Effects of Female-Specific Selection for Reproductive Investment on Male Fertility Traits

Chloe Mason, University of Sheffield, camason1@sheffield.ac.uk

School of Biosciences, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom

Barbara Tschirren, University of Exeter

Nicola Hemmings, University of Sheffield

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Conflict of Interest Statement

The authors declare no conflict of interest.

Ethical Approval

All procedures were conducted under licenses provided by the Veterinary Office of the Canton of Zurich, Switzerland (permit number 195/2010; 14/ 2014; 156).

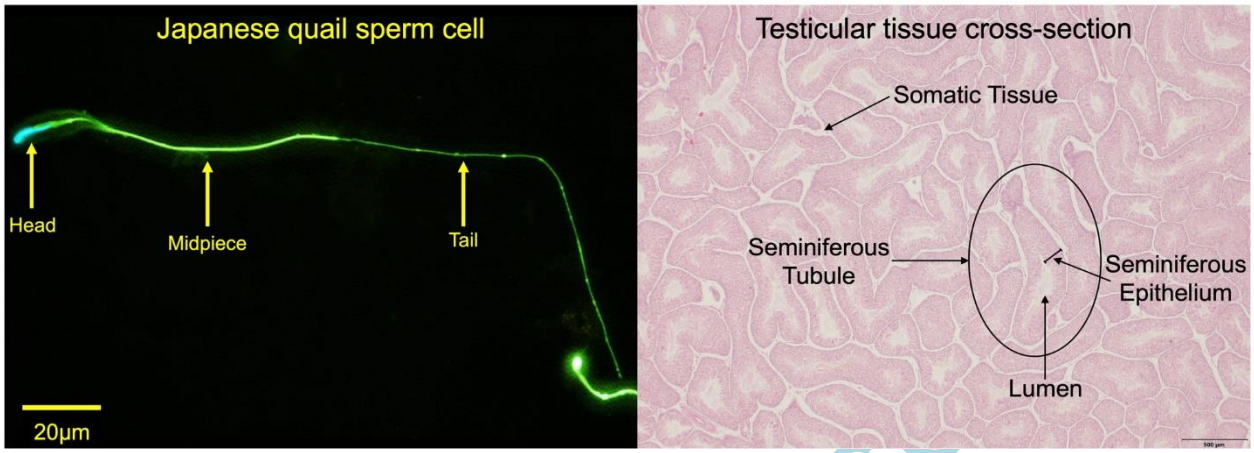
Abstract

Despite sharing an autosomal genome, the often divergent reproductive strategies of males and females cause selection to act in a sex-specific manner. Selection acting on one sex can have negative, positive, or neutral fitness consequences on the opposite sex. Here we test how female-limited selection on reproductive investment in Japanese quail (*Coturnix japonica)* affects male fertility-related traits. Despite there being no difference in the size of males' testes from lines selected for high female reproductive investment (H-line) or low female reproductive investment (L-line), in both lines, the left testis had a greater volume of sperm-producing tissue. Since H-line females have a larger left-side restricted oviduct, this suggests a positive genetic correlation between male and female gonad function, and that internal testis structure is a target of sexual selection. However, despite H-line males having previously been found to have greater fertilisation success in a competitive scenario, we found little evidence of a difference between the lines in sperm number, motility, velocity, length, or the number of sperm that reached the ova. Pre-copulatory cues and/or the role of seminal fluid in sperm motility may thus be more likely to contribute to the H-line male fertilisation advantage in this species.

Keywords

Egg size, fertility, sexual selection, sperm, spermatogenesis, testis.

Graphical abstract



Introduction

Due to the differential roles of males and females during reproduction, selection often acts in a sex-specific manner (Cox & Calsbeek, 2009). However, as males and females share an autosomal genome, selection acting on one sex can have profound effects on the phenotype and fitness of the opposite sex (Lande, 1980; Poissant et al., 2010). If the fitness optimum of a trait with a shared genetic architecture is concordant between the sexes, intersexual genetic correlations will result in an amplification of the selection response in both sexes (Whitlock & Agrawal, 2009). However, if the sexes have distinctly different selective optima for a trait with a shared genetic architecture, this can lead to intralocus sexual conflict (Bonduriansky & Chenoweth, 2009; Prasad et al., 2007). Such conflict can force the sexes to evolve a separate genetic basis and exhibit strong sexual dimorphism (Coyne et al., 2008; Lande, 1980; Poissant et al., 2010). Reproductive traits show the highest levels of sexual dimorphism across taxa (Birkhead & Pizzari, 2002), but whilst many studies have investigated the direct effect of selection on reproductive traits in one sex (Andersson, 1994; Hare & Simmons, 2018), few have studied the indirect effects of selection on the fitness of the opposite sex (e.g., Fischer et al., 2009; Pick et al., 2017). Such studies are crucial for understanding how male and female fitness evolve and the degree to which intersexual genetic correlations have or have not been broken down (Cox, 2014).

In oviparous species, egg size is a female-specific reproductive trait with profound effects on reproductive success (Krist, 2011). The size of a female's egg relative to her body size determines resource investment in each embryo and therefore represents an important portion of the overall energy she invests into reproduction (Blomqvist et al., 1997; Fox & Czesak, 2010; Martin, 2008; Pick et al., 2016a). It is assumed that egg size and ovary size are strongly related, and a positive linear

relationship (+0.61) between oviduct diameter (specifically the isthmus diameter) and egg width has been found in Galliform species (Montgomerie et al., 2021). As male and female gonads arise from the same developing tissue, selection acting on ovary or oviduct size is likely to affect testis size and structure (Fischer et al., 2009; Pick et al., 2017) with potential consequences for sperm numbers and morphology (Lüpold et al., 2009; Pitnick, 1996; Ramm & Schärer, 2014), and ultimately, fertilisation success (Gomendio & Roldan, 2008; Lüpold et al., 2020; Parker & Pizzari, 2010). Birds provide an interesting example: in many species, only the left ovary and oviduct reach full development in females due to germ cells concentrating on the left side of the oviduct during early embryogenesis whilst the right side regresses (Kinsky, 1971; Stanley & Witschi, 1940). Most bird species also have a larger left testis in males (Briskie & Montgomerie, 2007) which is thought to be a by-product of selection for the degeneration of the right ovary in females (Calhim & Montgomerie, 2015).

In the Japanese quail (*Coturnix japonica*), selection for maternal reproductive investment, measured as egg size relative to body size, has previously been shown to have a concordant effect on male reproductive success (Pick et al., 2017). In lines selected for high female reproductive investment (relatively large eggs compared to body size; H-line), males fertilised more eggs under both competitive and non-competitive scenarios compared to males from low female reproductive investment lines (L-line). Females from H-lines also exhibited higher reproductive success, measured as an increase in offspring growth rate and survival (Pick et al., 2016a), and had heavier reproductive organs (Pick et al., 2016b) compared to females from L-lines. This provided novel evidence for a positive genetic correlation between male and female reproductive traits despite a high degree of sexual dimorphism (Pick et al., 2017). However, the precise effects of female-specific selection for relative egg size on male primary fertility traits remain unknown, as does the mechanistic basis of the fertility advantage for males from H-lines.

This study aimed to assess the effect of female-specific selection for relative egg size on testis size and structure, as well as on the form and function of the sperm they produce. Japanese quail are a well-suited model bird species for laboratory evolutionary studies due to being precocial, having rapid reproductive maturation, and being moderate in size (Ainsworth et al., 2010). Using the same artificially selected population of Japanese quail as described above (Pick et al., 2016c), we predicted H-line males to have a larger left testis compared to their right (Pick et al., 2017). We also predicted H-line males to have a greater volume of sperm-producing tissue overall, thereby producing more sperm and/or sperm that are more likely to fertilise compared to L-line males, particularly from their larger left testis, underpinning the fertilisation advantage of H-line males previously observed by Pick et al. (2017).

