The relationships between testes asymmetry, seminiferous composition and the quality and quantity of sperm produced in a precocial bird

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I certify that all material in this dissertation which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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Abstract

Theory assumes that postcopulatory sexual selection favours increased investment into testes size, as larger testes produce ejaculates that provide a competitive advantage when rival males compete for fertilisation. However, the relationship between relative increases in testes size with the organisation of sperm-producing tissue, and how such changes influence sperm quality and quantity is not fully understood. Male Japanese quail (*Coturnix japonica*) originating from lines artificially selected for high female reproductive investment experienced higher fertilisation success, as well as a relatively larger left testis than males from low reproductive investment lines. The aim of this study was to determine the origin of this increased fertility from a morphological perspective. For males from both lines and in both testis, we measured the proportion and absolute amount of seminiferous tissue, as well as the quantity and quality of sperm within the sperm reserves, including sperm length and proportions that were alive and morphologically normal. The left testis of high line males had higher proportions of interstitial tissue, but not seminiferous tissue, and did not produce different sperm morphologies that could explain the enhanced fertilisation success. On the contrary, the right testis had higher proportions of seminiferous tissue and produced more alive sperm. There was no difference in sperm quantity or proportions of sperm with normal morphology in either testis of males from divergent lines. Independently of line origin, the right testis contained sperm with larger tails, total lengths, and shorter ratios of the head : tail, indicating a specialisation to produce faster sperm. Overall, we found no clear difference in the quality or quantity of sperm produced by high line males that could explain their reproductive success. However, because ejaculates are complex, interactive, multivariate traits, fertilisation success could have been influenced by other traits not measured here, such as the composition of copulatory fluids, functions of somatic cells in the testes, provisioning against oxidative stress or the regulation of sperm use by the females. Altogether, these results highlight unusual relationships between testes size with testes and sperm morphology and demonstrate that relatively larger testes do not necessarily have more seminiferous tissue or produce more, or better quality sperm.

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Introduction

Sperm competition occurs when males compete for fertilisation and is often the driving force underlying the evolution of ejaculate traits (Birkhead & Pizzari 2002). A common response to sperm competition is for males to invest more into testes size, which is commonly associated with adaptations that increase the likelihood of fertilisation, such as producing more sperm (Møller 1988; Hosken & Ward 2001; Lüpold et al 2009), or better quality sperm (Snook 2005; Rowe & Pruett-Jones 2011). However, key reproductive traits such as these are subject to various selective forces that may differentially influence their expression. For example, the environment during prenatal development can impact the fitness of individuals (Henry & Ulijaszek 1996; Lindström 1999). In female oviparous species, this environment can be represented by egg size (Bernardo 1996), as the resource allocation provided by mothers is key to offspring growth in early life (Pick et al 2016; Finkler et al 1998) and reproduction in adulthood (Lindström 1999). Additionally, the genotype of an individual is key to their phenotype (Hosken & Ward 2001; Birkhead et al 2005; Simmons & García-González 2008; Mossman et al 2009) and can impose a strong influence on reproductive success (Fowler et al 1997; Simmons & García-González 2008). However, because both sexes share most of their genome, adaptations to one sex may affect the phenotype of the other sex (Lande 1980). The fitness consequences of such adaptations would depend on whether the current phenotype of either sex is moved towards, or away from their fitness optimum (Parker 2006). If selection causes one sex to receive a benefit at the cost of the other, variation will be driven between the sexes as one sex evolves counter adaptations to improve their fitness (Parker 1979; Lund-Hansen et al 2019). On the other hand, if selection has a positive effect on the fitness of both sexes, there will be an amplified phenotypic response in males and females, alongside the removal of deleterious alleles from the population (Whitlock & Agrawal 2006). For internally fertilising species to successfully reproduce, sperm must traverse the female reproductive tract (FRT), the obstacles in which select for only sperm morphologies capable of completing the journey to the ova (Birkhead et al 1993; Hemmings et al 2016), which makes the male ejaculate a key target of selection that is closely tied to fertilisation success.

In the context of sperm competition, male fertilisation success is largely explained by ejaculate composition, which consists of both sperm and non-sperm components that contribute differently to the ability of sperm to reach and fertilise the ovum. For the sperm component, the quality and quantity produced are key, but influence fertilisation success in different ways, namely by: i) Transmitting greater numbers of sperm (Martin et al 1974), which increases the likelihood of a given sperm to reach the ovum (Immler et al 2011); ii) Transmitting greater proportions of sperm that are alive (Bilgili et al 1985; García-González & Simmons 2005), as sperm longevity could be positively associated with swimming endurance and therefore the duration sperm is viable and competitive (Birkhead et al 2009); iii) Transmitting greater proportions of sperm that are morphologically normal (Oettlé 1993; Gomendio et al 2007; Rowe & Pruett-Jones 2011), as abnormal sperm are impaired at traversing the FRT and fusing with the ova (Kruger et al 1988; Saacke et al 1994; Krzanowska et al 1995); and iv) Producing sperm with increased motility to improve sperm transport through the FRT (Birkhead et al 1999). Besides, these traits are often associated with sperm competition, as species with relatively larger testes can also produce more sperm (Møller 1988; Hosken & Ward 2001; Lüpold et al 2009), sperm with improved motility (Møller 1988), higher proportions of alive sperm (Rowe & Pruett-Jones 2011), and higher proportions of morphologically normal sperm (Rowe & Pruett-Jones 2011).

The higher amounts of sperm produced from relatively larger testes is often attributed to increasing amounts or proportions of seminiferous tissue therein (Ramm & Schärer 2014), but it may also expand beyond measures of seminiferous tissue, and could also be explained by the rate at which sperm are produced (Amann 1981). However, because increased proportions of seminiferous tissue can occur independent of changes to relative testes size (Firman et al 2018), testes mass and the organisation of seminiferous tissue may be under different selection pressures. Spermatogenesis is regulated by somatic cells in the testes, two of which that are of key importance are Sertoli and Leydig cells (Smith & Walker 2014). Sertoli cells lie within the seminiferous tissue providing structural support and nourishment for developing sperm (Griswold 1998). Whereas Leydig cells lie in-between the seminiferous tubules, within the interstitial tissue and supply developing sperm with androgens

(Desjardins 1978). Principally, Leydig cells produce testosterone, which is essential for Sertoli cell function (Smith & Walker 2014). The efficiency of Sertoli cells (i.e., the ratio between Sertoli cells and germ cells), can be higher in species with relatively larger testes, containing greater proportions of seminiferous tissue (delBarco-Trillo et al 2013), and could influence sperm production through efficient androgen diffusion into dense seminiferous tissue. Higher Sertoli cell efficiencies have been positively linked to sperm quantity (Russel & Peterson 1984), by both generating more spermatids per spermatogonium (Lüpold et al 2011) and completing faster cycles of spermatogenesis (delBarco-Trillo et al 2013). Additionally, higher Sertoli cell efficiencies have been linked to improved sperm quality, by increasing sperm length and possibly removing undesirable sperm during spermatogenesis (Lüpold et al 2011). Altogether this indicates that to maximize both sperm quality and quantity, testis may evolve adaptations that extend beyond measures of sperm-producing tissue.

Longer sperm are assumed to be faster than shorter sperm because they have longer midpieces (Immler & Birkhead 2007), which supply more energy (Gu et al 2019), and longer tails (Immler & Birkhead 2007), which provide stronger propulsion (Cardullo & Baltz 1991). Faster swimming sperm are assumed to have a competitive advantage when fertilising an ovum because they can traverse the FRT before slower sperm (Birkhead et al 1999). Indeed, studies have shown that longer sperm can be faster (Gomendio & Roldan 1991) and more capable of fertilising the ovum (Bennison et al 2015). Additionally, sperm motility can also be explained by the ratio of the head : tail, which represents drag : propulsion forces of sperm (Humphries et al 2008). Indeed, sperm with smaller ratios of the head : tail have been found to be faster (Helfenstein et al 2010) and have increased fertilisation success (Hemmings et al 2016). In addition to motility, sperm length could also be related to the longevity of sperm produced. Longer sperm typically die sooner, presumably due to the increased metabolic activity a of relatively longer flagellum draining sperm energy reserves more quickly (Stockley et al 1997; Helfenstein et al 2010). If such a relationship exists, it could pose an evolutionary trade-off between sperm size and longevity, as sperm endurance depends on the energy available which is determined by sperm size. In avian species, once sperm have traversed the

vagina, they are kept in sperm storage tubules at the uterovaginal junction (Bakst et al 1994). These tubules impose a strong selective force on sperm, as only sperm that are alive and morphologically normal can enter them (Allen & Grigg 1957). If longer sperm are faster and die sooner, then these sperm might be able to reach the ovum or occupy the storage tubules more quickly than smaller sperm to provide a fertile advantage, but only if fertilisation occurs soon after insemination. On the other hand, longer lived sperm may remain viable in the FRT for a longer duration, and provide a fertile advantage when fertilisation occurs a considerable time after insemination.

