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Mason, C. orcid.org/0009-0000-9622-0638, Tschirren, B. and Hemmings, N. orcid.org/0000-0003-2418-3625 (2024) Effects of female-specific selection for reproductive investment on male fertility traits. Journal of Evolutionary Biology, 37 (9). pp. 1113-1124. ISSN 1010-061X

https://doi.org/10.1093/jeb/voae095

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Journal of Evolutionary Biology, 2024, **37**, 1113–1124
https://doi.org/10.1093/jeb/voae095
Advance access publication 7 August 2024
Research Article

JOURNAL OF
Evolutionary Biology
eseb European Society for Evolutionary Biology

Effects of female-specific selection for reproductive investment on male fertility traits

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Handling editor: John Hunt, Associate editor: Clarissa Palma-Silva

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Abstract

Despite sharing an autosomal genome, the often divergent reproductive strategies of males and females cause the 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 fertilization 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. Precopulatory cues and/or the role of seminal fluid in sperm motility may thus be more likely to contribute to the H-line male fertilization advantage in this species.

Keywords: egg size, fertility, sexual selection, sperm, spermatogenesis, testis

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 the 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, fertilization 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).

Received March 28, 2024; revised July 5, 2024; accepted August 2, 2024
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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 fertilized more eggs under both competitive and noncompetitive 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 fertilize compared to L-line males, particularly from their larger left testis, underpinning the fertilization 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 nonrelated males and females (not sharing any grandparents) were established and housed in individual cages (112 cm × 50 cm × 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 focussed on a single replicate from the fourth generation to minimize 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 fertilization success, or testis size between this study system's line replicates at generation four (Pick et al., 2016c, 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 fertilization 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 euthanized 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 0.01 g (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 (Sigma-Aldrich, UK) for histological analysis (see below). After 24 hr, 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 1 ml of Ham's nutrient media (Invitrogen, UK) and heated to 38 °C. The distal end of each seminal glomerus was gently squeezed with forceps, and 20 µl of semen was extracted using a pipette. Of this, 5 µl was added to an Eppendorf with 15 µl Ham's media, allowed to "swim out" for 10 s, and then 4 µl of this solution was loaded into a slide chamber, prewarmed to 38 °C on a heat mat, for sperm motility and velocity analysis (see below). Two additional samples of 5 µl extracted semen were each added to a sample tube containing 50 µl 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 hr, and testis mass was recorded. Testes were put in a drying oven at 60 °C and weighed every 24 hr 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 0.01 g was recorded.

Testis sperm production capacity

The testes preserved for histological analysis were embedded in paraffin blocks and cut into five sections that were each stained with hematoxylin and eosin and then mounted onto

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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 0.01 mm by a single researcher using ImageJ (Schneider et al., 2012). Testis volume to the nearest 0.01 mm^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 five sections per testis was taken as the final measure of testis volume.

$$\text{Testis volume } \left(\text{mm}^3\right) = \frac{4}{3} \times \pi \times A \times B^2 \quad (1)$$

The testis sections were then imaged under a compound light microscope at 2× 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 (Supplementary 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 (Supplementary 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 the 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 20 µl 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 0.9 mm^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.

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

Sperm motility and velocity

Sperm motility and velocity were assessed using the Sperm Class Analyser Computer-Assisted Sperm Analysis software (Microptic, Barcelona). Four microlitres of semen were loaded into a 20 µl depth slide chamber (Leja, Netherlands) and allowed to equilibrate on the microscope heated stage (38 °C) for 30 s. 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 eight samples (4 H-line and 4 L-line), however, there were insufficient sperm to sample 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 analyzed the data both with and without them (see *Results* section 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 nonmotile 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 Supplementary Table S1 for full descriptions). Due to the colinearity of VAP, VCL, and VSL (Supplementary 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., 0–70 µm/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 4 µl 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 min. Using a fluorescence microscope (Leica DMBL) and darkfield filter at 400× magnification, five 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 Supplementary Figure S3) were measured to the nearest 0.1 µm using ImageJ (Schneider et al., 2012) by a single researcher. Measurements were taken three times and used to calculate