Materials and Methods

Study Population

Japanese quail (*Coturnix japonica*) males from the fourth generation of selective breeding for divergent maternal investment were used in this study. From a founder population, the 10 females producing the largest and smallest eggs (relative to their body size) were assigned to the high and low investment lines, respectively. Within each line, breeding pairs consisting of non-related males and females (not sharing any grandparents) were established and housed in individual cages (112 x 50 x 50 cm) in a breeding facility at the University of Zurich, Switzerland maintained on a 16:8 light:dark cycle at ~20°C. The birds had constant access to food, water, grit, and a source of calcium. The bottom of the cages

were lined with sawdust and there was a house and a raised sand bath in each cage. Two sons and 2 daughters of each of the 10 females producing the largest (20 sons and 20 daughters total) and 10 females producing the smallest (20 sons and 20 daughters total) eggs within their respective lines were selected for the next generation of breeding (20 breeding pairs per line). All individuals were only bred once. By generation four, there was a strong divergence in egg size and dried egg components (resource investment) between lines (difference in absolute egg size = 1.06 standard deviations; mean ± SDs: H-line = 12.46 ± 0.94g, L-line = 11.12 ± 0.91g; Pick et al., 2016c), but no difference in laying rate. Initially, two independent replicates per line were bred, controlling for seasonal and age effects (see Pick et al. 2016c for a detailed description of the founder population and selection procedure). In this study, however, we focused on a single replicate from the fourth generation to minimise the number of individual birds sacrificed for tissue samples. Whilst we acknowledge that sampling a single replicate introduces the possibility of detecting the effects of drift and mutation, rather than selection, previous work has found no difference in either female reproductive investment, male fertilisation success, or testis size between this study system's line replicates at generation four (Pick et al., 2016c; Pick et al., 2017), suggesting that selection has acted consistently on reproductive traits across the two replicates. It should be noted that other fourth generation males from these selection lines have been used in a previous study investigating differences in fertilisation success (Pick et al., 2017), but our study uses an independent set of individuals selected randomly from the same generation.

Testis and Sperm Sampling

Sample collection was carried out in 2016 using 20 randomly selected fourth generation males per outbred selection line from a single replicate. To collect sperm and testes, males were humanely euthanised and immediately dissected to remove the testes and seminal glomera (the site of male sperm storage before ejaculation). Excess connective tissue was cleaned from the testes and each testis (left and right) was weighed individually to the nearest 0.01g (wet mass). Testes from 10 males per line were frozen immediately after dissection to preserve for testis dry mass analysis (see below). For the remaining 10 males per line, the testicular capsule (outer casing) of each testis was pricked several times with a sharp needle and preserved in Bouin's solution (SigmaAldrich, UK) for histological analysis (see below). After 24h, the Bouin's solution was replaced with 70% ethanol and stored at room temperature until further processing.

Sperm samples were obtained via dissection of the seminal glomera, rather than from ejaculates, which are technically difficult to acquire in Japanese quail due to their production of a foam-like seminal fluid on ejaculation (Thélie et al., 2019). The left and right seminal glomera were placed separately into 1mL of Ham's nutrient media (Invitrogen, UK) and heated to 38ºC. The distal end of each seminal glomerus was gently squeezed with forceps and 20µl of semen was extracted using a pipette. Of this, 5µl was added to an Eppendorf with 15µl Ham's media, allowed to 'swim out' for 10 seconds, and then 4µl of this solution was loaded into a slide chamber, pre-warmed to 38ºC on a heat mat, for sperm motility and velocity analysis (see below). Two additional samples of 5µl extracted semen were each added to a sample tube containing 50µl 5% formalin, to preserve for later analysis of sperm morphology and concentration (see below).

Dry Testis Mass

The frozen testes were defrosted at room temperature for 24 hours and testis mass was recorded. Testes were put in a drying oven at 60°C and weighed every 24 hours until there was a 0% change in mass after additional drying time and constant mass had been achieved. Testes were removed from the drying oven and left to cool, and a final measure of dry mass to the nearest 0.01g was recorded.

Testis Sperm Production Capacity

The testes preserved for histological analysis were embedded in paraffin blocks and cut into 5 sections that were each stained with hematoxylin and eosin and then mounted onto glass slides. Slides were photographed using a Canon EOS 600D camera to measure testis length (at the longest point) and width (perpendicular to length) to the nearest 0.01mm by a single researcher using ImageJ (Schneider et al., 2012). Testis volume to the nearest 0.01mm^3 was calculated for each section using the formula for the volume of an ellipsoid (Equation 1, where A = length(mm)/2 and B = width(mm)/2; Lambert, 1951), and the mean average of the 5 sections per testis was taken as the final measure of testis volume.

$$Testis\ volume\ (mm^3) = \frac{4}{3} \times \pi \times A \times B^2 \qquad Equation\ 1$$

The testis sections were then imaged under a compound light microscope at 2x magnification, and the images were imported into ImageJ (Schneider et al., 2012) to measure the volume of sperm-producing tissue. Testis tissue covered the entire area of each image (Figure S1). The images were converted to greyscale so that seminiferous epithelium tissue (site of sperm production) was dark, and the lumen/somatic tissue was light. An intensity-based threshold was applied to highlight seminiferous epithelium pixels black, and the % area of black pixels (sperm-producing tissue) was measured. The mean average of 5 sections per testis was taken as the final measure of the proportion of sperm-producing tissue. Five measures gave high repeatability (R = 0.78, P <0.0001) and captured over 95% of within-testis variability in the proportion of sperm-producing tissue (Figure S2; see Supplementary Methods 1 for how repeatability was assessed). The volume of sperm-producing tissue was calculated as testis volume multiplied by the proportion of sperm-producing tissue.

To measure the density of seminiferous tubules, the testis sections were photographed at 10x magnification under a compound light microscope. The curvature pen tool in Adobe Photoshop CC (version 24.6.0) was used to outline the basal membrane of each imaged seminiferous tubule, and the fill path tool was then used to mask the tubule, including the lumen, leaving only the interstitial space. The % area cover of seminiferous tubules was measured using ImageJ (Schneider et al., 2012). Whilst volume of sperm-producing tissue is an important indicator of the number of sperm produced (Lüpold et al., 2009), males with similar volumes of sperm-producing tissue may differ in their seminiferous tubule density due to the width of their tubules (i.e., may have many small or fewer large tubules). This is important because it has been shown that males with wider tubules can produce longer sperm across bird species (Lüpold et al., 2009).

Sperm Concentration

Formalin-preserved semen samples were vortexed to disperse sperm clumps and 20µl was then loaded onto an Improved Neubauer chamber (Celeromics Technologies). All sperm were counted across the whole grid of both sides of the Neubauer chamber and an average of the two grids was calculated, giving the number of sperm found in 0.9mm^3 of semen, which was subsequently corrected for dilution and converted to sperm per mL (Equation 2). Data from 40 males (20 per selection line) were collected.

$$\textit{Sperm concentration (per mL)} = \left(\left(\frac{\textit{sperm count}}{0.9}\right) \times 1000\right) \times 100 \qquad \textit{Equation 2}$$

Sperm Motility and Velocity

Sperm motility and velocity were assessed using the Sperm Class Analyser® Computer-Assisted Sperm Analysis (CASA) software (Microptic, Barcelona). Four microlitres of semen was loaded into a 20µl depth slide chamber (Leja®, Netherlands) and allowed to equilibrate on the microscope heated stage (38ºC) for 30 seconds. Sperm were filmed swimming using a pseudo-negative phase at 200x magnification with a Basler acA780-75gc camera connected to an Olympus BX41 microscope. Multiple one-second video clips were recorded systematically for each sample with the aim of tracking at least 100 sperm per male. In 8 samples (4 H-line and 4 L-line), however, there were insufficient sperm to samples 100 cells, and additional clips were recorded over multiple fields of view to capture as many sperm as possible. Due to the short recording timeframe, it is unlikely that an individual sperm would be captured twice (in

different fields of view), but we cannot rule this out for any of the samples. To ensure our results were not biased by the inclusion of these samples, we analysed the data both with and without them (See Results and Supplementary Material). Cell debris and dead sperm were manually deleted from all videos before analysis, and the proportion of motile sperm was manually counted from videos to avoid the inclusion of non-motile but drifting sperm. Drifting sperm were manually identified during this process and removed from velocity data.