Furthermore, the ability of sperm to successfully fertilise the ova can be influenced by non-sperm portions of the ejaculate (Perry et al 2013) as well as the female regulation of sperm use (Eberhard 1966; Firman et al 2017). For example, non-sperm portions of the ejaculate drive sperm metabolic functions, enhance sperm motility, protect sperm from the harmful environment of the FRT, and allow for regular sperm functioning such as capacitation and the acrosome reaction (reviewed in Poiani 2006; Ramm 2020). Furthermore, male copulatory fluids indirectly contribute to fertilisation through interactions with female immune responses and reproductive processes (reviewed in Schjenken & Robertson 2020). For example, by allowing sperm access to sperm storage tubules (Neubaum & Wolfner 1999; Sasanami et al 2015) and inducing ovulation (Eberhard & Cordero 1995). In male Japanese quail (*Coturnix japonica*) most of the non-sperm copulatory fluid comes from the proctodeal gland which produces a foam that is separate from, but introduced alongside the ejaculate during copulation (Klemm et al 1973). This foam improves sperm motility (Farooq et al 2015), sperm transport through the FRT (Singh et al 2012) and decreases the fertilisation success of a rival males' sperm (Finseth et al 2013).

The relative testes sizes of insects can be manipulated by enforcing polygynous or monogamous mating systems (Hosken & Ward 2001), which in turn can influence reproductive success (Simmons & García-González 2008). Therefore, because relative testes size is linked to sperm quality and quantity (Møller 1988; Gage 1994; Rowe & Pruett-Jones 2011), and that polygynous avian species have larger testes (Pitcher et al 2005), it seems plausible that in species with asymmetric testes, the relative sizes of each testis may confer differences in

functionality. Testes asymmetry is especially pronounced amongst birds, where a relatively larger left testis is commonplace (Calhim & Montgomerie 2015), however the evolutionary causes and reproductive consequences of testes asymmetry are not fully understood.

During avian development, gonad differentiation depends on sex, in females the functional ovary develops only from the left gonad whilst the right regresses, whereas males' testis develop on both sides (Romanoff 1960). Indeed, avian testes asymmetry may result from selection on asymmetric development of female gonad morphology, however the existence of right-bias testes asymmetry in some species indicates that other factors are likely to be important (Calhim & Montgomerie 2015). The compensation hypothesis suggests that relatively smaller testis serve as a back-up, by retaining function if the larger one becomes incapacitated (Møller 1994), and studies have shown that castration of a single testis can cause the other one to grow (Calhim & Birkhead 2009). Alternatively, because avian testes are internal their growth might be limited by the space shared with other asymmetrical organs (Witschi 1935). Across avian species, increasing relative testes size has been associated with reduced asymmetry between the testes, but only for individuals with a larger left testis (Calhim & Montgomerie 2015). Whereas across *Maluridae* species, the direction of testes asymmetry was found to be dependent on the combined relative testes mass, those with smaller testes were left-biased, and those with larger testes were right-biased (Calhim et al 2019). Furthermore, some species responded to intense sperm competition by shifting from more symmetrical testes to right-biased asymmetry (Calhim et al 2019). However, testes asymmetry may not necessarily confer differences in functionality, as *Maluridae* for example appear to have no difference in the proportion of seminiferous tissue within or across species exhibiting divergent testes asymmetry (Calhim et al 2019), which indicates a functional equivalence between asymmetric testes. Altogether, there exists substantial variation between the causes and functions of asymmetric testes. To better understand these processes, for species whereby testes asymmetry is linked to reproductive success, it would be interesting to see whether morphological adaptations of asymmetric testes translate into variation in sperm quantity and

quality, and how these adaptations vary in response to different selective pressures.

Egg production is a female sex-limited trait that arises from asymmetrical gonad development in female ovaries. Female Japanese quail were selected to form lineages of high and low maternal reproductive investment, i.e., to produce large and small eggs, respectively. It was found that males born from the high maternal investment line experienced higher fertilisation success in both competitive and non-competitive scenarios (Pick et al 2017), suggesting that high-investment males produce ejaculates that are more capable of successfully fertilising a female. This result was linked to an increase in the relative size of the left testis (Pick et al 2017), indicating that selection for high maternal reproductive investment has shifted the shared asymmetrical gonad development in both sexes towards their optima for fertilisation success. To study the origin of this difference in fertility, we compared the left and right testis in males selected for divergent maternal reproductive investment for i) amounts and proportions of sperm producing tissue, ii) lengths of sperm cell components, and iii) proportions of alive and morphologically normal sperm. We also tested for relationships between variation in seminiferous tissue composition and sperm quality and quantity. In the high maternal investment line, we expect to see either increased amounts and/or proportions of sperm producing tissue, sperm that are longer or have shorter head : tail ratios, and higher proportions of sperm that are alive and morphologically normal. Furthermore, because larger testes are associated with improved key sperm traits (Møller 1988; Lüpold et al 2009; Rowe & Pruett-Jones 2011), within the high line we expect to see increased parameters of sperm quality or quantity in the relatively larger left testis, compared to the right.

Method

Animals and sample collection

Males for this study were from a captive population of Japanese quail (*Coturnix japonica*) selected for divergent maternal reproductive investment as described in (Pick et al 2016). This population had undergone divergent selection for high or low maternal reproductive investment; in generation one, eggs from 25% of females with the highest and lowest relative egg sizes were incubated to form

the high and low lines, then in subsequent generations, 50% of females producing extreme egg sizes were selected (Pick et al 2016). Breeding pairs consisted of individuals from the same line and replicate (Pick et al 2016). After 4 generations, absolute egg size between the high and low lines differed by 1.06 standard deviations (Pick et al 2016). Once the sixth generation was mature, 10 males from each of the high and low lines were euthanised and sperm was dissected directly out of the seminal glomera, with samples taken from both testis to obtain spermatozoa produced from each side. Both testis were fixed in Bouins solution and stored until sectioning, then embedded in paraffin blocks, cut into sections, and stained with Hematoxylin and Eosin.

Histological sections were imaged at x2 and x10 magnification using an Olympus BX61 microscope and CellSens software (Olympus RRID:SCR_01455). To obtain seminiferous tubule cross-sections from 20 males, 2 of the roundest seminiferous tubules were selected from each of the 5 slide sections of the left and right testis. Only round tubules displaying clear progression of spermatogenic development from the edge of the seminiferous tubule to the lumen were selected. Images were taken at 10x magnification for seminiferous tubule characteristics (areas of the tubule, lumen, and seminiferous tissue). 1 image of each section was taken at x2 magnification to measure variation in interstitial tissue throughout the testes. All measurements were conducted blindly to male treatments. All features were quantified using ImageJ (Schneider et al 2012).

Measures of seminiferous tissue

For every male, a total of 20 tubules were measured across 5 slide sections from each testis, i.e., 2 tubules per slide. Three measurements of each structure within the seminiferous tubule were recorded and averaged to the nearest 0.01 µm; The seminiferous tubule cross sectional area was measured from tracing tubule circumference (Fig 1.a). The lumen area was measured by tracing the circumference of the innermost area of the tubule (Fig 1.a). This technique yielded high repeatability of measurement error for both tubule area (r = 1, n = 1200, p = <0.001) and lumen area (r = 1, n = 1200, p = <0.001). The proportion of spermatogenic tissue throughout the testis (hereafter referred to as testicular seminiferous density) was derived from 10 images taken at x2 magnification per individual, i.e., 1 image per slide from each testis. Images where made

greyscale and set at a constant threshold, so proportions of coloured : non coloured pixels represented proportions of spermatogenic tissue (Fig 1.b)

Measurements were also checked for repeatability per male / side, yielding low/moderate repeatability of the tubule area ($r = 0.472$, $n = 400$, $p = <0.001$), seminiferous area ($r = 0.392$, $n = 400$, $p = <0.001$) and testis seminiferous density ($r = 0.609$, $n = 200$, $p<0.001$), and high repeatability of the seminiferous proportion ($r = 0.73$, $n = 400$, $p<0.001$) and lumen area ($r = 0.726$, $n = 400$, $p<0.001$). For repeatability of these traits per male, see appendix Table S1. Measurements were further averaged per testis side to test relationships with other testes and sperm parameters.

The tubule seminiferous area was calculated as (seminiferous tubule area - lumen area), the tubule seminiferous proportion was calculated as (tubule seminiferous area / tubule cross sectional area), and the testicular seminiferous density was calculated as (coloured pixels / coloured pixels + non-coloured pixels).