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the mean average length of each component. Three measures gave high repeatability for all components, and sperm morphology was consistent within males (see Supplementary 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 (112 cm × 50 cm × 50 cm; 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 10 µl Hoechst 33342 fluorescent dye, following the methods described in Birkhead et al. (2008). A 1 cm^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. The 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 analyze 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 LMMs and 1 MANOVA assessing differences in the testis and sperm traits between selection lines, (b) 5 LMMs assessing the effect of testis traits on sperm traits, (c) 6 LMMs 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 the testes did not differ between selection lines or between the right and left testis within lines, and there was no significant interactive effect of the 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 (Supplementary 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 (Supplementary 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 (Supplementary 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 (Supplementary Table S4).

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 (Supplementary Table S4).

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

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Table 1. Linear mixed models (LMMs) for the effect of selection line and testis side on measured testis and sperm traits.

Predictor	Selection line				Testis side				Interactive effect				
Response	χ^2	DF	*p*	FDR-*p*-value	χ^2	*df*	*p*	FDR-*p*-value	χ^2	*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 (mm^3)	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% of sperm (PC1)	0.31	1	0.58	0.82	0.16	1	0.69	0.82	2.76	1	0.10	0.38	53
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

Note. Initial *p*-values and False Discovery Rate (FDR)-corrected *p*-values are given. Significant results are shown in bold.
*$p < 0.05$.
***$p < 0.001$.

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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 and right testis. Coloured boxes represent 25% and 75% quantiles, whiskers 1.5 interquartile range, and the bold black line is the median. Significant effect of testis side on the volume of sperm-producing tissue: $\chi^2 = 14.17$, $df = 1$, $p < 0.001$, FDR-p-value = 0.02.

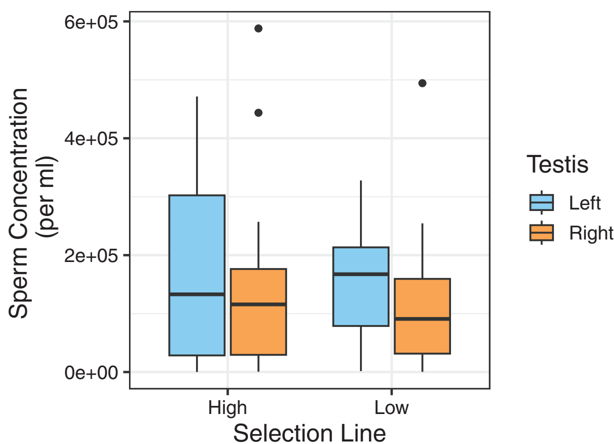

Figure 2. Sperm concentration: Effect of artificial selection for high or low female reproductive investment on the number of sperm per ml of semen taken from the seminal glomera of the left and right testis.

was consistent when four samples from each line with low sperm numbers were excluded (see *Materials and Methods* section and Supplementary Figure S4). Inter-male variation in sperm motility did not differ significantly between lines (Supplementary 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; Supplementary Figure S5). This result was also consistent when four samples from each line with low sperm numbers were excluded (see *Materials and Methods* section 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 (Supplementary Table S4).

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 LMMs 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 (Table 1; Figure 4). Inter-male variation in head length, midpiece length, tail length, and total sperm length did not differ significantly between lines (Supplementary Table S4). Each sperm component length was significantly positively correlated with total length (Supplementary Table S5). On average, the midpiece