The three kinematic parameters obtained from each sperm were: (i) average path velocity (VAP), (ii) curvilinear velocity (VCL), and (iii) straight line velocity (VSL; see Table S1 for full descriptions). Due to the co-linearity of VAP, VCL and VSL (Table S2; assessed using Pearson's correlations), a principal component analysis was used to calculate a single velocity index per sperm (PC1). Mean PC1 scores of sperm from each male testis (right and left) were used in analyses, instead of the raw data, to avoid inflated significance values due to measuring several intercorrelated velocity traits. However, there is considerable variation in sperm velocity within males (e.g., 0 - 70μm/s) meaning that average PC1 scores could be misleading. The dataset was therefore divided into four subpopulations: mean PC1 score of (i) all sperm, (ii) fastest 20% of sperm, (iii) fastest 10% of sperm, and (iv) fastest single sperm (as in Mossman et al., 2009). Due to a sampling error, motility data was not collected for one male. Data from 27 males (12 H-line and 15 L-line) were collected for motility, and data from 28 males (13 H-line and 15 L-line) were collected for velocity.

Sperm Morphology

Four microlitres of formalin-preserved sperm solution was pipetted onto a microscope slide, followed by 4µl each of MitoTracker Green (which stains the sperm midpiece and tail green) and Hoechst 33342 (which stains the nucleus blue) dye solutions, then covered with a coverslip and incubated in the dark for 5 minutes. Using a fluorescence microscope (Leica DMBL) and darkfield filter at x400 magnification, 5 morphologically normal and undamaged sperm (in some cases, sperm tails had degraded during storage and were unmeasurable) were photographed using an Infinity 3 camera (Luminera Corporation). The head, midpiece and tail (shown in Figure S3) were measured to the nearest 0.1µm using ImageJ (Schneider et al., 2012) by a single researcher. Measurements were taken three times and used to calculate the mean average length of each component. Three measures gave high repeatability for all components, and sperm morphology was consistent within males (see Table S3 for estimates and Supplementary Methods 1 for how repeatability was assessed). Data from 20 males (10 per selection line) were collected.

Sperm Numbers Reaching the Egg

To assess whether the number of sperm reaching ova differed between lines, before euthanasia and dissection, the males used for testis and sperm quantification (see above) were mated to a female from the same line. Breeding pairs were housed in individual cages (112x50x50cm; see Pick et al., 2017 for a detailed description of the mating procedure). For each pair, 3 - 7 eggs (depending on the laying frequency of the individual female) were collected on the day they were laid. Collected eggs were

dissected and examined for the presence and number of trapped sperm on the perivitelline layer (PVL): the PVL was removed from the yolk, cleaned in phosphate-buffered saline solution, and stained on a microscope slide with 10µl Hoechst 33342 fluorescent dye, following the methods described in Birkhead et al. (2008). A 1cm^2 area of the PVL was then examined under 400x magnification using a fluorescence microscope (Leica DMBL) with a darkfield filter, and the total number of sperm was counted. In total, 73 eggs from 36 mating pairs (17 H-line and 19 L-line line males) were examined. All procedures were conducted under licenses provided by the Veterinary Office of the Canton of Zurich, Switzerland (permit number 195/2010; 14/ 2014; 156).

Statistical Analysis

Linear mixed models (LMMs) were used to assess the difference in sperm and testis traits between the two selection lines and testis sides. Selection line (high or low), testis side (right or left), and their interaction were included as fixed effects, and male ID as a random effect to control for multiple measures per male. In addition, body size (tarsus length) was included as a covariate in models that evaluated differences in testis traits; and egg number (in laying sequence) was included as a covariate in the model that evaluated the difference in the number of sperm on the PVL. LMMs were also used to analyse the effect of testis traits on sperm traits with testis mass as an explanatory variable. See Supplementary Methods 2 for a full description of the models used.

A multivariate analysis of variance (MANOVA) was conducted to simultaneously assess the differences in sperm component lengths (head, midpiece and tail) between selection lines and

testis sides. Head length, midpiece length and tail length were included as response variables, and line and testis were predictors. To account for the fact that the dataset included multiple sperm from each male, male ID was included as an error term. Associations between the sperm component lengths and total sperm length were calculated using Pearson's correlations. To assess the relationship between sperm morphology and velocity, separate LMMs were used with either average: (i) component/total sperm length, (ii) ratio of the flagellum (midpiece plus tail) and head length (flagellum:head), or (iii) ratio of the midpiece and tail length (midpiece:tail), as an explanatory variable, and the average velocity of all sperm as the dependent variable. Male ID was included as a random effect to control for two measures per male (right and left testis). We used data from 11 males (5 H-line and 6 L-line) for which both traits (sperm morphology and velocity) were measured. Since trait variation can increase under stressful developmental conditions (Badyaev, 2005; Hoffmann & Schiffer, 1998), a Levene's test was used to assess the difference in variation between the two selection lines for each measured sperm and testis trait. The coefficient of variation was calculated as the standard deviation divided by the mean.

Significance was determined in general linear models using *F* statistics and mixed models by comparing nested models using likelihood-ratio tests. To control for the increased risk of Type 1 errors due to multiple comparisons, the Benjamini-Hochberg False Discovery Rate (FDR) correction was applied separately within each group of tests: (a) 15 linear mixed models and 1 MANOVA assessing differences in the testis and sperm traits between selection lines, (b) 5 linear mixed models assessing the effect of testis traits on sperm traits, (c) 6 linear mixed models assessing the relationship between sperm length and sperm velocity, and (d) 15 Levene's tests assessing differences in variation in sperm and testes traits between the selection lines. We

present both initial and FDR-corrected *p*-values for comparison in our results, but ultimately base our conclusions on the more conservative FDR-corrected *p*-values. Analyses were performed in R (version 4.4.0; R Core Team, 2024). Mixed models were performed using lme4 (Bates et al. 2015) and the MANOVA was performed using MANOVA.RM (Friedrich et al., 2023).

Results

Testis Size, Structure and Sperm Production Capacity

The wet mass of testes did not differ between selection lines or between the right and left testis within lines, and there was no significant interactive effect of testis side and selection line on testis wet mass (Table 1; Figure 1A). Inter-male variation in testis wet mass did not differ significantly between lines (Table S4). Similarly, there was no difference in testis dry mass between selection lines, or between the right and left testis (Table 1; Figure 1B), and no difference in inter-male variation in testis dry mass between lines (Table S4).

In terms of sperm-production capacity, there was no difference in the total volume of sperm-producing tissue between males from divergent selection lines. However, in both lines, males had a significantly greater volume of sperm-producing tissue in their left testis than in their right (Table 1; Figure 1C). Inter-male variation in the volume of sperm-producing tissue did not differ significantly between lines (Table S4). There was no difference in the density of the seminiferous tubules between selection lines or testis

sides (Table 1; Figure 1D), but there was greater inter-male variation in the density of seminiferous tubules in the L-line compared to the H-line (Table S4).

Figure 1. Testis traits: *Effects of artificial selection for high or low female reproductive investment on the (A) testis wet mass, (B) testis dry mass, (C) volume of sperm-producing tissue, and (D) density of seminiferous tubules, for the left (blue) and right (orange) testis. Coloured boxes represent 25% and 75% quantiles, whiskers 1.5 interquartile range, and the bold black line the median. Significant effect of testis side on volume of sperm-producing tissue:* $\chi^2 = 14.17$, $df = 1$, $p < 0.001$, *FDR-p-value = 0.02.*

Sperm Form and Function

Sperm concentration did not differ significantly between lines or testis sides (Table 1; Figure 2), and inter-male variation in sperm concentration did not differ significantly between lines (Table S4).

Figure 2. Sperm concentration: *Effect of artificial selection for high or low female reproductive investment on the number of sperm per mL of semen taken from the seminal glomera of the left (blue) and right (orange) testis.*

L-line males had a significantly greater proportion of motile sperm in their left testis compared to their right, but there was no difference in the proportion of sperm motile between the left and right testis of H-line males and no difference overall between selection lines (Table 1; Figure 3a). This result was

consistent when 4 samples from each line with low sperm numbers were excluded (See Methods and Supplementary Figure S4). Inter-male variation in sperm motility did not differ significantly between lines (Table S4).