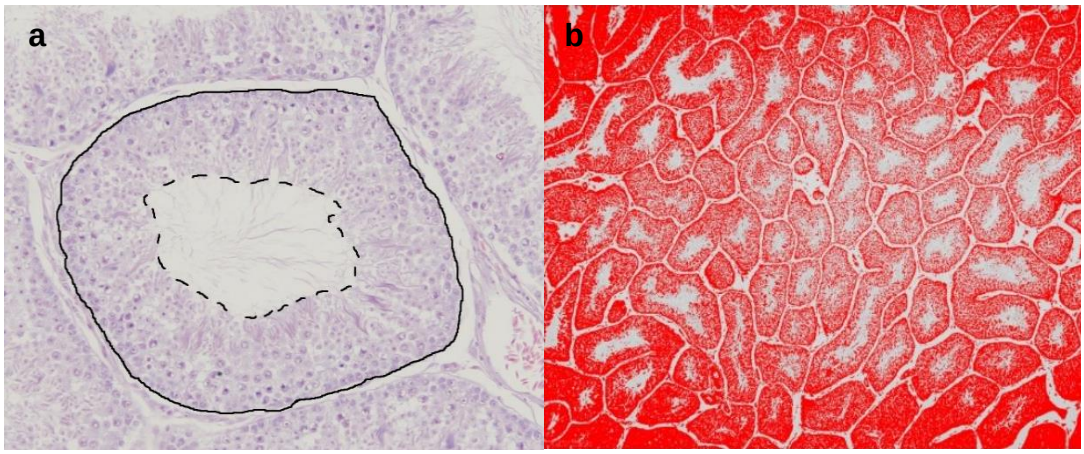

Fig 1. Examples of how measurements of seminiferous tissue were taken. **a**) The solid line delineates the seminiferous tubule area, and the dashed line delineates the lumen area. **b**) The testis seminiferous density was measured as the proportion of a binary image.

Assessment of sperm cell viability

Sperm viability was assessed using a live/dead sperm viability kit (SYBR 14 / Propidium Iodide (PI)) (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA), using a methodology like that described by (García-González & Simmons 2005). To create working solutions, stains were dissolved

in anhydrous dimethyl sulfoxide (DMSO). Working solutions for each stain were as follows: SYBR 14 (1µl : 10µl), PI (1µl : 200µl). 5µl of sperm sample was placed with 5µl of each working solution onto a coverslip, incubated in the dark for 5 minutes and images were taken at 250x magnification using darkfield microscopy (Leica DMBL) with an Infinity 3 camera (Luminera Corporation) and InfinityAnalyse software. Starting at the furthest edge, the coverslip was visually scanned from top to bottom, then moved to the side after reaching the bottom of the coverslip to avoid pseudo replication of sperm, and then scanned from bottom to top. This process was repeated until 100 sperm per testis (200 sperm per individual) were imaged. Sperm was classified as “live” if the head appeared completely stained green with SYBR 14 (Fig 2.a), or “dead” if any part of the head was stained red with Propidium iodide (Fig 2.b). Measurements were further averaged per testis side to test relationships with other testis and sperm parameters.

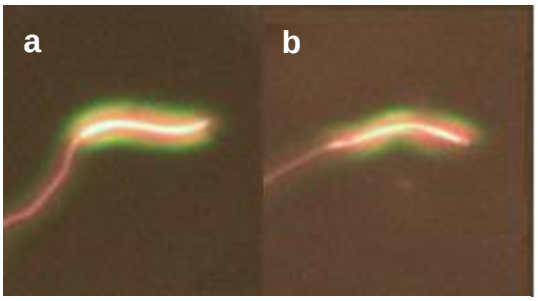

Fig 2. Examples of sperm considered **a**) Live and **b**) Dead.

Measures of sperm morphology

The proportion of morphologically normal sperm was assessed using the same samples used for sperm viability. Abnormalities were classified to all sperm cell components; any sperm deviating from typical cylindrical head shape or linear midpiece shape, and any sperm missing a head, midpiece or tail was considered abnormal.

To measure sperm length, 20 morphologically normal sperm from each testis (40 per individual, except one individual which had 17 from each testis) were measured for every male. DNA in the head was stained with Hoechst 33342, and mitochondria in the midpiece was stained with Mitotracker Green FM (Molecular Probes, Eugene, OR, USA) (Fig 3). For stock solutions, stains were dissolved in DMSO, and for each stain were as follows: Hoechst (1µl : 9µl), Mitotracker (50µl : 74 µl). For working solutions, stock solutions were further diluted in Phosphate-buffered saline (PBS), and for each stain were as follows: Hoechst (1µl :200 µl) and Mitotracker (1µl : 20µl). Sperm were photographed using darkfield microscopy at 400x mag and measured to the nearest 0.001 µm.

For each sperm, 3 measurements of the length of the head and midpiece were averaged, as well as the tail and acrosome when present. Total sperm length was calculated as the sum of the head, midpiece and tail. Flagellum length was calculated as the sum of the midpiece and tail. The ratio of the head : tail was calculated as (head / head + tail). This technique yielded high repeatability of measurement error for all sperm cell components, namely the head (r = 0.987, n = 2382, p = <0.001), midpiece (r = 1, n = 2364, p = 0.001), tail (r = 0.999, n = 1662, p = <0.001) and acrosome (r = 0.999, n = 1365, p = <0.001).

Repeatability was also calculated per male, per side, which yielded low/moderate repeatability of all sperm cell components, namely the head (r = 0.285, n = 794, p < 0.001), midpiece (r = 0.398, n = 788, p < 0.001), tail (r = 0.09, n = 554, p < 0.001), acrosome (r = 0.061, n = 445, p = 0.003), total length (r = 0.313, n = 553, p < 0.001), flagella (r = 0.298, n = 553, p < 0.001) and head : tail (r = 0.067, n = 554, p < 0.001). For repeatability of these traits per male, see appendix Table S1. Measurements were further averaged per testis side to test relationships with other testes and sperm parameters.

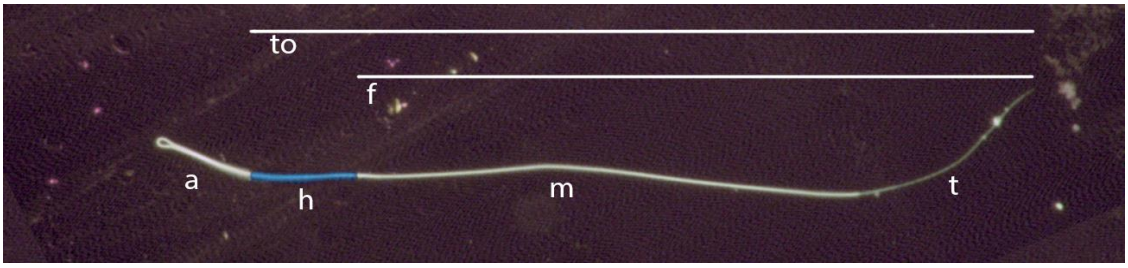

Fig 3. Example of Hersch and Mitotracker dyes superimposed on sperm to identify cell components; The nucleus is stained blue (h), and the acrosome (a), midpiece (m) and tail (t) are stained green. Total sperm length (to) and flagellum length (f) are also indicated.

Sperm concentrations

Concentrations of sperm were derived from averaging the sperm present in two coverslips of 10 µl sperm sample using a Leica DMBL microscope. Concentrations of live and normal sperm were calculated by multiplying the amounts of live or normal sperm with the average sperm concentrations.

Data analysis

Data were analysed in R v. 4.0.2 (R Core Team 2017) using the package lme4 (Bates et al 2015). The function ‘lmer’ was used to test Linear mixed models (LMM), and the function ‘glmer’ was used to test Generalized linear mixed models (GLMM). A Pearson’s correlation matrix was also conducted between key testis and sperm traits given in Table S2.

LMM were used to test whether lineage or testis side affected the areas or proportions of seminiferous tissue, lengths of sperm cell components, sperm concentration, and concentrations of live and normal sperm. For all LMM, line, testis side and the interaction between line and side were included as fixed effects, and male ID was fitted as a random effect. LMM were also used to test whether testis mass affected areas or proportions of seminiferous tissue, whereby area or proportion of seminiferous tissue was fitted as a dependant variable, testis mass was fitted as a predictor, and male ID was fitted as a random effect.

