014). All tests were two-tailed and considered significant at $\alpha < 0.05$, and all means are shown $\pm$ SD unless otherwise noted.

To quantify the effects of breeding experience and success on sperm swimming speed (i.e. VCL, velocity over the actual sperm track) for both all sperm and the top 10% fastest sperm, whole egg and yolk mass, male body mass, and delay to initiating egg laying (defined in days: from day paired or nest removal of previous clutch) we fitted separate linear mixed models (LMM) for each dependent variable. Generalized mixed models (GLMM) were used to assess the proportion of motile sperm (binomial logit) and the total number of sperm on the PVL (negative binomial). To assess the impact of success and experience on the number of sperm on the PVL, we used a maximum of the first 5 eggs (minimum clutch size 3 eggs), reflecting the average clutch size in this species (Griffith et al. 2008). However, all results were qualitatively the same if all eggs in clutch were used (data not shown). Fixed effects included breeding experience (three-level factor: clutch 1, 2, or 3 from Phase 1 and 3) and success class (two-level factor: successful or unsuccessful), as well as their 2-way interaction. Each

model also had pair identity as a random factor to account for repeated measures. The following additional random effects were added: to VCL models – Male ID_Clutch to control for multiple sperm tracked per sample, and to the proportion motile model an observation-level was added to correct for overdispersion. We used likelihood ratio tests to compare the fit of models with and without this interaction using maximum likelihood (ML) estimation. Non-significant interactions were removed from models, and all final LMM models were fitted with REML. The sperm swimming speed and proportion motile models had the additional fixed effect of total number of sperm analysed (i.e. the number of sperm identified in the analysed video) as a proxy for sample dilution, as this has been shown to impact sperm motility in studies of passerines (Cramer et al. 2019). In their final models, to correct scaling convergence issues, the fastest 10% VLC and the total number of sperm analyzed in the proportion motile model were log rescaled.

We investigated if sperm motile performance (i.e. sperm swimming speed and proportion of motile sperm) influenced the number of sperm reaching the site of fertilisation independent of breeding success or experience. To do this, we used a negative binomial GLMM, with PVL sperm number as the response variable, using the average of the entire clutch or the average of egg 4 and 5 (eggs potentially fertilized with sperm from a mating on day of sampling). Fixed factors included VCL (scaled: all or top 10%), proportion motile sperm, and total number of sperm analyzed. Male ID was included as a random effect to control for repeat sampling. Therefore, in total, four models were run.

We investigated hatching success during Phase 2 using two approaches. We calculated the total number of hatched and unhatched eggs each pair produced across this phase and examined the effect of success class on hatching success using a two-sided two-sample test for equality of proportions without continuity correction. In this way we tested for a difference in hatching success regardless of how many attempts it took a pair to be successful. To investigate whether hatching success (proportion of hatched eggs) per breeding attempt improved with experience (i.e. number of breeding attempts) we ran separate models for each success class of pair (successful, unsuccessful) with breeding attempt (i.e. 1-4) as a fixed factor and pair identity as a random factor. Separate models were run to control for the variable number of breeding attempts.

All mixed models were performed using the ‘*lme4*’ package (Bates et al. 2015). For all models, when main effects (with 3 or more factors) were significant. For LMMs, we generated p-values using the R package *lmerTest* (Kuznetsova et al. 2016), post hoc comparisons were done using

emmeans package (Russell 2019), *piecewiseSEM* (Lefcheck 2016) package to calculate R^2 , and repeatability calculated using *rptR* (Stoffel et al. 2017). All averages are reported with standard deviation.

Data accessibility

The dataset supporting this study is available in the Knowledge Network for Biocomplexity repository, <https://doi.org/10.5063/F1T43RFB>.

Results

General breeding success

Of the 20 pairs initially established, one pair member died in two pairs during the study and another was dropped due to a missed clutch collection. Consequently, all data from these pairs was excluded from analyses. For the remaining 17 pairs across the four breeding attempts in Phase 2, success varied: 12 pairs were successful in rearing chicks, and 5 pairs were unsuccessful (see Fig. 1).

All pairs reduced the number of days taken to lay a new clutch as they gained experience (supplementary Table S1), from an average of six days down to three days (from collected clutch 1 to clutch 3: $t_{32} = -2.28$, $p = 0.03$; n.b. all unsuccessful birds laid 4 clutches of eggs and successful laid between 2 and 5 clutches in the interim between these collected clutches). Further, there was no significant difference in delay to new clutch between success classes ($t_{15} = 0.80$, $p = 0.43$), with clutch initiation reducing from clutch 1 to clutch 3 by 56.4% (successful) and 31.4% (unsuccessful). We found a significant increase in egg investment with experience (Table 1). There was a significant increase in yolk mass between clutch 1 and 3, and an increase in egg mass with each subsequent clutch (Table 1). There was no consistent difference over attempts between the successful or unsuccessful pairs in yolk mass or egg mass (Table 1).

In Phase 2 of the work, successful pairs laid a total of 180 eggs across two to five clutches (5.29 ± 1.09 per clutch), of which 170 (94.4%) hatched, and 77 were successfully fledged (overall 42.8% of eggs resulted in fledglings, an average of 2.24 ± 1.91 per clutch). In contrast, unsuccessful pairs laid a total of 78 eggs (4.87 ± 1.45 per clutch) across four clutches, of which 68 (87.2%) hatched. All unsuccessful clutches produced resulted in at least two hatched chicks, but no chicks survived to fledging. Thus, in a simple comparison of success class, overall hatching success was significantly

higher in successful compared to unsuccessful pairs ($X^2_1 = 4.02$, $p = 0.04$). Hatching success was not significantly affected by breeding experience in unsuccessful birds ($X^2_3 = 1.85$, $p = 0.60$), with average hatching never rising above 90.3% (third breeding attempt). However, successful birds showed a significant increase in hatching success between breeding attempt 1 to 2 ($z = 2.42$, $p = 0.02$) and breeding attempt 1 to 3 ($z = 2.42$, $p = 0.01$), with 100% of their eggs hatching after successful rearing one set of chicks. Overall, hatching failure appeared to be caused primarily by early embryo mortality; 57.9% of eggs were classified as failed at < 4 days embryonic age, while 31.6% failed at > 10 days embryonic age, and 10.5% appeared infertile.