Sperm produced by males from divergent lines did not differ in velocity for any of the parameters measured, or between the left and right testis (Table 1; Figure 3b; Figure S5). This result was also consistent when 4 samples from each line with low sperm numbers were excluded (See Methods and Supplementary Figure S6). Inter-male variation in average velocity of all sperm, the fastest 20%, fastest 10%, and fastest single sperm did not differ significantly between lines (Table S4).

Figure 3. Sperm Motility and Velocity: *Effect of artificial selection for high or low female reproductive investment on the (A) proportion of sperm motile, and (B) mean average swimming velocity of all sperm (PC1) from the left (blue) and right (orange) testis. Significant interactive effect of selection line and testis side on the proportion of sperm motile:* $\chi^2 = 24.73$, $df = 1$, $p < 0.001$, *FDR-p-value = 0.02.*

The multivariate analysis revealed no difference in sperm component lengths between selection lines ($F_{3, 16} = 3.38$, $P = 0.04$, FDR-p-value = 0.18) and testis sides ($F_{3, 177} = 1.66$, $P = 0.18$, FDR-p-value = 0.45). Analysis using linear mixed models for individual sperm components (head, tail, midpiece, and total length) also showed no significant difference in sperm component lengths between selection lines or testis sides. Inter-male variation in head length, midpiece length, tail length and total sperm length did not differ significantly between lines (Table S4). Each sperm component length was significantly positively correlated with total length (Table S5). On average, the midpiece was 9.86 $\pm$ 4.10µm longer

and the tail was 6.09 ± 2.30µm longer, but the head was 0.79 ± 0.69µm shorter in the L-line compared to the H-line (see mean dimensions for each selection line in Table S6).

Figure 4. Sperm Morphology: : *Effect of artificial selection for high or low female reproductive investment on the (A) total length, (B) head length, (C) midpiece length, and (D) tail length of sperm from the left (blue) and right (orange) testis.*

There was no significant association between sperm velocity and sperm component lengths (head: χ^2 = 0.26, df = 1, *P = 0.61*, FDR-*p*-value = *0.78* ; midpiece: χ^2 = 1.01, df = 1, *P = 0.32,* FDR-*p*-value = *0.78*; tail: χ^2 = 0.33, df = 1, *P = 0.56,* FDR-*p*-value = *0.78*; Figure S7A,B,C) or total sperm length (χ^2 = 0.96, df = 1, *P = 0.33,* FDR-*p*-value = *0.78*; Figure S7D), and no significant association between velocity and flagellum:head ratio (χ^2 = 0.08, df = 1, *P = 07.78,* FDR-*p*-value *= 0.78*; Figure S5E), or midpiece:tail ratio (χ^2 = 0.20, df = 1, *P = 0.66,* FDR-*p*-value = *0.78*; Figure S7F).

Ultimately, the number of sperm reaching the PVL of eggs did not differ between lines (Table 1; Figure S8), and inter-male variation in sperm numbers reaching the egg did not differ significantly between lines either (Table S4). There was no effect of testis mass on sperm concentration, the proportion of sperm motile, average sperm velocity, total sperm length, or the number of sperm on the PVL of eggs (Table 2). Detailed results of all models are provided in Supplementary Tables S7 - S16.

Discussion

Using an artificial selection approach, we show that female-specific selection on reproductive investment resulted in few differences in sperm and testis traits linked to male fertility. These few changes in primary male fertility traits appear unlikely to explain the greater fertilisation success rate of males from the high investment line (H-line) previously demonstrated by Pick et al. (2017), suggesting that other traits beyond primary sexual characteristics may be subject to concordant selection.

Testis Asymmetry

We found no difference in the wet or dry mass of the right or left testis between selection lines, despite H-line females having a larger left-side restricted oviduct compared to L-line females (Pick et al., 2016b). This suggests that there is little intersexual genetic correlation in gonad development in this species. However, Pick et al. (2017) found that H-line males had increased testis asymmetry compared to L-line males in the same population of Japanese quail, and so while we find the mass of the testes to be similar, asymmetries in H-line males could lead to variations between selection lines in the proportion of the total testis mass that is the left testis. Selective breeding for divergence in female reproductive investment over future generations could eventually lead to H-line males having a significantly larger left testis compared to their right testis.

Despite there being no difference in testis mass, we found some evidence that the left testis may be more functional than the right, as the left testis contained more sperm-producing tissue than the right across both lines. The density of the seminiferous tubules remained consistent between the left and right testes in both lines, suggesting that the increased volume of sperm-producing tissue in the left testis results from the seminiferous tubules having a thicker epithelium (the site of spermatogenesis) and smaller lumen compared to those in the right testis, further indicating the increased functionality of the left testis. This is consistent with evidence that the left gonad has higher stem cell numbers and transcriptional activity in both sexes in chickens (*Gallus gallus*) (Intarapat & Stern, 2013). Typically, larger testes are associated with increased sperm competition and sexual selection (Amann, 1970; de Reviers & Williams, 1984; Schärer et al., 2004; Willett & Ohms, 1957). However, the fact that such variation in sperm-producing tissue can exist, independent of differences in total testis mass, suggests that measuring testis size alone may underestimate the intensity of postcopulatory sexual selection (Lüpold et al., 2009).

No evidence was found to suggest that the left testis produced more numerous or higher quality sperm, except that the left testis of L-line males had a greater proportion of motile sperm than their right. Increased motility could enable sperm to achieve superior placement in the female's reproductive tract (Birkhead et al., 1999; Froman et al., 2002). The right testis may compensate for having less sperm-producing tissue by increasing the number of sperm produced per unit of sperm-producing tissue, or the speed at which individual sperm cells are generated, so that sperm concentration is consistent between the testis sides (Amann 1970; Amann, 1981; Schärer & Vizoso, 2007; Ramm & Stockley, 2008; Sekii et al., 2013). In Icterids, the height of the seminiferous epithelium is positively correlated with sperm length, and tubule size increases/decreases accordingly when selection favours longer/shorter sperm (Lüpold et al., 2009). However, in Japanese quail, sperm length may be maintained independently of seminiferous

tubule structure, and ultimately, there was no effect of testis mass on any measured sperm trait. Unfortunately, we were only able to measure both sperm traits and the volume of sperm-producing tissue or density of seminiferous tubules in a small number of males, so the relationship between these traits remains unclear.

Differences Between Selection Lines

There was no significant difference in any of the measured testis and sperm traits between the selection lines individually and there was no significant effect of sperm size on velocity, which may be because smaller levels of variation in sperm size are found at the intraspecific level (Birkhead et al., 2005; Dziminski et al. 2009; Gage et al., 2004). Given that our original assumptions about which sperm traits are likely to enhance fertilisation success were wrong, it is possible that other factors drive the differences in fertilisation success previously detected between the lines. For example, several important molecules regulate avian fertilization, including proteases in sperm that hydrolze the egg PVL to create a path for sperm penetration and successful fertilization (Ichikawa et al., 2016). It is therefore possible that variation in sperm protease between males from the H-line and L-line, rather than differences in morphological traits, may lead to variation in fertilization success.

Pre- and Post-Copulatory Mechanisms

The lack of significant differences in primary male fertility traits between the selection lines suggests that alternative mechanisms are responsible for the elevated fertilisation success of H-line males that has been previously reported (Pick et al., 2017). We suggest three possibilities: firstly, fertilisation success in this system was measured after natural copulations (i.e., not artificial insemination; Pick et al., 2017), so it is therefore possible that pre-copulatory mechanisms, including female-mate choice, contributed to the fertilisation success of H-line males (Andersson, 1994). For example, females may have chosen to copulate with H-line males more frequently, or accepted a greater proportion of their sperm, due to physical cues before or during copulation. The vaginal fluid of females can facilitate sperm selection and reduce the sperm performance of undesirable males, or trigger an immune response, causing insufficient sperm to reach the site of fertilization (Assersohn et al., 2021). Studies have shown that female mating preference in Japanese quail is stimulated by male testosterone level (Hiyama et al., 2018), which could potentially be higher in the larger H-line males.