GLMM were used to test whether line or testis side affected proportions of live or normal sperm. Live and Dead, or Normal and Abnormal were fitted as binary dependant variables. Line, testis side and the interaction between line and side were included as fixed effects, and male ID was fitted as a random effect. GLMM were also used to test associations between proportions of live or normal sperm with sperm morphometry, sperm concentration or amounts and proportions of sperm producing tissue. Live and Dead or Normal and Abnormal were fitted as binary dependant variables, and either testis morphological parameter (seminiferous area / proportion / density), sperm concentration, or sperm cell component length were included as predictors, and male ID was fitted as a random effect. Seminiferous area and sperm concentration were standardised around 1 using the ‘scale’ function in R, and total sperm length and midpiece length where log transformed to solve convergence errors. For GLMM, data was modelled with a binomial error distribution and a logit link function. Overdispersion was accounted for by fitting male ID as a random effect to all GLMMs.

Significance of fixed effects was determined by comparing models with and without the fixed effect of interest using the anova function. Non-significant

effects were removed from the model in order of lowest significance, significant fixed effects ($p < 0.05$) remained in the model. For models with significant interactive effects, post-hoc pairwise comparisons between parameter estimates were performed using the 'glht' function from the multcomp package (Hothorn et al 2008); using Tukey Tests.

Results

Effects of selection line and testis side on testis and sperm parameters

Testis morphology

There was a trend for an interaction effect between line and testis side on the area of seminiferous tissue within tubules ($\chi^2 = 3.60$, $p = 0.058$) (Table S3). Post-hoc pairwise comparisons revealed differences in seminiferous area only in the high line, with larger areas in the right testis compared to the left ($z = 3.7$, $p < 0.001$). This effect was not present in low line individuals ($z = 1.02$, $p = 0.706$, Fig 4.a). No other post-hoc comparison was significant (Table S5). The seminiferous density was significantly affected by the interaction between line and side ($\chi^2 = 4.56$, $p = 0.033$, Fig 4.b) (Table S3). Post-hoc pairwise comparisons showed differences in high line males, with larger proportions in the right testis compared to the left testis ($z = 2.93$, $p = 0.014$). There was no difference in the testicular seminiferous density between testis of low line individuals ($z = -0.1$, $p = 1$), and no other post-hoc comparisons were significant (Table S5). There was no difference in the proportion of seminiferous tissue within tubules between the lines ($\chi^2 = 0.04$, $p = 0.845$), or testis sides ($\chi^2 = 0.06$, $p = 0.813$), or from the interaction between line and testis side ($\chi^2 = 0.04$, $p = 0.844$) (see Table S3).

Sperm viability

The proportion of live sperm was significantly affected by the interaction between line and side ($\chi^2 = 15.07$, $p < 0.001$) (Table S4). Post-hoc pairwise comparisons showed that in high line males, sperm in the left testis had lower probability to be alive than sperm in the right testis (34% vs 41%, $z = 3.80$, $p < 0.001$, Fig 4.c). This comparison was not significant in low line males ($z = -1.69$, $p = 0.282$). All other post-hoc comparisons were not significant (Table S5). There was no difference in the proportion of sperm with normal morphology

between the lines (χ^2 = 1.12, p = 0.290), testis sides (χ^2 = 1.34, p = 0.247), or from the interactions between line and side (χ^2 = 1.24, p = 0.265) (Table S5).

Sperm concentration

There was no difference in sperm concentration between the lines (χ^2 = 0.04, p = 0.843), testis sides (χ^2 = 1.47, p = 0.225), or from the interaction between line and testis side (χ^2 = 0.39, p = 0.534) (Table S4). There was no difference in the concentration of live sperm between the lines (χ^2 = 0.00, p = 0.996), testis sides (χ^2 = 1.21, p = 0.272), or from the interaction between line and testis side (χ^2 = 0.24, p = 0.624) (Table S4). There was no difference in the concentration of normal sperm between the lines (χ^2 = 1.72, p = 0.189), testis sides (χ^2 = 0.28, p = 0.598), or from the interactions between line and testis side (χ^2 = 0.57, p = 0.451) (Table S4).

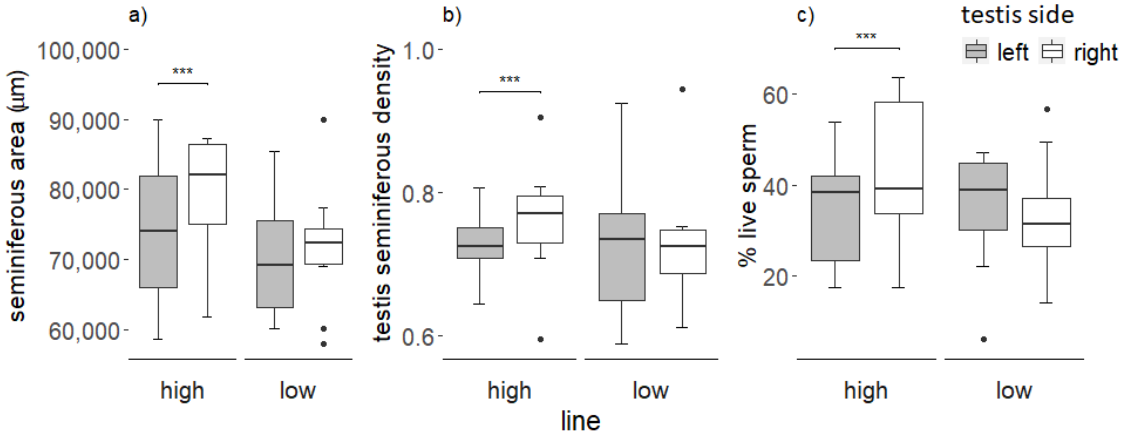

Fig 4. **a)** The right testes of high line males had a higher seminiferous area within the tubule, there was no difference in seminiferous area between testes of low line males. **b)** The right testes of high line males had a higher proportion of testes seminiferous tissue, there was no difference in the testis seminiferous density between testis sides in low line males. **c)** The right testes of high line males has a higher proportion of live sperm, there was no difference in the amount of live sperm between testes of low line males. The midlines of the boxes represent median values, the lower and upper edges represent the first and third quartiles respectively, and the whiskers represent the minimum and maximum values. Outliers are represented as black dots. Statistically significant differences between sides are shown with an asterisk; *** = p<0.001. Data comprise 40 testicles from 20 males.

Sperm length

There was no difference in any measure of sperm morphometry (head, midpiece, tail, total, flagellum, or head : tail ratios) between the lines (all p-values > 0.311) or from the interaction between line and side (all *p*-values > 0.175) (Table S6). However, the right testis had larger tails (χ^2 = 5.97, *p* = 0.015), total lengths (χ^2 = 4.91, *p* = 0.027), flagella (χ^2 = 4.63, *p* = 0.031), as well as smaller ratios of the head : tail (χ^2 = 4.28 *p* = 0.039) (Table S6, Fig 5). There was no effect of testis side on lengths of the sperm head (χ^2 = 0.62, *p* = 0.432) or midpiece (χ^2 = 1.38, *p* = 0.220) (Table S6).

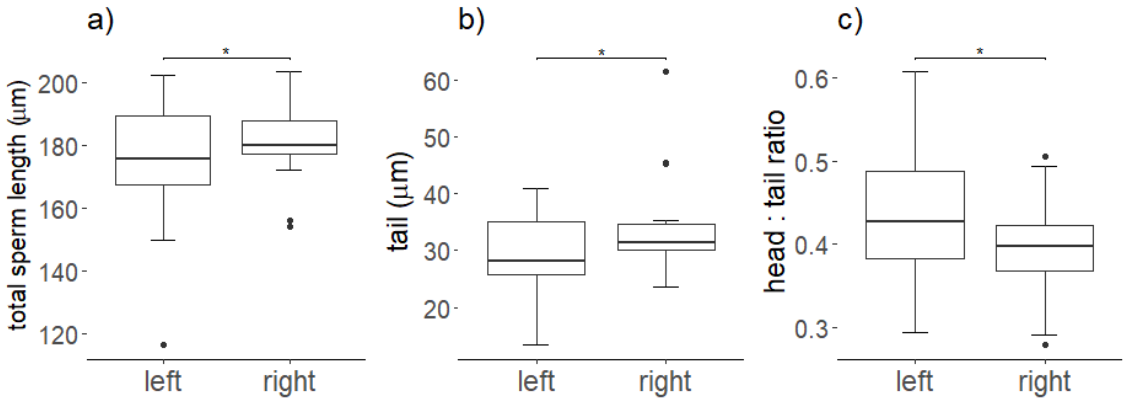

Fig 5. Across all males the right testis produced sperm with **a)** longer total lengths, **b)** longer tail lengths, and **c)** smaller ratios of the head : tail. * = *p* < 0.05.