PVL Sperm numbers

The relationship between the number of sperm on the PVL of eggs differed significantly between success classes and clutch (Table 2, Fig. 2). In this model, clutch number and success class accounted for 10% of the variance, and pair identity was significantly repeatable ($p < 0.001$, $R = 0.32$, $CI = 0.11 - 0.43$). In successful pairs, the average number of sperm on the PVL was significantly lower at clutch 3 compared to both clutch 1 ($z = 4.74$, $p < 0.001$) and clutch 2 ($z = 4.96$, $p < 0.001$). In contrast, unsuccessful pairs showed no difference in the average number of sperm on the PVL between clutch 1 and 3 ($z = -0.04$, $p = 0.99$) or clutch 1 and 2 ($z = -0.53$, $p = 0.86$). Comparing total PVL sperm number, successful pairs had a significantly lower number of sperm on the PVL compared to unsuccessful pairs in clutch 3 ($z = -2.06$, $p = 0.04$), but did not differ at clutch 1 ($z = -0.07$, $p = 0.94$) or clutch 2 ($z = 0.34$, $p = 0.73$).

Sperm motile performance and male mass

In the model examining the VCL of all motile sperm, we found no significant effect of success class, experience, or the number of sperm identified in sample on sperm swimming speed (Table 3). In contrast, using the fastest 10% subpopulation of sperm, we found a significant interaction between success class and clutch number, but no effect of number of sperm in the sample (Table 3). Post-hoc testing showed that while successful pairs did not start with significantly faster sperm than unsuccessful pairs (clutch 1: $t_{23,6} = 1.23$, $p = 0.23$), there was a significant decrease in sperm swimming speed over time in the successful pairs; most notably between clutch 1 and clutch 2 sampling (Fig. 3: $t_{367} = 3.20$, $p = 0.004$). In contrast, the sperm of males in unsuccessful pairs significantly increased

swimming speed between clutch 2 and 3 ($t_{366} = -4.51$, $p < 0.001$). However, at clutch 3, there was no significant difference in the swimming speed of the top 10% subpopulation of sperm between the successful and unsuccessful pairs ($t_{17.4} = -1.84$, $p = 0.08$), despite the significant difference in slope direction. Finally, there was no effect of either success class ($z = 0.15$, $p = 0.88$) or breeding experience (all $z < 0.4$, all $p > 0.69$) on the proportion of motile sperm in a sample (percentages Table 4, full model supplementary Table S2). Similarly, there was no effect of sperm number (i.e. dilution) on the proportion of motile sperm in a sample ($z = 0.56$, $p = 0.58$: supplementary Table S2).

Both successful and unsuccessful males significantly increased their body mass across the experiment (clutch 1 to 3 $z = 3.44$, $p = 0.002$: supplementary Table S3), with post-hoc testing showing body mass increasing in both success groups from Clutch 1 to 3 (successful: $t_{30} = -3.44$, $p = 0.005$, unsuccessful: $t_{30} = -4.60$, $p < 0.001$). Importantly, there was no difference in male body mass between the success classes, nor was the interaction between success class and clutch number significant (all $z < 2.0$, all $p > 0.06$: supplementary Table S3).

PVL sperm numbers and sperm motile performance

Total number of sperm present on the PVL (i.e. average across total clutch, at egg4, and egg 5) was not influenced by sperm swimming speed (either all sperm or just the top 10%), proportion motile sperm, or total number of sperm analyzed (Table 4). Finally, the proportion of motile sperm was positively correlated with sperm swimming speed when all tracked sperm were used in the analysis ($r = 0.45$, $t_{44} = 3.31$, $p\text{-value} = 0.002$), but not when sperm swimming speed was assessed with just the fastest top 10% of sperm ($r = 0.06$, $t_{44} = 0.39$, $p\text{-value} = 0.69$).

Discussion

We investigated a number of parameters that likely reflect the reproductive coordination of a socially monogamous partnership, including one of the most fundamental aspects of sexual reproduction in internal fertilisers - the extent to which sperm reach the site of fertilisation. We found that, after they gained breeding experience, pairs that successfully fledged offspring had a lower number of sperm on the yolk membrane within the egg (PVL sperm number) compared to unsuccessful pairs (i.e. those that failed to fledge chicks). Furthermore, in successful pairs, the number of PVL sperm decreased, while hatching success increased, as they gained experience breeding together as a pair. In contrast,

381 unsuccessful pairs showed no change in PVL sperm number or hatching success with experience, such
382 that PVL sperm numbers were greater, and hatching success lower, than that of successful pairs by the
383 end of the experiment. These changes were not influenced by egg or yolk mass, as both increased in all
384 pairs over progressive breeding attempts. These results support the idea that there is an optimal number
385 of supernumerary sperm on the PVL, and that this number was often exceeded in unsuccessful pairs.
386 This could contribute to the high percentage of early embryo failure seen in unhatched eggs both in this
387 study and more generally across birds (Christensen et al. 2005; Forstmeier and Ellegren 2010). As we
388 measured PVL sperm numbers and hatching success of different clutches, we were unable to directly
389 assess the direct relationship between the two, particularly as the repeatability of PVL within a pair was
390 significant, but low (0.32). This low repeatability meant that it would be inappropriate to relate PVL
391 measured on one clutch against the hatching success of a later clutch.

392 Our finding that successful pairs have fewer sperm on the PVL may at first seem counter
393 intuitive, given that previous studies have invoked the idea that poor quality males might be
394 functionally infertile (Sheldon 1994) and that poor fertilisation success may be driven by the lack of
395 sperm on the PVL (Birkhead and Fletcher 1998). However, as our study is the first to examine this
396 aspect of pair coordination, the literature offers no indication as to whether one would predict a greater
397 or lesser number of sperm on the egg membrane in the most coordinated pairs. Indeed, ejaculate-
398 female/sperm-egg interactions are likely to be evolutionarily dynamic (Hasson and Stone 2009; Pitnick
399 et al. 2009), and in other species it may indeed be the case that unsuccessful pairs may fail through a
400 lack of sperm, while successful pairs have higher numbers of sperm on the PVL. That is because the
401 number of sperm on the egg membrane will depend upon a range of parameters, such as the number of
402 sperm transferred during copulation, number of copulations, sperm quality, and female sperm storage
403 and usage (Brillard and Bakst 1990; Hemmings et al. 2016), as well as a number of complex
404 physiological and molecular interactions between male and female cells, tissues, and proteins critical to
405 successful fertilisation. As such, the optimal sperm number for fertilisation and successful development
406 may vary across species (Hurley et al. 2017), though in the case of the zebra finch our findings here
407 suggest this to be around 70.0 ± 7.2 sperm (average total sperm for clutch 3 for successful pairs). We
408 suggest this as this number is lower than, but similar to, earlier reported averages for this species of
409 around 87.4 ± 28.9 (Birkhead et al. 1993) and 106.3 ± 6.5 (Hurley et al. 2017) sperm trapped in the
410 PVL; although these earlier studies did not discriminate between successful and unsuccessful pairs, and

as such may have examined eggs produced by pairs that would have had low hatching/fledgling success. Further, it is 3 times higher than the ~20 PVL sperm needed to achieve a 50% chance of successful fertilisation (Birkhead and Fletcher 1998).