Secondly, since sperm samples were obtained via dissection of the seminal glomera (male sperm storage organ) in our study, rather than from natural ejaculates, the number of sperm that we obtained may differ from that which is actually transferred to females during copulation. There is evidence that males can strategically allocate more or less sperm to certain females, either by changing ejaculate size or inseminating more or less frequently. Males can also vary in their ability to replenish sperm stores and inseminate females with more sperm (Perry & Rowe, 2010; Kelly & Jennions, 2011). It is possible that these factors differ between the selection lines, potentially contributing to the heightened reproductive success of H-line males. However, we found no difference in the number of sperm reaching the PVL

layer of females' ova following mating with either L-line or H-line males, suggesting sperm numbers alone do not drive the heightened fertilisation success of H-line males.

Finally, other components of the ejaculate may contribute to male fertilisation success including seminal fluid proteins that undergo high rates of adaptive evolution (Clark et al., 2006; Swanson & Vacquier, 2002) and have been demonstrated to play a role in sperm function/selection within the oviduct across species (Heriberto et al., 2011; Perry et al., 2013; Poiani, 2006; Ram & Wolfner, 2007). Our knowledge of avian seminal fluid has grown in the last decade (e.g., Labas et al., 2015; Borziak et al., 2016; Rowe et al., 2020; Santiago-Moreno & Blesbois, 2020; Tang et al., 2022). Japanese quail transfer a substantial volume of a unique foam from a specialised cloacal gland along with their sperm into the female's oviduct upon ejaculation. The function of the foam is not fully understood (Fujihara, 1990), but research has shown the presence of the foam secreted during natural copulations has a positive effect on male fertilisation success (Cheng at al., 1989a), and this effect is enhanced when there is sperm competition from rival males (Finseth et al., 2013) suggesting ejaculatory fluid is subject to sexual selection in this species. The foam has shown to enhance and prolong sperm motility in vitro (Biswas et al., 2010; Cheng et al., 1989b), disaggregate clumps of sperm (Singh et al., 2012), and extend the duration of the female's fertile period (Cheng et al., 1989a; Singh et al., 2012). It is therefore feasible that the foam contributes to the fertilisation advantage of H-line males in this system and this possibility warrants further investigation.

Conclusion

This study provides some evidence of a positive correlation between males and females in the functionality of the left-side of their gonads, and suggests measuring testis internal structure, rather than testis size, is a better indicator of postcopulatory sexual selection. Although there was a difference in sperm length between males from lines selected for divergent female reproductive success, it is unlikely to explain the fertilisation advantage of H-line males, as there was ultimately no difference in the number of sperm able to reach the PVL of a female's egg. Pre-copulatory cues and/or the role of seminal fluid in sperm motility may therefore be more likely to explain the previously observed H-line male fertilisation advantage in this system.

Table 1: Linear mixed models for the effect of selection line and testis side on measured testis and sperm traits. Initial *p*-values and FDR-corrected *p*-values are given. Significant results are shown in bold: *p<0.05**, *p<0.01***, *p<0.001****.

Predictor	**Selection line**				**Testis side**				**Interactive effect**				
Response	***χ2***	**DF**	***P***	**FDR-*p*-value**	***χ2***	**DF**	***P***	**FDR-*p*-value**	***χ2***	**DF**	***P***	**FDR-*p*-value**	***n***
Testis wet mass (g)	0.13	1	0.72	0.82	0.33	1	0.57	0.82	4.11	1	0.04*	0.18	80
Testis dry mass (g)	0.75	1	0.39	0.67	1.13	1	0.29	0.56	0.94	1	0.33	0.61	40
Volume of sperm-producing tissue (mm3)	1.53	1	0.22	0.46	14.17	1	<0.001***	**0.02***	0.17	1	0.68	0.82	40
Density of seminiferous tubules (%)	6.30	1	0.01*	0.13	2.08	1	0.15	0.44	0.42	1	0.52	0.79	200
Sperm concentration (number/mL)	0.002	1	0.96	0.97	4.12	1	0.04*	0.18	0.42	1	0.52	0.79	80
Proportion of sperm motile (%)	1.49	1	0.22	0.46	5.31	1	0.02*	0.18	24.73	1	<0.001***	**0.02***	52
Velocity of all sperm (PC1)	0.19	1	0.67	0.82	0.14	1	0.71	0.82	0.27	1	0.61	0.82	53
Velocity of fastest 20% of sperm (PC1)	0.25	1	0.62	0.82	1.64	1	0.20	0.45	4.11	1	0.04*	0.18	53
Velocity of fastest 10%	0.31	1	0.58	0.82	0.16	1	0.69	0.82	2.76	1	0.10	0.38	53

of sperm (PC1)													
Velocity of fastest single sperm (PC1)	0.12	1	0.73	0.86	1.93	1	0.16	0.44	0.90	1	0.34	0.61	53
Total sperm length (µm)	4.43	1	0.04 *	0.18	1.39	1	0.24	0.48	1.67	1	0.20	0.45	200
Head length (µm)	0.70	1	0.40	0.67	1.89	1	0.17	0.45	0.002	1	0.97	0.97	200
Midpiece length (µm)	2.15	1	0.14	0.44	2.28	1	0.13	0.44	2.02	1	0.16	0.44	200
Tail length (µm)	4.17	1	0.04 *	0.18	0.14	1	0.71	0.82	0.11	1	0.74	0.82	200
Sperm number on PVL	0.01	1	0.91	0.97	---	---	---	---	---	---	---	---	73

Table 2: Linear mixed models for the effects of testis mass on measured sperm traits. Initial *p*-values and FDR-corrected *p*-values are given.

Predictor	**Testis mass**				
Response	χ^2	**DF**	***P***	**FDR-*p*-value**	***n***
Sperm concentration (number/mL)	0.77	1	0.38	0.53	80
Proportion of sperm motile (%)	0.50	1	0.48	0.53	52
Velocity of all sperm (PC1)	1.60	1	0.21	0.53	53
Total sperm length (µm)	0.40	1	0.53	0.53	200
Sperm number on PVL	1.16	1	0.28	0.53	73

References

Ainsworth, S., Stanley, R.L. & Evans, D.J.R. (2010). Developmental stages of the Japanese quail. *Journal of Anatomy*, 216(1), 3-15. https://doi.org/10.1111/j.1469-7580.2009.01173.x

Amann, R.P. (1970). Sperm production rates. In A. D. Johnson, W.R. Gomes & N. L. Vandemark (Eds.), *The Testis* (pp. 433-482). Academic Press, Inc.

Amann, R.P. (1981). A critical review of methods for evaluation of spermatogenesis from seminal characteristics. *Journal of Andrology*, 2(1), 37-58. https://doi.org/10.1002/j.1939-4640.1981.tb00595.x

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

Assersohn, K., Brekke, P. & Hemmings, N. (2021). Physiological factors influencing female fertility in birds. *Royal Society of Open Science*, 8(7), 202274. https://doi.org/10.1098/rsos.202274

Badyaev, A.V. (2005). Stress-induced variation in evolution: From behavioural plasticity to genetic assimilation. *Proceedings of the Royal Society B: Biological Sciences,* 272(1566), 877-886. https://doi.org/10.1098/rspb.2004.3045

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. http://doi.org/10.18637/jss.v067.i01

Birkhead, T. R., Hall, J., Schut, E., & Hemmings, N. (2008). Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis*, 150(3), 508-517. https://doi.org/10.1111/j.1474-919X.2008.00813.x

Birkhead, T. R., Martínez, J. G., Burke, T., & Froman, D. P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society B: Biological Sciences*, *266*(1430), 1759–1764. https://doi.org/10.1098/rspb.1999.0843

Birkhead, T.R., Pellatt, E.J., Brekke, P., Yeates, R. & Castillo-Juarez, H. (2005). Genetic effects on sperm design in the zebra finch. *Nature*, 434(7031), 383-387. https://doi.org/10.1038/nature03374

Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews Genetics*, *3*(4), 262–273. https://doi.org/10.1038/nrg774

Biswas, A., Ranganatha, O.S. & Mohan, J. (2010). The effect of different foam concentration on sperm motility in Japanese quail. *Veterinary Medicine International*. Article 564921. https://doi.org/10.4061/2010/564921

Blomqvist, D., Johansson, O. C., & Gotmark, F. (1997). Parental quality and egg size affect chick survival in a precocial bird, the lapwing *Vanellus vanellus*. *Oecologia*, *110*(1), 18–24. https://doi.org/10.1007/s004420050128

Bonduriansky, R. & Chenoweth, S.F. (2009). Intralocus sexual conflict. *Trends in Ecology & Evolution,* 24(5), 280-288. https://doi.org/10.1016/j.tree.2008.12.005

Borziak, K., Álvarez-Fernández, A., Karr, T.L., Pizzari, T. & Dorus, S. (2016). The seminal fluid proteome of the polyandrous red junglefowl offers insights into the molecular basis of fertility, reproductive ageing and domestication. *Scientific Reports,* 6(1), Article 35864. https://doi.org/10.1038/srep35864

Briskie, J. V. & Montgomerie, R. (2007). Testis size, sperm size and sperm competition. In: B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds: Vol 6A. Phylogeny, Morphology, Hormones, Fertilization* (pp. 513 – 551). Science Publishers.