Relationships between testis and sperm parameters

There was no relationship between testis mass and the area of seminiferous tissue in tubules (*t* = 1.15, df = 29.67, *p* = 0.258) or on the seminiferous density throughout the testis (*t* = -*1*.56, df = 35.73, *p* = 0.127) (Table S7). However, there was a significant negative association between the seminiferous proportion of tubules and testis mass (b = -0.06 ± 0.12, *t* = -4.48, df = 37.82, *p* < 0.001). Larger testis had lower proportions of sperm producing tissue in their tubules (Table S7). There was no effect of testis mass on sperm concentration (t = -0.13, df = 29.57, *p* = 0.896) (Table S7).

There was no relationship between the proportion of live sperm and the tubule seminiferous area (χ^2 = 1.34, *p* = 0.246, Fig 6.a), or the testis seminiferous density (χ^2 = 0.01, *p* = 0.926, Fig 6.c) (Table S8). However, there was a significant positive association between the tubule seminiferous proportion and

the proportions of live sperm (b = 3.39 ± 1.46, χ^2 = 5.09, p = 0.024, Fig 6.b) (Table S8).

There was a significant negative relationship between the proportion of sperm with normal morphology and the seminiferous density throughout the testis (b = 3.54 ± 1.03, χ^2 = 12.38, p < 0.001, Fig 6.f), as well as the seminiferous area of tubules (b = -0.15 ± 0.07, χ^2 = 5.08, p = 0.024, Fig 6.d) (Table S8). Conversely, there was a significant positive association between the proportion of normal sperm and the seminiferous proportion of tubules (b = 3.88 ± 1.63, χ^2 = 5.58, p = 0.018, Fig 6.e) (Table S8).

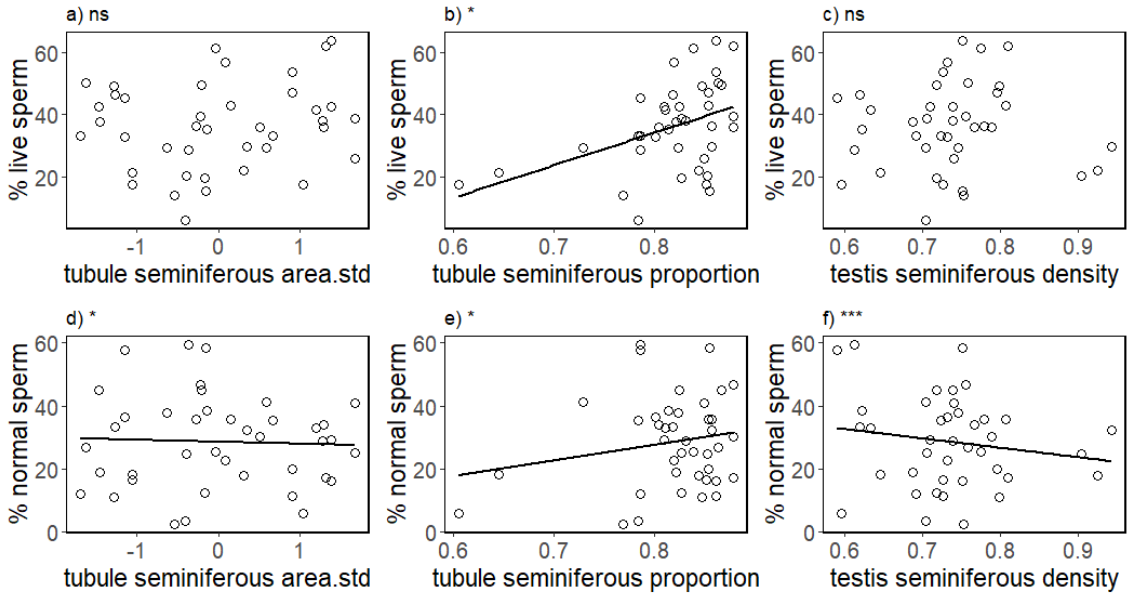

Fig 6. The proportion of live sperm was not affected by the **a)** tubule seminiferous area, or the **c)** testis seminiferous density but increased with the **b)** tubule seminiferous proportion. The proportion of normal sperm increased with **e)** tubule seminiferous proportion but decreases with the **d)** seminiferous area and **f)** testis seminiferous density. Fitted lines show linear regression. Data comprise 5132 sperm for Live/Dead and 5285 sperm for Normal/Abnormal from 20 males. *** = p<0.001, * = p < 0.05, ns = not significant.

Relationships between sperm parameters

There was a positive trend between proportions of live sperm and total sperm length (b = 1.41 ± 0.75, χ^2 = 3.46, p = 0.063, Fig 7.a) (Table S9), and a significant positive regression between proportions of live sperm and midpiece length (b = 1.88 ± 0.55, χ^2 = 10.31, p = 0.001, Fig 7.b) (Table S9). Conversely,

there was a significant negative regression between tail length and proportions of live sperm (b = -0.02 ± 0.01, χ^2 = 7.08, p = 0.008) (Table S9). There was no significant relationship between total sperm length and the amount of sperm produced (χ^2 = 0.10, p = 0.749) (Table S9). The proportion of live sperm was negatively linked to the amount of sperm produced (b = -0.15 ± 0.06, χ^2 = 7.23, p = 0.007, Fig 7.d) (Table S8).

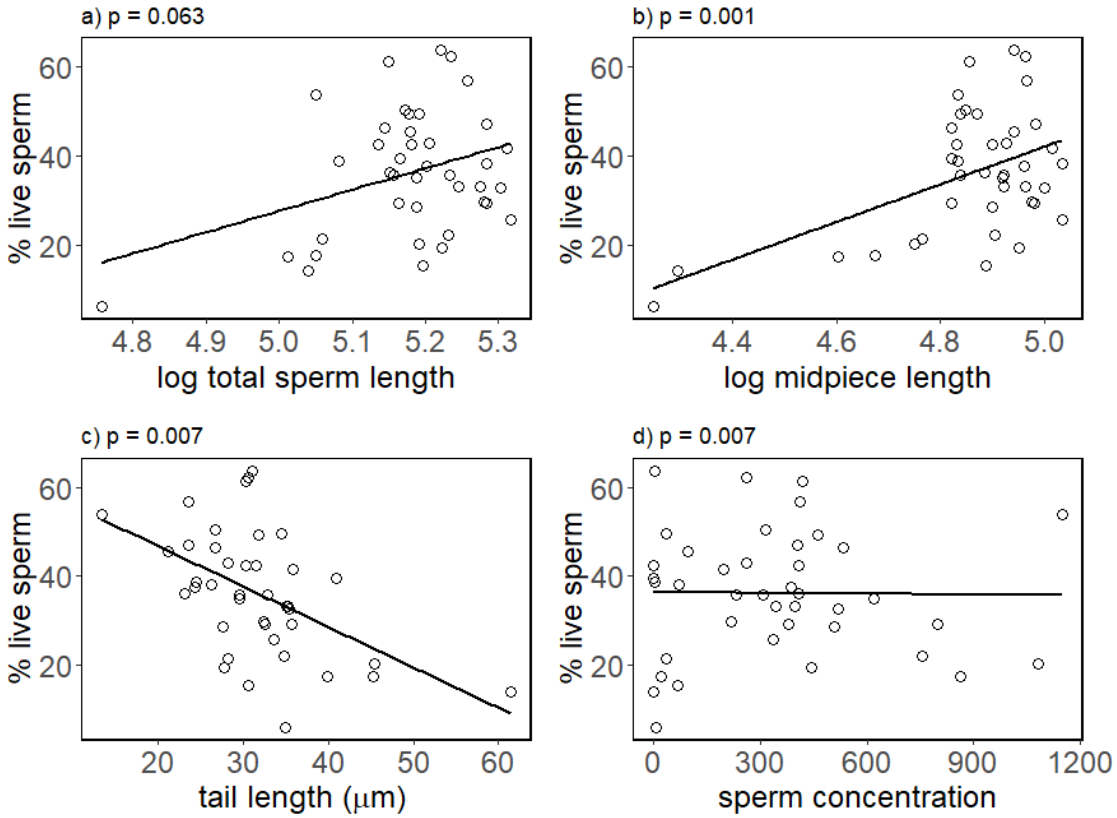

Fig 7. The proportion of live sperm was positively associated with **a)** total sperm length (trend) and significantly positively associated with **b)** midpiece length. Conversely, proportions of live sperm were significantly negatively related to **c)** tail length and **d)** sperm concentration.