The complex and cryptic nature of ejaculate-female and sperm-egg interactions make it difficult to determine specific causal factors explaining the lower number of PVL sperm in successful and more experienced pairs in our study. We suggest that social interactions between the pair, such as a change in copulation rate, likely plays a large part in breeding success beyond female- or male-specific processes, although this would require future study to tease apart. Nonetheless, the post-mating interactions and the female will play a critical role, given that: the vagina is thought to be an active site of sperm selection (Birkhead and Brillard 2007); that sperm may spend several days in sperm storage tubules and that maintenance of stored sperm appears to be influenced by the microenvironment of the female (Sasanami et al. 2013); and finally that female-derived substances might support sperm movement up the reproductive tract (Matsuzaki and Sasanami 2017). There is certainly strong selection on the female to ensure that enough sperm, but not too many sperm, reach the egg to ensure fertilisation and embryo development (Hemmings and Birkhead 2015) but also reduce the risk of early development failure from genetic abnormality (Fechheimer 1981; Christensen et al. 2005; Forstmeier and Ellegren 2010). While we are currently unable to draw many conclusions about the precise nature of the female role in the patterns we found here, our work highlights the considerable scope for future research in this area.

From the male perspective, some proportion of the differences we observed across success classes and over the period of breeding experience may be attributable to intrinsic differences among males. In zebra finches, sperm morphology has been shown to be highly variable among-males, though within-male repeatability of sperm length is high (Birkhead et al. 2005). Moreover, the success of sperm in reaching the ovum has been linked to both total sperm length (Bennison et al. 2015), as well as the absolute and relative length of the sperm head (Hemmings et al. 2016), with longer faster sperm fertilising more eggs (Kim et al. 2017; Knief et al. 2017). Longer sperm tend to swim faster (Mossman et al. 2009; Bennison et al. 2015), so slower sperm swimming speed could cause a reduction in the number of sperm reaching the ova. However, we found no significant relationship between sperm swimming speed and the number of sperm reaching the ova. These results, contrast somewhat with those found in a competitive mating experiment in which longer, faster swimming sperm were more

likely to reach the PVL and fertilise eggs (Bennison et al. 2015; Kim et al. 2017). However, in this previous work, the social partnership was disrupted by the experimental design (Bennison et al 2015), and therefore, perhaps the lack of a relationship in our study (between swimming speed and PVL sperm) indicates that the social partnership obfuscates any effect of intrinsic differences in sperm traits *per se*. Nonetheless, it is possible that certain male traits could help to explain the differences in the number of sperm reaching the PVL across success classes of males observed in our study.

Interestingly, sperm morphology has been shown to change in response to changes in a male's social situation in at least two passerine species (e.g. Immler et al. 2010; Girndt et al. 2019). In our study, the data collected after the pairs were given the opportunity to breed and rear offspring will likely have been affected by the social differences between the groups of males, resulting from the different reproductive history of the males up to that point. For example, successful males are by definition in a good partnership and they will have been investing fully in parental behaviour and chick rearing. In contrast, unsuccessful males are in a poor partnership in which they have not invested in any chick rearing. Presumably at some level, individuals will value these unproductive partnerships differently and males or females in unsuccessful partnerships might respond through a deterioration of the social bond on the basis that it would probably be adaptive for one or both of them to find a new partner (although our study suggests that zebra finches do not seem to attribute breeding failure to mate choice; Ihle et al. 2015).

Though we did not measure sperm morphology in this study, we did measure sperm swimming speed. We show that sperm swimming speed fluctuates over the course of the study, but that these fluctuations followed different patterns in males that displayed different levels of success. In particular, sperm swimming speed declined significantly in males in successful pairs, whereas sperm swimming speed of males in unsuccessful pairs increased. Importantly, these changes were not related to differences in body condition; all males significantly increased their mass over the duration of the experiment and at the same rate across both groups. Similar short-term fluctuations in sperm quality have been linked to changes in male social status in domestic fowl: sperm mobility declined over time in socially dominant males while it remained relatively constant in subdominant males (Pizzari et al. 2007). In that study, it was suggested that the observed fluctuations in sperm quality might be explained by trade-offs between pre- and post-copulatory trait investment (Pizzari et al. 2007). Although the zebra finch is socially monogamous, we believe that changes in sperm swimming speed

over time in relation to the social context of a partnership, are in line with the idea of a trade-off between fertilisation effort and parental effort, i.e. increased allocation to parental effort required for raising offspring constrains allocation to future investment in post-copulatory traits.

Alternatively, the changes we observed in sperm motile performance may be related to post-meiotic sperm aging and variation in copulation rates across success classes. For example, the social context (within the partnership) of an unsuccessful pair might result in an increased rate of copulation by the pair, as they try harder to produce fertile eggs, with the increase in copulation rate resulting in the release of higher quality or younger sperm. However, male zebra finches suffer from sperm depletion when copulation rates are high, and indeed ejaculates sampled just one hour apart show a nearly 50% reduction in sperm motile performance (Birkhead et al. 1995). Though we were unable to determine the role of copulation history in our study, our findings suggest future work aimed at understanding phenotypic plasticity of sperm traits in response to changes in social context, beyond those associated with risk and intensity of sperm competition, are warranted.

A related issue that is worthy of further work is the extent to which higher or lower copulation rates, or adjustments by the female (cf. Hemmings and Birkhead 2015) may be counter-productive and lead to egg failure as a result of too much, or too few sperm, present on the PVL. Restriction of copulation, and thus post-meiotic aging of sperm in the male or female, has been shown to impact number of sperm reaching PVL, embryonic development, and hatching rate (Lodge et al. 1971; Birkhead and Fletcher 1998; White et al. 2008). We suggest a valuable question to ask is: to what extent are the higher levels of sperm seen on the PVL of unsuccessful pairs a result of a male-driven process, a female-driven process, or a lack of mutual coordination?

The differences that we have demonstrated across classes of successful and unsuccessful pairs, as well as the changes observed across sequential reproductive attempts have identified a number of new questions about the mechanisms and adaptive consequences of reproductive synchrony between socially monogamous partners. We acknowledge that our definition of pair success encompasses potential influencing factors beyond those of sperm quality, fertilisation, and embryonic development, but involves broader parental effects such as the ability to effectively incubate eggs and brood and provision offspring. Nonetheless, our measure of success is evolutionarily relevant, and future work could partition these quite different determinates of pair fitness by experimentally cross-fostering eggs or offspring to examine infertility, developmental failure, and parental care separately.