Calhim, S., & Montgomerie, R. (2015). Testis asymmetry in birds: The influences of sexual and natural selection. *Journal of Avian Biology*, *46*(2), 175–185. https://doi.org/10.1111/jav.00503

Cheng, K.M. Hickman, A.R. & Nichols, C.R. (1989a). Role of the proctodeal gland foam of male Japanese quail in natural copulations. *The Auk*, 106(2), 279-285. https://doi.org/10.1093/auk/106.2.279

Cheng, K.M., McIntyre, R.F. & Hickman, A.R. (1989b). Proctodeal gland foam enhances competitive fertilization in domestic Japanese quail. *The Auk*, 106(2), 286-291. https://doi.org/10.1093/auk/106.2.286

Clark, N. L., Aagaard, J. E., & Swanson, W. J. (2006). Evolution of reproductive proteins from animals and plants. *Reproduction*, 131(1), 11-22. https://doi.org/10.1530/rep.1.00357

Cox, R. M. (2014). Integrating Costs of Reproduction between the Sexes. In L. Martin, C. Ghalambor, & A. Woods (Eds.), *Integrative Organismal Biology* (pp. 153–168). John Willey & Sons, Inc. https://doi.org/10.1002/9781118398814.ch10

Cox, R. M., & Calsbeek, R. (2009). Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. *American Naturalist*, *173*(2), 176–187. https://doi.org/10.1086/595841

Coyne, J. A., Kay, E. H., & Pruett-Jones, S. (2008). The genetic basis of sexual dimorphism in birds. *Evolution*, *62*(1), 214–219. https://doi.org/10.1111/j.1558-5646.2007.00254.x

De Reviers, M. & Williams, J.B. (1984). Testis development and production of spermatozoa in the cockerel (*Gallus domesticus*). In: F. J. Cunningham, P. E. Lake & D. Hewitt (Eds.), *Reproductive Biology of Poultry* (pp. 183-202). British Poultry Science.

Dziminski, M. A., Dale Roberts, J., Beveridge, M., & Simmons, L. W. (2009). Sperm competitiveness in frogs: Slow and steady wins the race. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1675), 3955–3961. https://doi.org/10.1098/rspb.2009.1334

Finseth, F.R., Iacovelli, S.R., Harrison, R.G. & Adkins-Regan, E.K. (2013). A nonsemen copulatory fluid influences the outcome of sperm competition in Japanese quail. *Journal of Evolutionary Biology*, 26(9), 1875-1889. https://doi.org/10.1111/jeb.12189

Fischer, K., Zimmer, K., & Wedell, N. (2009). Correlated responses to selection on female egg size in male reproductive traits in a butterfly. *Evolutionary Ecology*, *23*(3), 389–402. https://doi.org/10.1007/s10682-007-9233-1

Fox, C. W., & Czesak, M. E. (2010). Evolutionary ecology of progeny size in arthropods. *Science*, *45*(1), 341–369. https://doi.org/10.1146/annurev.ento.45.1.341

Friedrich, S., Konietschke, F. & Pauly, M. (2023). MANOVA.RM: Resampling-based analysis of multivariate data and repeated measures designs. R package version 0.5.4. https://CRAN.R-project.org/package=MANOVA.RM

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H., & Birkhead, T. R. (2002). Sperm mobility: Mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus. Proceedings of the Royal Society B: Biological Sciences*, *269*(1491), 607–612. https://doi.org/10.1098/rspb.2001.1925

Fujihara, N. (1990). Structures and functions of the accessory reproductive organs in the domestic birds. In: H. Iwasawa & K. Ishida (Eds.), *Biomechanism of the Gonads* (pp. 56-71). IPC.

Gage, M. J. G., Macfarlane, C. P., Yeates, S., Ward, R. G., Searle, J. B., & Parker, G. A. (2004). Spermatozoal traits and sperm competition in Atlantic salmon. *Current Biology*, 14(1), 44–47. https://doi.org/10.1016/j.cub.2003.12.028

Gomendio, M., & Roldan, E. R. S. (2008). Implications of diversity in sperm size and function for sperm competition and fertility. *International Journal of Developmental Biology*, *52*(5–6), 439–447. https://doi.org/10.1387/ijdb.082595mg

Hare, R. M. & Simmons, L. W. (2018). Sexual selection and its evolutionary consequences in female animals. *Biological Reviews,* 94(3), 929-956. https://doi.org/10.1111/brv.12484

Heriberto, R., Kvist, U., Ernerudh, J. Sanz, L. & Calvete, J.J. (2011). Seminal plasma proteins: What role do they play? *American Journal of Reproductive Immunology*, 66(1), 11-22. https://doi.org/10.1111/j.1600-0897.2011.01033.x

Hiyama, G., Mizushima, S., Matsuzaki, M., Tobari, Y., Choi, J., Ono, T., Tsudzuki, M., Makino, S., Tamiya, G., Tsukahara, N., Sugita, S. & Sasanami, T. (2018). Female Japanese quail visually differentiate testosterone-dependent male attractiveness for mating preferences. *Scientific Reports*, 8(1), 10012. https://doi.org/10.1038/s41598-018-28368-z

Hoffmann, A.A. & Schiffer, M. (1998). Changes in the heritability of five morphological traits under combined environmental stresses in *Drosophila melanogaster. Evolution,* 52(4), 1207-1212. https://doi.org/10.1111/j.1558-5646.1998.tb01847.x

Ichikawa, Y., Matsuzaki, M., Hiyama, G., Mizushima, S. & Sasanami, T. (2016). Spem-egg interaction during fertilization in birds. *Journal of Poultry Science*, 53(3), 173-180. https://doi.org/ 10.2141/jpsa.0150183

Intarapat, S., & Stern, C. D. (2013). Sexually dimorphic and sex-independent left-right asymmetries in chicken embryonic gonads. *PLoS ONE*, *8*(7), 1–8. https://doi.org/10.1371/journal.pone.0069893

Kelly, C. D., & Jennions, M. D. (2011). Sexual selection and sperm quantity: Meta-analyses of strategic ejaculation. *Biological Reviews*, *86*(4), 863–884. https://doi.org/10.1111/j.1469-185X.2011.00175.x

Kinsky, F. C. (1971). The consistent presence of paired ovaries in the Kiwi (*Apteryx*) with some discussion of this condition in other birds. *Journal Für Ornithologie*, *112*(3), 334–357. https://doi.org/10.1007/BF01640692

Krist, M. (2011). Egg size and offspring quality: A meta-analysis in birds. *Biological Reviews*, *86*(3), 692–716. https://doi.org/10.1111/j.1469-185X.2010.00166.x

Labas, V., Grasseau, I., Cahier, K., Gargaros, A., Harichaux, G., Teixeira-Gomes, A., Alves, S., Bourin, M., Gérad, N. & Blesbois, E. (2015). Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *Journal of Proteomics*, 112, 313 – 335. https://doi.org/10.1016/j.jprot.2014.07.024