Discussion

Here we found that selection for increased female reproductive investment impacted testes composition and sperm characteristics. Among high line males, the right testis had a larger area and density of seminiferous tissue and produced more alive sperm than the left one, meanwhile males from the low line did not show this difference between left and right testis. Independently of the selection lines, we found a significant difference in the length of sperm between

testis sides, with the right testis producing sperm with longer total lengths, and shorter ratios of the head : tail than the left testis. Sperm concentration was not linked to the area, proportion, or density of seminiferous tissue, nor was it linked to sperm length. However, sperm concentration was negatively linked to the proportion of alive sperm. Here, we discuss possible regulating mechanisms controlling sperm quality and quantity in the testes, the potential role of seminal fluids and female regulation on sperm functions, as well as the extent to which genes and nutrition may influence the development of male reproductive morphology.

Previously, it was shown that males from lines selected for high maternal reproductive investment experienced greater fertilisation success, linked to a relatively larger left testis (Pick et al 2017). We found that testes composition also differed between the left and right testis of high line males. However, the larger size of the left testis appeared to be attributed to increased amounts of interstitial tissue between seminiferous tubules, rather than a larger amount of sperm producing tissue within them. This was surprising because higher measures of interstitial tissue are typically associated with relatively smaller testes sizes (Lüpold et al 2009; Rowe & Pruett-Jones 2011), and periods of sexual inactivity (Shil et al 2015). We found no difference in the proportion of sperm producing tissue within the seminiferous tubules between the lines, indicating that selection for high female reproductive investment increased the proportions of interstitial tissue in relatively larger testes, but had no effect on the proportion of spermatogenic tissue within the tubules.

Interstitial tissue may affect sperm quality through the secretions of androgens that are vital for sperm development (Smith & Walker 2014; Heinrich & DeFalco 2020). For example, testosterone produced by Leydig cells can reduce the oxygen uptake of sperm (Sexton 1974), which in turn may improve sperm longevity. Testosterone diffusion from the interstium into seminiferous tubules would be expected to be more diluted in larger interstitial spaces, therefore could be higher in the densely packed seminiferous tissue of the smaller right testis of high line males. Likewise, a lack of androgen signalling by Sertoli cells can result in oxidative stress-induced DNA damage (Stanton et al 2012), which may compromise the survival of developing sperm (Moskovtsev et al 2007), possibly explaining the reduced longevity of sperm in the left testis of high line

males. Counterintuitive to our predictions, the relatively smaller right testis of high line males had higher areas of seminiferous tissue and testicular seminiferous densities, as well as higher proportions of alive sperm. We also found a negative association between testis mass and the proportion of seminiferous tissue within tubules, and that the proportion of seminiferous tissue within tubules was positively associated with the proportions of alive sperm, as well as morphologically normal sperm. Overall, this indicates that there may be a benefit to sperm quality associated with higher proportions of within-tubule seminiferous tissue, possibly regulated by somatic cells in the testes.

A similar relationship between increased testes size and the proportion of interstitial tissue has been reported in Capybaras, where the larger testes of adult males have approximately 50% more interstitial tissue than juveniles (Moreira et al 1997), as well as closely packed Leydig cells (Fawcett et al 1973). Furthermore, there is a positive relationship between size of the testosterone-dependant snout scent gland that is used in the establishment of social hierarchies, with the proportion of interstitial tissue in the testes (López et al 2008). It therefore appears that testosterone is driving testes size in this species, which invest into a reproductive strategy centred around dominance of social status, rather than sperm competition. If the smaller right testis of high line males in this study does experience higher androgen production, this could stem from either efficient androgen diffusion into dense seminiferous tissue, or from correlated increases in Leydig cell numbers in relatively larger testis. However, because the larger left testis of males in this study also produced lower proportions of alive sperm in high line males, as well as smaller sperm across all males, this suggests that there may be a stronger effect of androgen diffusion in the smaller right testis responsible for the beneficial adaptations to sperm quality, however this is highly speculative. To decipher whether increased testosterone originates from increased Leydig cell counts, or efficient diffusion into dense seminiferous tissue, future studies should determine the amount and efficiencies of Leydig and Sertoli cells in asymmetric testes and see if they are linked to testosterone levels, and whether such variation influences sperm quality or fertilisation success.

High line male fertilisation success could be due asymmetric testes specializing in providing different benefits to sperm (Abdul-rahman et al 2018), which act in conjunction to improve male fertilisation success. For example, high line males experienced asymmetry in the amount and density of seminiferous tissue, and the proportion of alive sperm between the left and right testis. It could be that selection for increased female reproductive investment in Japanese quails promotes sex hormone production, which in males is differentially expressed between their asymmetric testes. The right testis may specialize to produce a high amount of alive sperm, whereas the left testis may specialize in producing non-spermatic features originating from increased interstitial tissue. For example, interstitial cells can contain higher levels of certain antioxidant enzymes than germ cells (Bauché et al 1994), so the interstitium of high line males might be better protected against oxidative stress. Whereas, such hypothetical testes specialization would be absent among low line males, as both testis would generate sperm in similar ways. Moreover, the effects of testes asymmetry can extend to other aspects of male reproductive morphology, for example it has been shown to be positively related to seminal glomerus size (Calhim et al 2019). So, it could be that the fertilisation success associated with testes asymmetry is due to a correlated response to other aspects of male reproductive morphology. For example, the foam producing proctodeal gland of Japanese quail is androgen dependant (Sachs 1969), linked to testes mass (Siopes & Wilson 1975), and has significant effects on sperm function. The copulatory foam produced in this gland can improve sperm motility (Farooq et al 2015), sperm transport through the FRT (Singh et al 2012; Sasanami et al 2015) and allow sperm access to storage tubules in the female (Sasanami et al 2015). Indeed, high line males produced higher amounts of this foam than low line males (Tschirren et al unpublished data), which could explain their fertilisation success (Farooq et al 2015) and may be linked to the efficiency of androgen-producing somatic cells in the testes (Biswas et al 2007).

Theory predicts that sperm competition selects for sperm quantity over sperm length in species with long reproductive tracts such as in birds and mammals, when compared to those with short reproductive tracts such as in insects, to compensate for sperm diluting in the larger environment (Immler et al 2011). A positive relationship between seminiferous tissue and relative testes size has

been associated with increased sperm production across species (Møller 1988; Hosken & Ward 2001; Lüpold et al 2009; delBarco-Trillo et al 2013). However, we found no difference between the divergent lines in the sperm concentration, and no measure of sperm producing tissue explained variation in sperm concentrations. This suggests that any sperm traits conferring fertilisation success of high line males are likely qualitative, and highlights that the regulation of sperm quantity is complex and may expand beyond morphological measures of seminiferous tissue. However, the ways in which high line males may improve their reproductive success are not limited to the qualitative and quantitative aspects of sperm we measured here. For example, reproductive success could be influenced by other sperm-related features such as antioxidant provisioning (Ahmadi et al 2016; More et al 2017), DNA damage (Venkatesh et al 2011; Ahmadi et al 2016) and motility (Birkhead et al 1999; Mora et al 2017).

Converse to our predictions, we found that high line males did not produce sperm with different lengths, including lengths of any sperm cell component or ratios between the head : tail. Additionally, high line males had no difference in the proportion of sperm that were morphologically normal, which both indicate that these qualitative traits were not significantly contributing to their improved reproductive success. Independently of the selection lines however, the right testis across all males appeared to be specialised in producing longer sperm, and therefore potentially faster and more competitive sperm (Humphries et al 2008). Besides, theory predicts that sperm competition should reduce variation in sperm lengths by shifting the sperm phenotype towards its fitness optima (Calhim et al 2007), and we found that the right testis produced sperm with less variable lengths (see Fig 5), suggesting that this testis may produce sperm closer to an optimum phenotype. Although we did not find an effect of maternal lineage or the interaction of testis side and line on sperm lengths, the variation in sperm length between testis sides supports the idea of a specialisation to produce different sperm traits between the left and right testis.

We found no trade-off between sperm length and the amount produced, but there was a negative relationship between proportions of live sperm and the total amount produced. This suggests that trade-offs regulating sperm quantity might not be limited to just sperm lengths (Immler et al 2011) but could extend

to other qualitative aspects of sperm such as longevity, indicating that even in species with long reproductive tracts, sperm competition can select for sperm quality over quantity. However, the trade-off between sperm length and longevity was the opposite of our predictions. The proportion of live sperm trended to correlate positively with total sperm length and was significantly positively related to midpiece length. Additionally, we also found that tail length was significantly negatively related to proportions of live sperm. This suggests that the reduced lifespan arising from an increased metabolism of larger sperm found in other species (Stockley et al 1997; Helfenstein et al 2010) may not apply to quails and could depend on the sperm region: the midpiece would provide energy for increasing longevity, meanwhile the tail would use this energy for increasing motility. Indeed, across pheasant species (*Phasianidae, Galliformes*), longer sperm have been found to have increased longevity, and appear to display a negative relationship between midpiece size and sperm storage duration (Immler et al 2007). However, a latter study failed to find such a relationship between sperm size and longevity, but rather found a positive relationship between the number of sperm produced and the proportion of sperm both alive and normal (Liao et al 2019). Furthermore, the amount of sperm produced varied positively with the duration of female sperm storage, suggesting that the trade-off between sperm length and longevity might be itself regulated by sperm storage patterns, selecting for enough sperm to overcome the amount lost during storage (Liao et al 2019). Further investigation would be required to determine whether sperm morphology or number are influenced by female sperm storage in Japanese Quail, but the interplay between sperm morphology and female storage could explain the proportions of live sperm and reproductive success of high line males.