We believe that our results are relevant to most avian species, because polyspermy is ubiquitous and while the number of sperm on the perivitelline layer is related to the size of the egg (Birkhead et al. 1994), there are consistent differences in the number of sperm across species that are similar in size (Hurley et al. 2017), suggestive of a species-specific optima. While the zebra finch is a species with a low level of genetic polyandry (Griffith et al. 2010), the dunnoek, a highly polyandrous species (Burke et al. 1989), has a much lower number of sperm present on the egg membranes (Birkhead et al. 1994), suggesting that there is no straightforward relationship between sperm competition and sperm numbers on the membrane, and indeed we have limited understanding of the source or consequences of variation in sperm numbers of the egg (but see Girndt et al. 2019). Our data provide the most detailed examination to date of variation in the number of sperm on eggs across pairs both in the context of success and variation over a series of reproductive opportunities. It is true that globally, many species do not have the opportunity to fine-tune coordination over a number of repeated attempts, and so we might not expect the sequential changes that we have observed here. The zebra finch, like most Australian birds, typically has a breeding seasons that is more than twice as long as those of species in the seasonally constrained temperate areas of the northern hemisphere (Englert Duursma et al. 2017), where many species get just a single breeding attempt. In such constrained species, where the cost of reproductive failure is actually greater (due to the lack of opportunity to try again), it could be argued that there should be greater selection for a male and female to coordinate effectively before laying their first, and often only, clutch. It is certainly possible that the relationship between pair coordination, or experience together, and egg sperm numbers, is stronger in short-lived and highly seasonal breeders of the northern hemisphere temperate zone. Although the zebra finch does indeed have a quite different life history to such species, it is more representative of birds in Australian and sub-tropical and tropical areas of the world and in which we typically see a higher expression of other traits that indicate higher levels of pair coordination such as female song and duetting (Odom et al. 2014).

We believe that our work highlights the importance of a renewed focus on the quality of the pair together rather than just the quality of the two individuals involved. As with the study of sexual selection more broadly, the research on sperm biology has disproportionately focused on sexual conflict and the idea of large scale variation in the extent of additive genetic variation in genes relating to fitness, i.e. good genes (Slatyer et al. 2012), and good sperm (Sheldon 1994), or coercive males (Arnqvist and Kirkpatrick 2005). Interestingly, more than a century ago, in one of the earliest studies of

reproductive behaviour in a monogamous bird, the great-crested grebe *Podiceps cristatus*, Huxley (1914) suggested that the elaborate courtship display of that species was likely because “birds have obviously got to a pitch where their psychological states play an important part in their lives”. Huxley’s (1914) conclusion was that the courtship in that species was more about developing and maintaining the pair-bond rather than selecting a partner. Whilst there are many avenues to improved fitness for coordinated partners, such as improved incubation, and nestling provisioning our study provides a first examination of the extent to which coordination between a male and female achieve successful fertilisation. Our findings thus add a new dimension to help revitalise Huxley’s (1914) long-neglected perspective on the value of good partnerships in socially monogamous birds.

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Compliance with ethical standards

Ethical standards. 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.

Conflict of interest. The authors declare that they have no conflict of interest.

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Table 1 Models comparing yolk and egg masses

Yolk Mass					
Fixed effects	Estimate	Std. Error	df	t	p
Intercept	0.25	0.01	18.86	38.32	< 0.001
Success	0.00	0.01	15.06	0.08	0.94
Clutch 2	0.00	0.00	234.90	0.84	0.40
Clutch 3	0.02	0.00	234.50	4.42	< 0.001
	Variance	SD	n		
Marginal R ²	0.05				
Pair ID	0.00	0.02	17		
Residual	0.00	0.02	253	Conditional R ²	0.46
Egg Mass					
Fixed effects:	Estimate	Std. Error	df	t	p
Intercept	0.98	0.02	17.95	50.21	< 0.001
Success	0.00	0.03	15.02	0.08	0.94
Clutch 2	0.04	0.01	234.70	3.62	< 0.001
Clutch 3	0.13	0.01	234.40	13.70	< 0.001
	Variance	SD	n		
Marginal R ²	0.29				
Pair ID	0.00	0.06	17		
Residual	0.00	0.06	253	Conditional R ²	0.64

Table 2 Model of total number of sperm on the PVL

Total number of sperm on PVL				
Fixed effects	Estimate	Std. Error	z	p
Intercept	4.78	0.18	26.17	< 0.001
Success	0.02	0.34	0.07	0.94
Clutch 2	0.04	0.14	0.27	0.79
Clutch 3	-0.65	0.14	-4.74	< 0.001
Success:Clutch 2	-0.14	0.28	-0.50	0.62
Success:Clutch 3	0.66	0.27	2.47	0.01
	Variance	SD	n	
Marginal R ²	0.09			
PairID	0.29	0.54	17	
			232	Conditional R ² 0.38

Table 3 Model of all and top 10% fastest VCL single cell recordings

VCL All Recorded Sperm					
Fixed effects	Estimate	Std. Error	df	t	p
Intercept	109.06	5.77	38.01	18.91	<0.0001
Success	-1.33	7.62	16.15	-0.18	0.86
Clutch 2	-1.65	5.16	27.48	-0.32	0.75
Clutch 3	5.53	5.13	27.49	1.08	0.29
Total number of sperm analysed	0.02	0.02	30.44	0.91	0.37
	Variance	SD	n		
Marginal R ²	0.01				
Male ID_Clutch	169.2	13.01	46		
Pair ID	128.7	11.35	17		
Residual	1050.6	32.41	4030	Conditional R ²	0.23
Fastest 10% of VCL					
Fixed effects:	Estimate	Std. Error	df	t	p
Intercept	5.18	0.04	43.65	117.98	<0.0001
Success	-0.15	0.07	42.43	-2.13	0.04
Clutch 2	-0.09	0.05	24.98	-1.94	0.06

Clutch 3	-0.10	0.05	24.90	-2.19	0.04
Total number of sperm analysed	0.00	0.00	30.81	0.39	0.70
Success:Clutch 2	0.15	0.09	26.57	1.72	0.10
Success:Clutch 3	0.26	0.09	26.16	2.79	0.01
	Variance	SD	n		
Marginal R ²	0.13				
Male ID_Clutch	0.01	0.10	46		
Pair ID	0.00	0.05	17		
Residual	0.01	0.07	379	Conditional R ²	0.37

Table 4 Average percentage motile sperm (average $\pm$ SD) by success class for clutches 1, 2, and 3, as well as an overall average of all samples collected within each success class

	Clutch 1	Clutch 2	Clutch 3	Overall
Successful	54.0 $\pm$ 22.6%	54.9 $\pm$ 26.3%	65.3 $\pm$ 19.1%	58.1 $\pm$ 22.7%
Unsuccessful	60.8 $\pm$ 28.9%	66.1 $\pm$ 25.4%	48.9 $\pm$ 24.2%	58.5 $\pm$ 25.1%