Lambert, B. (1951). The frequency of mumps and of mumps orchitis and the consequences for sexuality and fertility. *Acta Genetica et Statistica Medica*, *2* (Suppl. I), 1–166. https://www.jstor.org/stable/45103094

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic Characters. *Evolution*, *34*(2), 292–305. https://doi.org/10.2307/2407393

Lüpold, S., Linz, G. M., Rivers, J. W., Westneat, D. F., & Birkhead, T. R. (2009). Sperm competition selects beyond relative testes size in birds. *Evolution*, *63*(2), 391–402. https://doi.org/10.1111/j.1558-5646.2008.00571.x

Lüpold, S., de Boer, R. A., Evans, J. P., Tomkins, J. L., & Fitzpatrick, J. L. (2020). How sperm competition shapes the evolution of testes and sperm: A meta-analysis: Sperm competition meta-analysis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1813). https://doi.org/10.1098/rstb.2020.0064

Martin, T. E. (2008). Egg size variation among tropical and temperate songbirds: An embryonic temperature hypothesis. *PNAS,* 105(27), 9268-9271. https://doi.org/10.1073/pnas.0709366105

Montgomerie, R., Hemmings, N., Thompson, J.E. & Birkhead, T. (2021). The shapes of birds' eggs: evolutionary constraints and adaptations. *The American Naturalist*, 198(6), 215-231. https://doi.org/10.1086/716928

Mossman, J., Slate, J., Humphries, S., & Birkhead, T. (2009). Sperm morphology and velocity are genetically codetermined in the zebra finch. *Evolution*, *63*(10), 2730–2737. https://doi.org/10.1111/j.1558-5646.2009.00753.x

Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews*, *85*(4), 897–934. https://doi.org/10.1111/j.1469-185X.2010.00140.x

Perry, J. C., & Rowe, L. (2010). Condition-dependent ejaculate size and composition in a ladybird beetle. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1700), 3639–3647. https://doi.org/10.1098/rspb.2010.0810

Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: How to compose an ejaculate. *Trends in Ecology and Evolution*, 28(7), 414–422. https://doi.org/10.1016/j.tree.2013.03.005

Pick, J. L., Ebneter, C., Hutter, P., & Tschirren, B. (2016a). Disentangling Genetic and Prenatal Maternal Effects on Offspring Size and Survival. *The American Naturalist*, *188*(6), 628–639. https://doi.org/10.5061/dryad.40jp4

Pick, J.L., Hutter, P., Ebneter, C., Ziegler, A., Giordano, M. & Tschirren, B. (2016b). Artificial selection reveals the energetic expense of producing larger eggs. *Frontiers in Zoology*, 13(38), 1-10. https://doi.org/10.1186/s12983-016-0172-y

Pick, J. L., Hutter, P., & Tschirren, B. (2016c). In search of genetic constraints limiting the evolution of egg size: Direct and correlated responses to artificial selection on a prenatal maternal effector. *Heredity*, *116*(6), 542–549. https://doi.org/10.1038/hdy.2016.16

Pick, J. L., Hutter, P., & Tschirren, B. (2017). Divergent artificial selection for female reproductive investment has a sexually concordant effect on male reproductive success. *Evolution Letters*, *1*(4), 222–228. https://doi.org/10.1002/evl3.21

Pitnick, S. (1996). Investment in testes and the cost of making long sperm in Drosophila. *The American Naturalist*, *148*(1), 57–80. https://doi.org/10.1086/285911

Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioural Ecology and Sociobiology*, 60(3), 289–310. https://doi.org/10.1007/s00265-006-0178-0

Poissant, J., Wilson, A. J., & Coltman, D. W. (2010). Sex-specific genetic variance and the evolution of sexual dimorphism: A systematic review of cross-sex genetic correlations. *Evolution*, *64*(1), 97–107. https://doi.org/10.1111/j.1558-5646.2009.00793.x

Prasad, N. G., Bedhomme, S., Day, T., & Chippindale, A. K. (2007). An evolutionary cost of separate genders revealed by male-limited evolution. *American Naturalist*, *169*(1), 29–37. https://doi.org/10.1086/509941

R Core Team (2024). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/

Ram, K. R., & Wolfner, M. F. (2007). Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. *Integrative and Comparative Biology*, *47*(3), 427–445. https://doi.org/10.1093/icb/icm046

Ramm, S. A. & Schärer, L. (2014). The evolutionary ecology of testicular function: Size isn't everything. *Biological Reviews*, *89*(4), 874–888. https://doi.org/10.1111/brv.12084

Ramm, S.A. & Stockley, P. (2008). Adaptive plasticity of mammalian sperm production in response to social experience. *Proceedings of the Royal Society B: Biological Sciences,* 276(1657), 745-751. https://doi.org/10.1098/rspb.2008.1296

Rowe, M., Whittington, E., Borziak, K., Ravinet, M., Eroukhmanoff, F., Sætre, G. & Dorus, S. (2020). Molecular diversification of the seminal fluid proteome in a recently diverged passerine species pair. *Molecular Biology & Evolution*, 37(2), 488-506. https://doi.org/10.1093/molbev/msz235

Santiago-Moreno, J. & Belsbois, E. (2020). Functional aspects of seminal plasma in bird reproduction. *International Journal of Molecular Sciences*, 21(16), 5564. https://doi.org/10.3390/ijms21165664

Schärer, L., Ladurner, P. & Rieger, R. M. (2004). Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. *Behavioural Ecology and Sociobiology,* 56, 420-425. https://doi.org/10.1007/s00265-004-0802-9

Schärer, L., Vizoso, D.B. (2007). Phenotypic plasticity in sperm production rate: there's more to it than testis size. *Evolutionary Ecology,* 21, 295-306. https://doi.org/10.1007/s10682-006-9101-4

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671-675. https://doi.org/10.1038/nmeth.2089

Sekii, K., Vizoso, D.B., Kuales, G., De Mulder, K., Ladurner, P. & Schärer, L. (2013). Phenotypic engineering of sperm-production rate confirms evolutionary predictions of sperm competition theory. *Proceedings of the Royal Society B: Biological Siences*, 280(1757), 20122711. https://doi.org/10.1098/rspb.2012.2711

Singh, R.P., Sastry, K.V.H., Pandey, N.K., Singh, K.B., Malecki, I.A., Farooq, U., Mohan, J., Saxena, V.K. & Moudgal, R.P. (2012). The role of the male cloacal gland in reproductive success in Japanese quail (*Coturnix japonica*). *Reproduction, Fertility and Development,* 24(2), 405-409. https://doi.org/10.107he1/RD11057

Stanley, A. J., & Witschi, E. (1940). Germ cell migration in relation to asymmetry in the sex glands of hawks. *The Anatomical Record*, *76*, 329–342. https://doi.org/10.1002/ar.1090760310

Swanson, W.J. & Vacquier, V.D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics*, 3(2), 137-144.

Tang, B., Xie, G., Hu, X., Xhang, X., Hu, S., Hu, J., Hu, B., Li, L. & Wang, J. (2022). A comparative proteomic study of high and low semen quality seminal plasma in drakes. *Poultry Science*, 101(11), 102130. https://doi.org/10.1016/j.psj.2022.102130

Thélie, A., Grasseau, I., Grimaud-Jottreau, I., Seigneurin, F. & Blesbois, E. (2019). Semen biotechnology optimization for successful fertilization in Japanese quail (*Coturnix japonica*). *Theriogenology,* 139, 98-105. https://doi.org/10.1016/j.theriogenology.2019.07.028

Whitlock, M. C., & Agrawal, A. F. (2009). Purging the genome with sexual selection: Reducing mutation load through selection on males. *Evolution*, *63*(3), 569–582. https://doi.org/10.1111/j.1558-5646.2008.00558.x

Willet, E.L. and Ohms, J.I. (1957). Measurement of testicular size and its relation to production of spermatozoa by bulls. *Journal of Dairy Science*, 40(12), 1559-1569. https://doi.org/10.3168/jds.S0022-0302(57)9467