On the other hand, the increased fertilisation success of high line males could be an indirect result of low line males having reduced fertilisation success. Ejaculate composition can be influenced by nutritional condition (Perry & Rowe 2010), and sperm traits such as viability and motility can be improved by nutritional supplementation (Safari Asl et al 2018; Fouad 2020). Low line males developed in smaller eggs, and likely experienced poorer nutritional conditions than high line males (Finkler 1998). This could have reduced the ability of low line males to alleviate the morphological consequences of a stressful

developmental environment on testes morphology (Türk et al 2016) and constrained the investment of resources necessary for generating improved ejaculates (Rowe & Houle 1996; Poiani 2006; Simmons 2012). Such constraints could reduce the capability of sperm to successfully fertilise the ova, for example by producing sperm with more DNA damage (Venkatesh et al 2011, Wright et al 2014), or producing copulatory fluids that are less beneficial to fertilisation success (Macartney et al 2019).

Due to energetic costs of producing and maintaining large testes (Deviche et al 2011), the poorer early environment of males from the low line, when compared to the high line, could explain the lack of testes asymmetry among low line males, despite its advantage in terms of fertility. However, it should be also mentioned that offspring can inherit testes traits (Hosken & Ward 2001; Simmons & García-González 2008) and sperm traits (Birkhead et al 2005; Mossman et al 2009). So, beyond the increased prenatal resource provisioning or in addition to that, the asymmetry on the size and functioning of high line males' testes could have a genetic origin. This would imply that the genes determining female egg size share a concordant intersexual relationship with genes determining those male traits (Pick et al 2017). To disentangle this, future studies should incorporate a hybrid-breeding design comparing pure-bred and hybrid offspring, which should help disentangle the effect of resource provisioning and genetic quality on the expression of reproductive characteristics.

In conclusion, we found that the testes asymmetry of males arising from lines of high maternal reproductive investment was associated with variation in seminiferous content and sperm quality, however the relationships between these traits were the opposite of our predictions. Previous work has demonstrated positive associations between relative testes size with proportions of seminiferous tissue and sperm quality and quantity, however we found that relatively larger testis were linked to a reduction in the proportion of seminiferous tissue, as well as the proportion of alive sperm, and had no association with the quantity of sperm produced. We also found that the left and right testis produced sperm of different lengths across all males. These findings support the idea of a functional specialization between the left and right testis. The unexpected relationships we found between testes size, seminiferous

content and sperm quality could be because changes in relative testis sizes, and the subsequent sperm production formed as a correlated response to artificial selection on female reproductive investment, rather than from naturally occurring male competition to produce more competitive sperm. Future studies would benefit from investigating whether testes asymmetry and proportions of seminiferous tissue are associated with levels of oxidative stress, or other aspects of male reproductive morphology such as somatic cells in each testis, or the foam of the proctodeal gland or ejaculate composition, and to assess whether these traits affect sperm development and post-copulatory performance.

Appendix

Table S1. Repeatability (*r*), standard error (*SE*), confidence intervals (CI) and *p*-values of sperm and testis traits per male, *p*-values derived from likelihood ratio tests.

Trait	*r*	*SE*	CI	*p*
Tubule Area	0.472	0.089	0.283, 0.628	<0.001
Seminiferous Area	0.392	0.085	0.202, 0.537	<0.001
Seminiferous Proportion	0.73	0.073	0.558, 0.832	<0.001
Lumen Area	0.726	0.073	0.543, 0.826	<0.001
Testis Seminiferous Density	0.609	0.095	0.376, 0.746	<0.001
Head Length	0.285	0.073	0.143, 0.417	<0.001
Midpiece Length	0.398	0.084	0.222, 0.552	<0.001
Tail Length	0.09	0.037	0.02, 0.169	<0.001
Acrosome Length	0.061	0.033	0.003, 0.13	0.003
Total Sperm Length	0.313	0.078	0.159, 0.453	<0.001
Flagella Length	0.298	0.076	0.144, 0.433	<0.001
Head : Tail ratio	0.067	0.032	0.013, 0.131	<0.001

Table S2. Means (*M*), standard deviations (*SD)* and Pearson correlations of key reproductive traits with confidence intervals. Values in square brackets indicate the 95% confidence interval for each correlation. * $p < .05$, ** $p < .01$.

Variable	*M*	*SD*	1	2	3	4	5	6	7	8	9	10	11	12
1. Tubule Area (µm)	90884	13755												
2. Seminiferous Area (µm)	74054	9503	.83** [.69, .90]											
3. Tubule Seminiferous Proportion	0.82	0.06	-.50** [-.70, -.22]	.07 [-.25, .37]										
4. Testis Seminiferous Density	0.74	0.08	-.18 [-.46, .14]	.14 [-.18, .43]	.56** [.30, .74]									
5. Live Sperm (count)	46.00	17.02	-.13 [-.43, .19]	.15 [-.17, .44]	.46** [.17, .68]	.12 [-.20, .41]								
6. Dead Sperm (count)	82.30	28.42	-.03 [-.34, .28]	-.07 [-.37, .25]	-.08 [-.38, .24]	.14 [-.18, .43]	-.29 [-.55, .02]							
7. Normal Sperm (count)	36.90	17.90	-.14 [-.44, .18]	-.04 [-.34, .28]	.17 [-.14, .46]	-.11 [-.41, .21]	.12 [-.20, .42]	.31* [.00, .57]						
8. Abnormal Sperm (count)	95.22	34.96	.01 [-.30, .32]	-.10 [-.40, .22]	-.15 [-.44, .17]	.08 [-.24, .38]	.18 [-.14, .46]	.46** [.17, .67]	-.40* [-.63, -.10]					
9. Midpiece (µm)	130.56	18.03	.02 [-.30, .33]	.24 [-.08, .51]	.31 [-.00, .57]	.08 [-.24, .38]	.64** [.41, .79]	.14 [-.18, .43]	.51** [.24, .71]	.07 [-.25, .37]				
10. Tail (µm)	31.64	7.89	.12 [-.20, .41]	-.06 [-.37, .26]	-.27 [-.54, .04]	.14 [-.18, .43]	-.50** [-.70, -.22]	.28 [-.04, .54]	-.19 [-.47, .13]	.05 [-.27, .36]	-.53** [-.72, -.26]			
11. Total Sperm Length (µm)	178.41	17.01	-.01 [-.32, .30]	.20 [-.12, .48]	.30 [-.01, .56]	.23 [-.08, .51]	.55** [.28, .73]	.30 [-.01, .56]	.50** [.22, .70]	.12 [-.20, .41]	.90** [.82, .95]	-.15 [-.44, .16]		
12. Testis_Mass (g)	2.84	0.56	.63** [.40, .79]	.33* [.02, .58]	-.63** [-.79, -.40]	-.32* [-.58, -.01]	-.02 [-.33, .30]	.13 [-.19, .43]	.09 [-.23, .39]	.14 [-.17, .44]	.26 [-.05, .53]	-.17 [-.46, .15]	.15 [-.17, .44]	
13. SQF	219.34	175.60	-.32* [-.57, -.01]	-.21 [-.49, .10]	.23 [-.08, .51]	.10 [-.22, .40]	.31 [-.00, .57]	.06 [-.26, .36]	.18 [-.14, .46]	.13 [-.19, .42]	.20 [-.11, .48]	-.31 [-.56, .01]	.16 [-.16, .45]	-.10 [-.40, .22]

Sperm Quality Factor (SQF) = ((proportion of live sperm + proportion of normal sperm) * sperm concentration)

Table S3. Effects of selection line and testis side on **a)** seminiferous area, **b)** tubule seminiferous proportion and **c)** total seminiferous density. Factor level of comparison in brackets.