Table 5 Model of how PVL sperm number (clutch or egg 4 and 5 average) are influenced by average total and top 10% VCL (scaled), proportion motile, and total sperm recorded

Average	All recorded sperm VCL				Fastest 10% VCL			
	Estimate	SE	z	p	Estimate	SE	z	p
Total Clutch								
Intercept	5.46	0.73	7.50	< 0.001	4.80	1.02	4.72	< 0.001
VCL scaled	-0.17	0.91	-0.19	0.85	0.57	0.99	0.58	0.56
Proportion motile	-0.87	0.65	-1.33	0.18	-0.93	0.55	-1.71	0.09
Total sperm recorded	< -0.001	0.001	-0.30	0.76	<-0.001	0.001	-0.44	0.65
	Variance	SD	n		Variance	SD	n	
Pair ID	0.07	0.27	17		0.06	0.24	17	
Egg 4 & 5								
Intercept	4.62	0.68	6.79		4.41	1.10	4.02	
VCL scaled	0.33	0.89	0.38	0.70	0.61	1.13	0.54	0.59
Proportion Motile	-0.27	0.62	-0.43	0.66	-0.28	0.55	-0.51	0.61
Total sperm recorded	< -0.001	0.001	-0.96	0.34	-0.001	0.001	-1.08	0.28
	Variance	SD	n		Variance	SD	n	
Pair ID	< 0.001	0.001	17		< 0.001	0.001	17	

Figure Legends

Fig. 1 Experimental design. Pairs were formed using virgin birds. Phase 1: Egg collection for PVL analysis and sperm sampling for quantification of sperm motile performance was undertaken for the first two clutches of eggs produced by each pair. Phase 2: Breeding and chick production phase. Pairs were given four breeding attempts to produce a clutch of eggs that resulted in fledglings. Pairs were deemed 'successful' (denoted by chick head) if the reared chicks to fledge on their first or any subsequent attempt (in box) after failing (X) previous attempts. Pairs that never reared chicks were deemed 'unsuccessful'. Once pairs successfully reared a clutch to fledging they were allowed to produce and raise a second clutch. Phase 3: After breeding attempts were completed, a third clutch of eggs was collected for PVL analysis and sperm samples collected for assessment of sperm motile performance

Fig. 2 Total number of sperm on outer perivitelline layer (PVL) across clutches (1-3) in each of the two classes of breeding pairs: successful (white bar), unsuccessful (light grey bar). Clutch 1 and clutch 2 were first ever produced by the pair, and clutch 3 was produced after attempts to rear chicks to fledge. Circles represent raw data points with bean smooth density outline, bar denotes mean, box shows standard error

Fig. 3 Change in Top 10% or All sperm swimming speed (VCL: μms^{-1}) in successful (white bar), unsuccessful (light grey bar) breeding pairs with breeding experience (clutch). Clutch 1 and clutch 2 were those clutches first produced by a pair, and clutch 3 was produced after allowing attempts to rear chicks. Circles represent raw data points with bean smooth density outline, bar denotes mean, box shows standard error

Supplementary Material S1

Reproductive coordination breeds success: the importance of the partnership in avian sperm biology

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Full Descriptive Methods

Egg investment and PVL sperm analysis description:

Eggs were collected and weighed (to nearest 0.001g) on the day they were laid. Eggs were maintained at 4°C until the entire clutch was collected, after which they were frozen and stored at -20°C until processing. To process eggs, we thawed them at 4 °C and then carefully cut eggs open and discarded the shell and albumen. The yolk was rinsed, dried by gently rolling it across filter paper, and weighed (to nearest 0.001g). The yolk was cut approximately in half with the germinal disc centred on one side (following Hurley et al. 2017). Both PVL sections were then separated from the yolk and washed in phosphate buffered saline (PBS) using a hair loop. The washed sections were 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. We systematically scanned the slide at 200x magnification and quantified the total number of PVL sperm by counting all stained sperm on both halves of the PVL.

Sperm sampling and motile performance

Fresh sperm samples were obtained by cloacal massage (Wolfson, 1952), and were immediately used to assess sperm motile performance (swimming speed and proportion of motile sperm) (following Rowe et al 2015). Briefly, sperm were collected and immediately diluted in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Ltd.) pre-heated to 40°C [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 videoed for six unique fields of view (5s each for total 30s) at 400x using phase contrast microscopy (CX41, Olympus, Japan) connected to a digital video 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 40°C throughout.

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 set at 50 Hz and 25 frames (i.e. sperm cells were tracked for 0.5 s per field of view). Each analysis was visually examined and cell detection parameters established to exclude non-sperm particles as follows: 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 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 blind to both male identity and the status of the pair.

Following this filtering process, we excluded samples with less than 10 motile sperm tracks from analyses of sperm swimming speed, based on cumulative variance analysis of single cell data (see Hurley et al 2018). After this exclusion, the total number of motile sperm tracked for each male ranged from 15 to 458 (median = 96, mean = 121.3 ± 15.8). 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 used 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 recorded cells.

References:

- Calder WA. 1964 Gaseous metabolism and water relations of the zebra finch, *taeniopygia castanotis*. *Physiol Zool* **37**(4), 400-413.
- Hurley LL, Fanson KV, Griffith SC. 2017 Variation in the number of sperm trapped on the perivitelline layer of the egg in three species of estrildid finch. *Auk* **134**(4), 832-841. (doi:10.1642/auk-17-41.1).
- Hurley LL, McDiarmid CS, Friesen CR, Griffith SC, Rowe M. 2018 Experimental heatwaves negatively impact sperm quality in the zebra finch. *Proc R Soc B* **285**(1871). (doi:10.1098/rspb.2017.2547).
- Rowe M, Griffith SC, Hofgaard A, Lifjeld JT. 2015 Subspecific variation in sperm morphology and performance in the long-tailed finch (*poephila acuticauda*). *Avian Research* **6**, 1-10. (doi:Artn 23 10.1186/S40657-015-0032-Z).
- Wolfson A. 1952 The cloacal protuberance: A means for determining breeding condition in live male passerines. *Bird-banding* **23**(4), 159-165.