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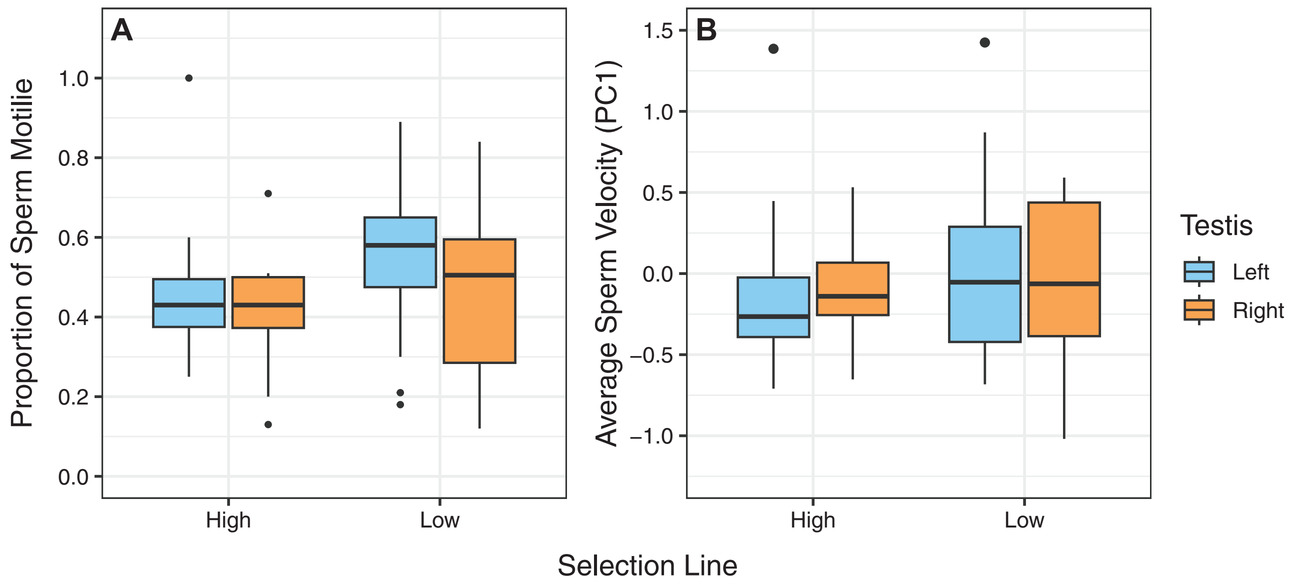

Figure 3. Sperm Motility and Velocity: Effect of artificial selection for high or low female reproductive investment on the (A) proportion of sperm motile and (B) mean average swimming velocity of all sperm (PC1) from the left and right testis. Significant interactive effect of selection line and testis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

was 9.86 ± 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 Supplementary Table S6).

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

Ultimately, the number of sperm reaching the PVL of eggs did not differ between lines (Table 1; Supplementary Figure S8), and inter-male variation in sperm numbers reaching the egg did not differ significantly between lines either (Supplementary 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 fertilization 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; Willet & 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

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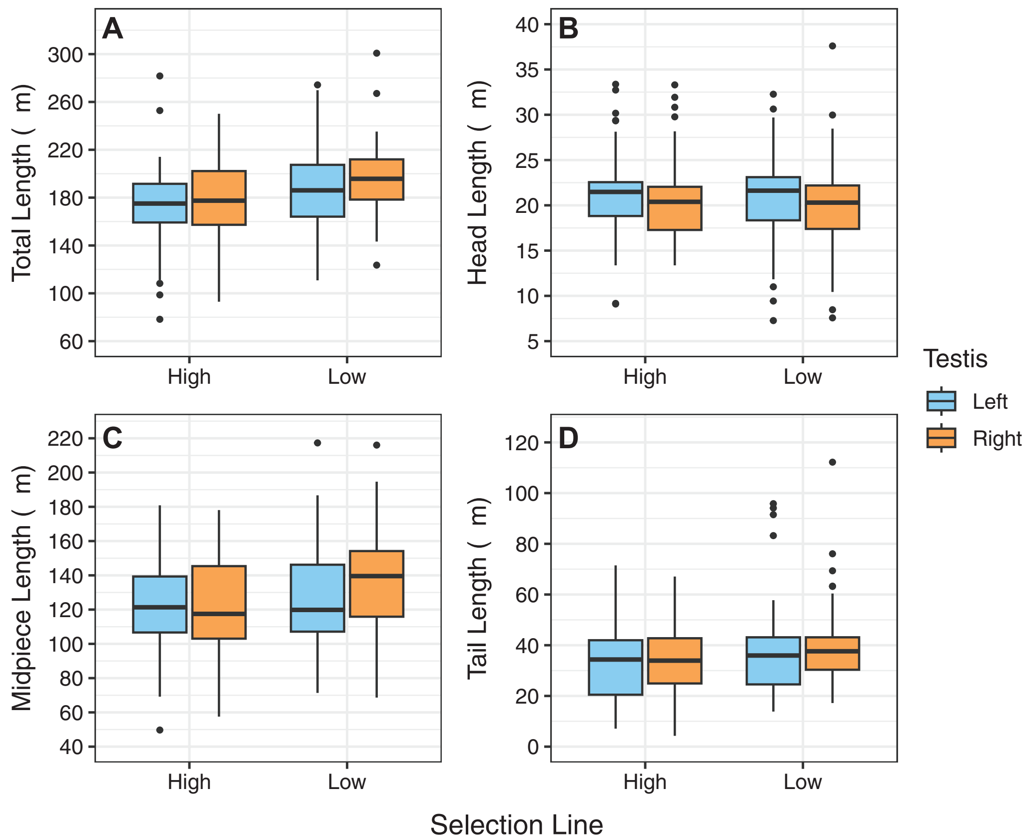