Response Variable	*Estimate*	*SE*	χ^2	*P*
a) Seminiferous Area (µm)				
(Intercept)	74495	2684	-	-
Side (Right)	5132	1387	10.92	**<0.001**
Line (Low)	-4152	3795	2.79	0.095
Line : Side (Low, Right)	-3722	1961	3.60	0.058
b) Tubule Seminiferous Proportion				
(Intercept)	0.82	0.02	-	-
Side (Right)	0.00	0.00	0.06	0.813
Line (Low)	0.00	0.03	0.04	0.845
Line : Side (Low, Right)	0.00	0.01	0.04	0.844
c) Testis Seminiferous Density				
(Intercept)	0.73	0.02	-	-
Side (Right)	0.03	0.01	-	-
Line (Low)	0.00	0.03	-	-
Line : Side (Low, Right)	-0.04	0.02	4.56	**0.033**

Table S4. Effects of selection line and testis side on **a)** proportions of live sperm, **b)** proportions of normal sperm, **c)** sperm concentrations, **d)** concentrations of live sperm and **e)** concentrations of normal sperm. Factor level of comparison in brackets.

Response Variable	*Estimate*	*SE*	χ^2	*P*
a) Live / Dead				
(Intercept)	-0.68	0.18	-	-
Line (Low)	0.03	0.26	-	-
Side (Right)	0.32	0.09	-	-
Line : Side (Low, Right)	-0.47	0.12	15.07	**<0.001**
b) Normal / Abnormal				
(Intercept)	-1.11	0.22	-	-
Line (Low)	0.26	0.32	1.12	0.290
Side (Right)	-0.15	0.09	1.34	0.247
Line : Side (Low, Right)	0.14	0.13	1.24	0.265
c) Sperm Concentration				
(Intercept)	352.35	95.05	-	-
Line (Low)	60.60	134.42	0.04	0.843
Side (Right)	-38.20	91.45	1.47	0.225
Line : Side (Low, Right)	-76.75	129.33	0.39	0.534
d) Live Sperm Concentration				
(Intercept)	17550.90	4941.78		
Line (Low)	1640.60	6988.73	0.00	0.996
Side (Right)	-2059.60	4871.39	1.21	0.272
Line : Side (Low, Right)	-3217.60	6889.18	0.24	0.624
e) Normal Sperm Concentration				
(Intercept)	10068.50	3609.99		
Line (Low)	7311.05	5105.29	1.72	0.189
Side (Right)	711.95	4752.13	0.28	0.598
Line : Side (Low, Right)	-4841.65	6720.53	0.57	0.451

Table S5. Post-Hoc pairwise comparisons between line and testis side on **a)** tubule seminiferous area and **b)** testis seminiferous density and **c)** proportion of live sperm. Factor level of comparison in brackets.

Response Variable	Estimate	*SE*	*z*	*P*
a) Seminiferous Area (µm)				
High (Right) - High (Left)	5132	1387	3.70	**<0.001**
Low (Left) - High (Left)	-4152	3796	-1.09	0.655
Low (Right) - High (Left)	-2743	3796	-0.72	0.870
Low (Left) - High (Right)	-9284	3796	-2.45	0.055
Low (Right) - High (Right)	-7875	3796	-2.08	0.134
Low (Right) - Low (Left)	1409	1387	1.02	0.706
b) Testis seminiferous density				
High (Right) - High (Left)	0.03	0.01	2.93	**0.014**
Low (Left) - High (Left)	0.00	0.03	-0.06	1.000
Low (Right) - High (Left)	0.00	0.03	-0.09	1.000
Low (Left) - High (Right)	-0.04	0.03	-1.04	0.691
Low (Right) - High (Right)	-0.04	0.03	-1.07	0.670
Low (Right) - Low (Left)	0.00	0.01	-0.10	1.000
c) Live / Dead				
High (Right) - High (Left)	0.32	0.09	3.80	**<0.001**
Low (Right) - Low (Left)	-0.14	0.09	-1.69	0.282
Low (Right) - High (Left)	-0.12	0.26	-0.45	0.964
High (Right) - Low (Left)	0.29	0.26	1.14	0.619
Low (Left) - High (Left)	0.03	0.26	0.12	0.999
Low (Right) - High (Right)	-0.44	0.26	-1.71	0.274

Table S6. Effects of selection line and testis side on the lengths of sperm cell components. Factor level of comparison in brackets.

Response Variable	*Estimate*	*SE*	χ^2	*P*
Head (µm)				
(Intercept)	17.53	0.28	-	-
Line (Low)	-0.40	0.39	0.56	0.454
Side (Right)	-0.05	0.13	0.62	0.432
Line : Side (Low, Right)	0.25	0.19	1.84	0.175
Midpiece (µm)				
(Intercept)	127.65	5.74	-	-
Line (Low)	4.10	8.13	0.23	0.629
Side (Right)	2.24	2.16	1.38	0.240
Line : Side (Low, Right)	-0.89	3.07	0.08	0.773
Tail (µm)				
(Intercept)	29.80	2.15	-	-
Line (Low)	0.18	3.05	0.06	0.806
Side (Right)	4.29	2.01	5.97	**0.015**
Line : Side (Low, Right)	-1.60	2.79	0.32	0.573
Total Length (µm)				
(Intercept)	172.51	5.17	-	-
Line (Low)	7.51	7.32	0.90	0.344
Side (Right)	5.39	2.78	4.91	**0.027**
Line : Side (Low, Right)	-2.20	3.86	0.32	0.569
Flagellum (µm)				
(Intercept)	154.92	5.04	-	-
Line (Low)	7.97	7.14	1.03	0.311
Side (Right)	5.49	2.80	4.63	**0.031**
Line : Side (Low, Right)	-2.58	3.88	0.44	0.507
Head : Tail (µm)				
(Intercept)	0.43	0.02	-	-
Line (Low)	0.00	0.03	0.33	0.568
Side (Right)	-0.04	0.02	4.28	**0.039**
Line : Side (Low, Right)	0.02	0.03	0.71	0.400

Table S7. Effects of testis mass on **a)** seminiferous tubule area, **b)** seminiferous area of tubules **c)** seminiferous proportion of tubules, **d**) seminiferous density of the testis and **e)** the sperm concentration.

Response Variable	*Estimate*	*SE*	*t*	*df*	*P*
Tubule Area (µm)					
(Intercept)	50796.1	9776.13	-	-	-
Testis Mass	14092.06	3367.93	4.18	24.18	**<0.001**
Seminiferous Area (µm)					
(Intercept)	64434.15	8534.61	-	-	-
Testis Mass	3381.85	2932.12	1.15	29.67	0.258
Tubule Seminiferous Proportion					
(Intercept)	0.98	0.04	-	-	-
Testis Mass	-0.06	0.01	-4.48	37.82	**<0.001**
Testis seminiferous density					
(Intercept)	0.84	0.07	-	-	-
Testis Mass	-0.04	0.02	-1.56	35.73	0.127
Sperm Concentration					
(Intercept)	379.61	274.45	-	-	-
Testis Mass	-12.39	94.29	-0.13	29.57	0.896

Table S8. Effects of seminiferous area, tubule seminiferous proportion, total seminiferous density, and average sperm concentration on **a)** likelihood of sperm being live. The effect of seminiferous area, tubule seminiferous proportion, and total seminiferous density on **b)** likelihood of sperm being normal.

Response Variable	*Estimate*	*SE*	χ^2	*P*
a) Live / Dead				
(Intercept)	-0.62	0.13	-	-
Seminiferous Area.std	0.07	0.06	1.34	0.246
(Intercept)	-3.4	1.2	-	-
Tubule Seminiferous Proportion	3.39	1.46	5.09	**0.024**
(Intercept)	-0.57	0.66	-	-
Testis Seminiferous Density	-0.08	0.88	0.01	0.926
(Intercept)	-0.62	0.13	-	-
Sperm Concentration.std	-0.15	0.06	7.23	**0.007**
b) Normal / Abnormal				
(Intercept)	-1.02	0.17	-	-
Seminiferous Area.std	-0.15	0.07	5.08	**0.024**
(Intercept)	-4.2	1.35	-	-
Tubule Seminiferous Proportion	3.88	1.63	5.58	**0.018**
(Intercept)	-3.63	0.78	-	-
Testis Seminiferous Density	3.54	1.03	12.38	**< 0.001**

Table S9. The effects of sperm length on **a)** sperm concentration, and **b)** the proportion of live sperm produced.

Response Variable	*Estimate*	*SE*	χ^2	*P*
a) Sperm Concentration				
(Intercept)	191.3	528.62	-	-
Total Sperm Length	0.86	2.95	0.1	0.749
b) Live / Dead				
(Intercept)	-7.92	3.91	-	-
Total Sperm Length.log	1.41	0.75	3.46	0.063
(Intercept)	-0.83	1.55	-	-
Head	0.01	0.09	0.02	0.894
(Intercept)	-9.77	2.69	-	-
Midpiece.log	1.88	0.55	10.31	**0.001**
(Intercept)	0.02	0.26	-	-
Tail	-0.02	0.01	7.08	**0.008**

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