Supplemental Tables

Table S1. Model of lay delay

Lay Delay					
Fixed effects	Estimate	Std. Error	df	t-value	p-value
Intercept	5.63	1.00	41.24	5.62	< 0.001
Success	1.04	1.30	15.00	0.80	0.43
Clutch 2	-0.41	1.24	32.00	-0.33	0.74
Clutch 3	-2.82	1.24	32.00	-2.28	0.03

	Variance	SD	n		
Marginal R ²	0.11				
Pair ID	1.62	1.27	17		
Residual	12.99	3.60	51	Conditional R ²	0.21

Table S2. Model of proportion motile

Proportion Motile					
Fixed effects	Estimate	Std. Error	z-value	p-value	
Intercept	-0.12	0.98	-0.12	0.90	
Success	0.05	0.34	0.15	0.88	
Clutch 2	0.10	0.38	0.25	0.80	
Clutch 3	0.15	0.38	0.40	0.69	
log Total number of sperm analysed	0.11	0.20	0.56	0.58	
	Variance	SD	n		
Marginal R ²	0.003				
Observation-level	9.96E-01	0.998	46		
Pair ID	3.04E-07	0.001	17		
			46	Conditional R ²	0.19

Table S3. Model of male mass

Male Mass					
Fixed effects	Estimate	Std. Error	df	z-value	p-value
Intercept	13.49	0.39	19.84	34.83	< 0.001
Success	-0.99	0.71	19.84	-1.39	0.18
Clutch 2	0.48	0.25	30.00	1.96	0.06
Clutch 3	0.85	0.25	30.00	3.44	0.002
Success:Clutch2	-0.14	0.46	30.00	-0.31	0.76
Success:Clutch3	0.91	0.46	30.00	2.00	0.06
	Variance	SD	n		
Marginal R ²	0.17				
Pair ID	1.43	1.20	17		
Residual	0.37	0.61	51	Conditional R ²	0.83

Table S4. Experimental details as per suggestion of Griffith et al. 2017. Ethology.

N experimental groups	1 group started – 2 formed at end by categorising groups by being ‘successful’ or ‘unsuccessful’ in fledging at least one chick
N control groups	0
Nature of replication	
N adult males used	20
N adult females used	20
N males with opportunity to reproduce	20
N females with opportunity to reproduce	20
N females that laid eggs	20
N females that had chicks	20
N females that fledged young	15

N males for which data is presented	17
N females for which data is presented	17
N individuals that died or removed	2 pairs removed from analysis as one pair member died before end of experiment, 1 pair removed as an egg collection was missed.
Other reasons for missing data	na
Nature of any experimental manipulation	None
Nature of any invasive work	Sperm and Blood collection of males
Duration given for breeding opportunity	Up to 4 clutching attempts to produce chicks
Domesticated or wild stock	Domestic
Source population	Australia domestic
Variety	Wild type
Age	12-18 months
Average mass of adults	13.6
Prior breeding experience	No
Allocation of breeding partners	Force-paired
Any bias in selection of individuals	Virgins
Cage/aviary size	4.1 x 1.85 x 2.24 m
N individuals per cage	2
Sex ratio present in each cage	0.5
Food provided <i>ad libitum</i>	ad lib
Supplemental food provided	Hard boiled egg and green pea mix (peas, spinach, micro/macro nutrient supplement) , daily
Any restriction in provision of food	None
Type of nest site provided	Wooden box
Nesting material provided	November grass, emu feathers, hessian
Environmental enrichment or shelter	Natural branch perches, tree and/or grass in aviary
Indoors or outside	Outside
Temperature control	Local natural conditions
Humidity control	Natural
Light/dark cycle	Natural
Average clutch size $\pm$ SD	Successful pairs: 5.29 ± 1.09 , Unsuccessful: 4.87 ± 1.45
Average number of fledglings $\pm$ SD	Successful pairs: 2.24 ± 1.91 , Unsuccessful: 0

Griffith SC, Crino OL et al. (2017) Variation in Reproductive Success Across Captive Populations: Methodological Differences, Potential Biases and Opportunities.

Supplementary Material S2:

R code for “Reproductive coordination breeds success: the importance of the partnership in avian sperm biology”

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This is an R Markdown output. It includes all the R code to generate the main results in the paper

Libraries

```
library(lme4)
library(MASS)
library(car)
library(emmeans) #was lsmeans
library(lmerTest)
library(ggplot2)
library(tidyr)
library(dplyr)
library(doBy)
library(MuMIn) ##Marginal R2 and random factors
library(rptR)
library(piecewiseSEM)
```

The data

```
dat <- read.csv("General_Success")

Hatch <- read.csv("Hatch")

ALLVCL <- read.csv("VCL_Single_Cell")
ALLVCL <- ALLVCL[ALLVCL$Include>=10,]

Top10 <- read.csv("VCL_Top10")
```

Over dispersion tests

```
#GLMM FAQ https://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#overdispersion
```

```

overdisp_fun <- function(model) {
  rdf <- df.residual(model)
  rp <- residuals(model,type="pearson")
  Pearson.chisq <- sum(rp^2)
  prat <- Pearson.chisq/rdf
  pval <- pchisq(Pearson.chisq, df=rdf, lower.tail=FALSE)
  c(chisq=Pearson.chisq,ratio=prat,rdf=rdf,p=pval)
}
#####
# Quantifying Point Estimate of Overdispersion
#Harrison XA. 2014. Using observation-level random effects to model
overdispersion in count data in ecology and evolution. PeerJ 2:e616 DOI
10.7717/peerj.616.
# value >1 indicates overdispersion
#####
od.point<-function(modelobject){
  x<-sum(resid(modelobject,type="pearson")^2)
  rdf<-summary(modelobject)$AICtab[5]
  return(x/rdf)
}

#od.point(model)

#####
# Quantifying Overdispersion Through Parametric Bootstrap
#####

#Function to pass to parametric bootstrap function 'bootMer' that calculates
the sum of squared Pearson residuals (required for 'od' function)
FUN <- function(fit) {
  #return(fixef(fit))
  x<-resid(fit,type="pearson")
  return(sum(x^2))
}

#Function To Calculate Ratio of Model SS to Mean Parametric Bootstrap SS
('bias')
od<-function(bootobject){
  biasvals<-bootobject $t0/bootobject[2]$t
  bias<-mean(biasvals,na.rm=T)
  intervals<-quantile(biasvals,c(0.025,0.975),na.rm=T)
  dat<-c(bias,intervals)
  return(dat)
}

```

Delay to Lay

```

dat1 <- dat[dat$EggNumberF=="E1",] #simplifies to one row of data per Clutch
1, 2, 3 for variables with only one value