Figure 1

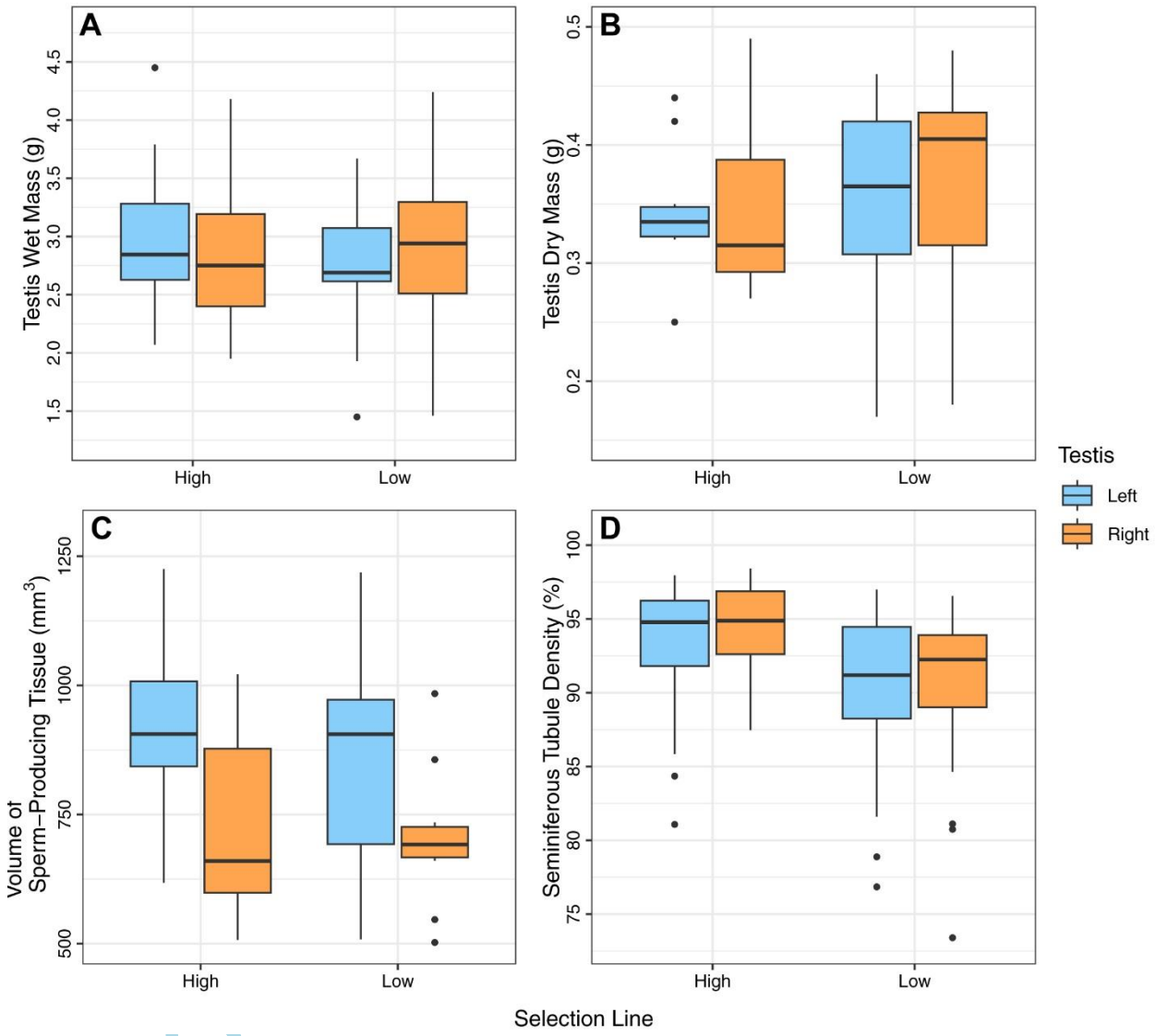


Figure 2

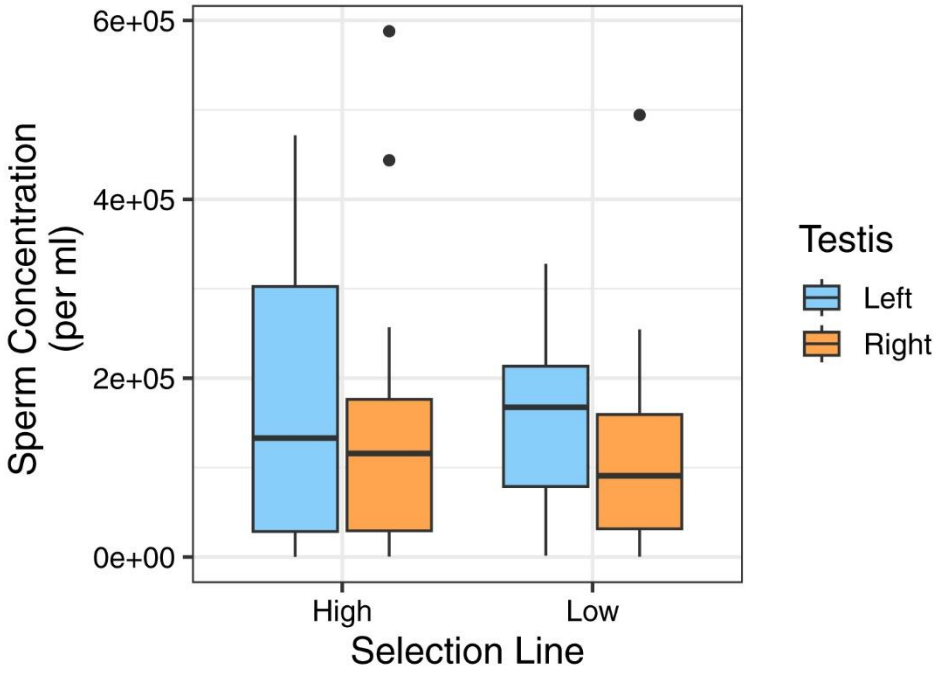


Figure 3

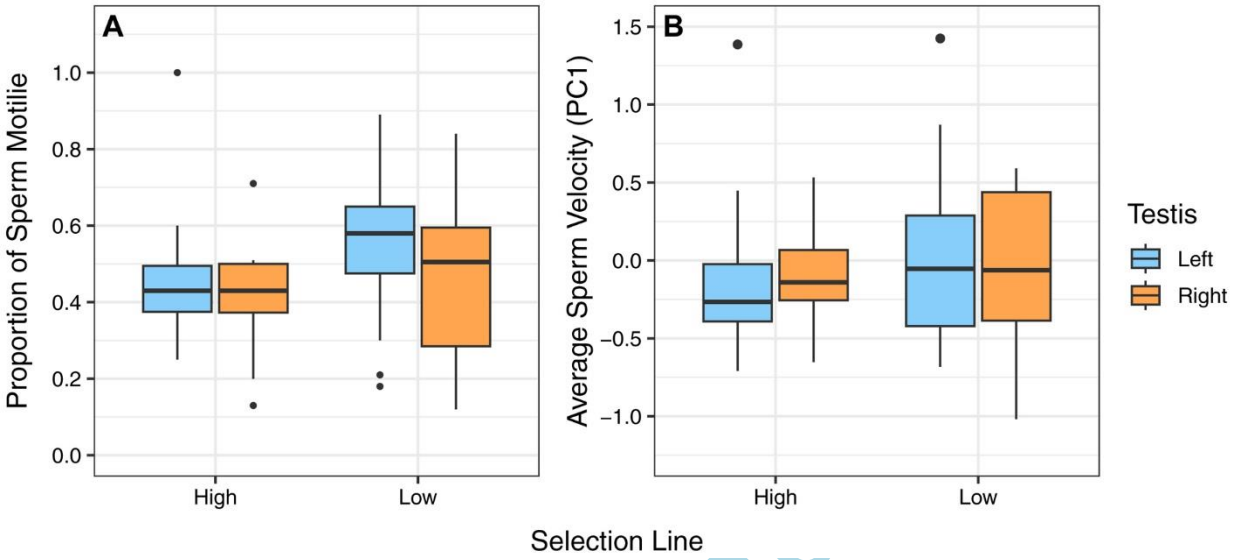


Figure 4