Figure 4. Sperm morphology: Effect of artificial selection for high or low female reproductive investment on the (A) total length, (B) head length, (C) midpiece length, and (D) tail length of sperm from the left and right testis.

Table 2. Linear mixed models (LMMs) for the effects of testis mass on measured sperm traits.

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

Note. Initial *p*-values and False Discovery Rate (FDR)-corrected *p*-values are given.

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, 1981; Ramm & Stockley, 2008; Schärer & Vizoso, 2007; 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 the 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 fertilization success were wrong, it is possible that other factors drive the differences in fertilization success previously detected between the lines. For example, several important molecules regulate avian fertilization, including proteases in sperm that hydrolyze the egg PVL to

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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 postcopulatory mechanisms

The lack of significant differences in primary male fertility traits between the selection lines suggests that alternative mechanisms are responsible for the elevated fertilization success of H-line males that have been previously reported (Pick et al., 2017). We suggest three possibilities: firstly, fertilization success in this system was measured after natural copulations (i.e., not artificial insemination; Pick et al., 2017), so it is therefore possible that precopulatory mechanisms, including female-mate choice, contributed to the fertilization 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 (Kelly & Jennions, 2011; Perry & Rowe, 2010). 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 fertilization success of H-line males.

Finally, other components of the ejaculate may contribute to male fertilization 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., Borziak et al., 2016; Labas et al., 2015; Rowe et al., 2020; Santiago-Moreno & Belsbois, 2020; Tang et al., 2022). Japanese quail transfer a substantial volume of a unique foam from a specialized 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 fertilization success (Cheng et 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 been 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 fertilization 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 fertilization 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. Precopulatory cues and/or the role of seminal fluid in sperm motility may, therefore, be more likely to explain the previously observed H-line male fertilization advantage in this system.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The data underlying this article are available in the Dryad Digital Repository, at https://dx.doi.org/10.5061/dryad.x69p8czt5.

Author contributions

Chloe Mason (Data curation [lead], Formal analysis [lead], Funding acquisition [equal], Investigation [equal], Methodology [equal], Visualization [lead], Writing—original draft [lead]), Barbara Tschirren (Conceptualization [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal]), and Nicola Hemmings (Conceptualization [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal])

Funding

C.M. was funded by a NERC ACCE studentship (NE/S00713X/I), B.T. was funded by the Swiss National Science Foundation (PP00P3_128386 and PP00P3_157455), and N.H. was funded by a Royal Society Dorothy Hodgkin Fellowship (DH160200).

Acknowledgments

We thank Pascale Hutter at the University of Zurich for technical assistance during sample and data collection and Mark Kinch at the University of Sheffield Medical School for expert assistance with histological sectioning.

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Conflicts of interest

None declared.

Ethical statement

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

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