```

```

Delay <- lm(LayDelay~SucessfulF*ClutchID, data=dat1)
Delay1 <- lmer(LayDelay~SucessfulF*ClutchID+(1|PairID), data=dat1,
REML=FALSE)
anova(Delay1, Delay)
summary(Delay1)

Delay2 <- lmer(LayDelay~SucessfulF+ClutchID+(1|PairID), data=dat1,
REML=FALSE)
anova(Delay2, Delay1)
summary(Delay2)

DelayTrue <- lmer(LayDelay~SucessfulF+ClutchID+(1|PairID), data=dat1, REML=T)
summary(DelayTrue) #PVL1-first

rsquared(DelayTrue)

#emmeans contast
warp.emmD <- emmeans(DelayTrue, ~ SucessfulF|ClutchID)
pairs(warp.emmD, simple = "each", combine = F) #contrasts all

LDr <- resid(DelayTrue)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(LDr)
qqnorm(LDr); qqline(LDr, col = 2)
hist(LDr, xlab = "LayDelay Residuals", main = "")
plot(dat1$NewClass, LDr, xlab = "Sucess", ylab = "LayDelay Residuals")
plot(dat1$ClutchID, LDr, xlab = "ClutchID", ylab = "LayDelay Residuals")
par(op)
#normality of residuals
shapiro.test(LDr)
#Bartlett test of homogeneity of variance
bartlett.test(LDr, dat1$NewClass)

```

Yolk mass

```

Yolk <- lm(YolkWeight~SucessfulF*ClutchID, data=dat)
Yolk1 <- lmer(YolkWeight~SucessfulF*ClutchID+(1|PairID), data=dat,
REML=FALSE)
anova(Yolk1, Yolk)
summary(Yolk1)

YolkTrue2 <- lmer(formula = YolkWeight ~ SucessfulF + ClutchID + (1 |
PairID), data = dat,
REML = TRUE)
summary(YolkTrue2)

rsquared(YolkTrue2)

```

```

#emmeans contrast
warp.emmY <- emmeans(YolkTrue2, ~ SucessfulF|ClutchID)
pairs(warp.emmY, simple = "each", combine = F) #contrasts all

YWr <- resid(YolkTrue2)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(YWr, add.smooth = FALSE, with=1)
qqnorm(YWr); qqline(YWr, col = 2)
hist(YWr, xlab = "Yolk Weight Residuals", main = "")
plot(dat$NewClass, YWr, xlab = "Sucess", ylab = "Yolk Weight Residuals")
plot(dat$ClutchID, YWr, xlab = "ClutchID", ylab = "Yolk Weight Residuals")
par(op)
#normality of residuals
shapiro.test(YWr)
#Bartlett test of homogeneity of variance
bartlett.test(YWr, dat$NewClass)

```

Egg mass

```

Egg <- lm(EggWeight~SucessfulF*ClutchID, data=dat)
Egg1 <- lmer(EggWeight~SucessfulF*ClutchID+(1|PairID), data=dat, REML=FALSE)
anova(Egg1, Egg)
summary(Egg1)

EggTrue2 <- lmer(formula = EggWeight ~ SucessfulF + ClutchID + (1 | PairID),
data = dat,
REML = TRUE)
summary(EggTrue2)

rsquared(EggTrue2)

#emmeans contrast
warp.emmE <- emmeans(EggTrue2, ~ SucessfulF|ClutchID)
pairs(warp.emmE, simple = "each", combine = F) #contrasts all

YWr <- resid(EggTrue2)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(YWr, add.smooth = FALSE, with=1)
qqnorm(YWr); qqline(YWr, col = 2)
hist(YWr, xlab = "Egg Weight Residuals", main = "")
plot(dat$NewClass, YWr, xlab = "Sucess", ylab = "Egg Weight Residuals")
plot(dat$ClutchID, YWr, xlab = "ClutchID", ylab = "Egg Weight Residuals")
par(op)
#normality of residuals
shapiro.test(YWr)
#Bartlett test of homogeneity of variance
bartlett.test(YWr, dat$NewClass)

```

Hatching success

#Simple comparison of success class on overall hatching success using total laid and hatched in Phase 2 of the experiment

#2-sample test for equality of proportions without continuity correction

#<http://www.sthda.com/english/wiki/two-proportions-z-test-in-r>

```
res<- prop.test(x = c(170, 68), n = c(180, 78), alternative = "two.sided",  
correct = F)
```

```
res
```

```
res$p.value
```

```
res$estimate
```

```
res$conf.int
```

```
fisher.test(matrix(c(170, 180-170, 68, 78-68), ncol=2))
```

#Change in hatching success by group

SucHatch <- Hatch[Hatch\$Sucessful=="Suc ",] #restricting data to just Successful pairs

```
Hatch3 <- glmer(cbind(SucHatch$hatch, SucHatch$laid-  
SucHatch$hatch)~Clutch+(1|PairID), family=binomial(logit), data=SucHatch)  
summary(Hatch3)  
rsquared(Hatch3)
```

NeverHat <- Hatch[Hatch\$Sucessful=="Un",] #restricting data to just Unsuccessful pairs

```
Hatch4 <- glmer(cbind(NeverHat$hatch, NeverHat$laid-NeverHat$hatch)~Clutch  
+(1|PairID), family=binomial(logit), data=NeverHat)  
summary(Hatch4)  
rsquared(Hatch4)
```

PVL sperm - egg 1-5 only

dat2 <- dat[dat\$EggNumber<=5,] #removing any eggs Laid beyond egg 5

```
PVL0 <- glmer.nb(TotalPVL~SucessfulF*ClutchID+(1|PairID),data = dat2)  
summary(PVL0)  
rsquared(PVL0)
```

```
interaction.plot(dat2$ClutchID, dat2$SucessfulF,dat2$TotalPVL, fixed=T)#
```

#emmeans contast

```
warp.emmP <- emmeans(PVL0, ~ SucessfulF|ClutchID)  
pairs(warp.emmP, simple = "each", combine = F) #contrasts all
```

```
PVLrn <- resid(PVL0)
```

```
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
```

```
plot(PVLrn)
```

```
qqnorm(PVLrn); qqline(PVLrn, col = 2)
```

```

hist(PVLrn, xlab = "PVL Residuals", main = "")
par(op)
#normality of residuals
shapiro.test(PVLrn)
#Bartlett test of homogeneity of variance
bartlett.test(PVLrn, dat2$SucessfulF)

#repeatability using rptR
repPVL<- rpt(TotalPVL ~ ClutchID + (1 | PairID), grname = c("PairID",
"Fixed"), data = dat2, datatype = "Poisson", nboot = 1000, npermut = 0,
adjusted = FALSE)

```

Male Mass compared across Phase 1 and 3 - Clutch 1,2,3

```

dat1 <- dat[dat$EggNumberF=="E1",] #simplifies to one row of data per Clutch
1, 2, 3 for variables with only one value
MaleM0 <- lmer(MaleMass~SucessfulF*ClutchID+(1|PairID),data = dat1, REML=T)
summary(MaleM0)

MaleM0_T <- lmer(MaleMass~SucessfulF*ClutchID+(1|PairID),data = dat1, REML=T)
summary(MaleM0_T)
rsquared(MaleM0_T)

interaction.plot(dat1$ClutchID, dat1$SucessfulF, dat1$MaleMass, fixed=T)

warp.emmM <- emmeans(MaleM0_T, ~ SucessfulF|ClutchID)
pairs(warp.emmM, simple = "each", combine = F) #contrasts all

Massrn <- resid(MaleM0_T)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(Massrn)
qqnorm(Massrn); qqline(Massrn, col = 2)
hist(Massrn, xlab = "Mass Residuals", main = "")
par(op)
#normality of residuals
shapiro.test(Massrn)
#Bartlett test of homogeneity of variance
bartlett.test(Massrn, dat1$SucessfulF)

```

Single cell VCL - All sperm recorded minus cases with < 10 sperm swimming

```

ALLVCL2 <- ALLVCL[ALLVCL$Include>=10,] #removes cases with less than 10
motile sperm

VCL <- lm(VidVCL~SucessfulF * ClutchID, data=ALLVCL2)
VCL1 <- lmer(VidVCL~SucessfulF * ClutchID + VidTotalSperm +
(1|PairID)+(1|MaleClutch), data=ALLVCL2, REML = F)

```

```

anova(VCL1, VCL)
summary(VCL1)

VCL2 <- lmer(VidVCL ~ SucessfulF + ClutchID + VidTotalSperm + (1|
PairID)+(1|MaleClutch), data = ALLVCL2, REML = F)
anova(VCL2)

VCLTrue <- lmer(VidVCL ~ SucessfulF + ClutchID + VidTotalSperm +
(1|PairID)+(1|MaleClutch), data = ALLVCL2, REML = TRUE)
anova(VCLTrue)

overdisp_fun(VCLTrue)
rsquared(VCLTrue)

interaction.plot(ALLVCL2$ClutchID, ALLVCL2$SucessfulF, ALLVCL2$VidTotalSperm,
fixed=T)#

#emmeans new contrast
emm_options(pbkrtest.limit = 4030)
warp.emm1 <- emmeans(VCLTrue, ~ SucessfulF|ClutchID)
pairs(warp.emm1, simple = "each", combine = F) #contrasts all

VCLr <- resid(VCLTrue)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(VCLr)
qqnorm(VCLr); qqline(VCLr, col = 2)
hist(VCLr, xlab = "VCL Residuals", main = "")
plot(ALLVCL2$SucessfulF, VCLr, xlab = "Sucess", ylab = "VCL Residuals")
plot(ALLVCL2$ClutchID, VCLr, xlab = "ClutchID", ylab = "VCL Residuals")
par(op)
#normality of residuals
shapiro.test(VCLr)
#Bartlett test of homogeneity of variance
bartlett.test(VCLr, ALLVCL2$SucessfulF)

```

Single cell VCL - Top 10% recorded of those with > 10 sperm swimming

```

Top10$log.VidVCL <- log(Top10$VidTotalSperm)

VCLT10 <- lm(log.VidVCL~SucessfulF * ClutchID + VidTotalSperm , data=Top10)
VCLT10_1 <- lmer(log.VidVCL~SucessfulF * ClutchID + VidTotalSperm +
(1|PairID)+(1|MaleClutch), data=Top10, REML = F)
anova(VCLT10_1, VCLT10)
summary(VCLT10_1)

overdisp_fun(VCLT10_1)
od.point(VCLT10_1)

VCLT10_True <- lmer(log.VidVCL~SucessfulF * ClutchID + VidTotalSperm +
(1|PairID)+(1|MaleClutch), data=Top10, REML = T)

```



```

summary(VCLT10_True)
rsquared(VCLT10_True)

interaction.plot(Top10$ClutchID, Top10$SucessfulF, Top10$log.VidVCL, fixed=T)

VCLr <- resid(VCLT10_True)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(VCLr)
qqnorm(VCLr); qqline(VCLr, col = 2)
hist(VCLr, xlab = "VCL Residuals", main = "")
plot(Top10$SucessfulF, VCLr, xlab = "Sucess", ylab = "VCL Residuals")
plot(Top10$ClutchID, VCLr, xlab = "ClutchID", ylab = "VCL Residuals")
par(op)
#normality of residuals
shapiro.test(VCLr)
#shapiro.test(Top10$VidVCL)

#Bartlett test of homogeneity of variance
bartlett.test(VCLr, Top10$SucessfulF)

```

Proportion motile

```

dat3 <- dat1[dat1$VidMotileSperm>=10,] #removes cases with less than 10
motile sperm
dat3$log.VidTotalSperm <- log(dat3$VidTotalSperm)

PMotile0 <- glmer((cbind(VidMotileSperm, VidTotalSperm-VidMotileSperm))
~SucessfulF*ClutchID+log.VidTotalSperm+(1|PairID), data=dat3,
family=binomial(logit))
summary(PMotile0)

od.point(PMotile0) #df.residual = 23.72 = overdispersed

dat3$Obs <- factor(1:46) # After Harrison 2015 -
https://peerj.com/articles/1114/ add observation level random effect as to
mitigate overdispersion

PMotile1 <- glmer((cbind(VidMotileSperm, VidTotalSperm-VidMotileSperm))
~SucessfulF*ClutchID+log.VidTotalSperm+(1|PairID)+(1|Obs), data=dat3,
family=binomial(logit))
summary(PMotile1)

od.point(PMotile1) #0.0655

PMotile2 <- glmer((cbind(dat3$VidMotileSperm, dat3$VidTotalSperm-
dat3$VidMotileSperm))
~SucessfulF+ClutchID+log.VidTotalSperm+(1|PairID)+(1|Obs), data=dat3,
family=binomial(logit))
anova(PMotile2, PMotile1)
summary(PMotile2)

```

```

rsquared(PMotile2)

od.point(PMotile2) #0.0610

#emmeans new contrast
warp.emm2 <- emmeans(PMotile2, ~ SucessfulF|ClutchID)
pairs(warp.emm2, simple = "each", combine = F) #contrasts all

PMr <- resid(PMotile2)
op <- par(mfrow = c(2, 2), mar = c(5, 4, 1, 2))
plot(PMr)
qqnorm(PMr); qqline(PMr, col = 2)
hist(PMr, xlab = "PMotile Residuals", main = "")
plot(dat3$SucessfulF, PMr, xlab = "Sucess", ylab = "PMotile Residuals")
plot(dat3$ClutchID, PMr, xlab = "ClutchID", ylab = "PMotile Residuals")
par(op)
#normality of residuals
shapiro.test(PMr)
#Bartlett test of homogeneity of variance
bartlett.test(PMr, dat3$NewClass)

```