Sperm traits of the three genetic morphs in the ruff sandpiper

Martin Bulla[1,2]✉, Clemens Küpper[1], David B Lank[3], Jana Albrechtová[4], Jasmine L Loveland[1,5], Katrin Martin[1], Kim Teltscher[1], Margherita Cragnolini[1], Michael Lierz[6], Tomáš Albrecht[4,7], Wolfgang Forstmeier[1] & Bart Kempenaers[1]✉

[1]Max Planck Institute for Biological Intelligence, Eberhard-Gwinner-Str., 82319 Seewiesen, Germany
[2]Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic
[3]Department of Biological Sciences, Simon Fraser University, Burnaby, BC V5A 1S6, Canada
[4]Institute of Vertebrate Biology, Czech Academy of Sciences, Brno, Czech Republic
[5]Department of Cognitive and Behavioral Biology, University of Vienna, Vienna, Austria
[6]Clinic for Birds, Reptiles, Amphibians and Fish, Justus Liebig University Giessen, Giessen, Germany
[7]Department of Zoology, Faculty of Science, Charles University, Prague, Czech Republic
✉bulla.mar@gmail.com, b.kempenaers@orn.mpg.de

Abstract

The ruff sandpiper *Calidris pugnax* is a Palearctic lekking shorebird with three genetic morphs determined by an autosomal inversion. Male morphs differ strikingly in body size, ornaments, endocrinology and mating behavior. Aggressive Independents represent the ancestral haplotype, semi-cooperative Satellites and female-mimicking Faeders the inverted haplotypes. Since one inversion breakpoint is homozygous lethal, the inverted haplotypes (Satellite and Faeder) cannot recombine and are expected to accumulate mutations. The inversion regions also harbor genes involved in spermatogenesis. However, it is unknown whether the genetic difference between the morphs also translate into differences in sperm traits. Here, we use a captive-bred population of ruffs to compare sperm velocity and morphology among the morphs. Faeder sperm moved the slowest, which is in line with expectations from mutation accumulation and the idea that Faeders might fare worse than Satellites, as Satellite haplotype arose through a rare recombination event that may have re-instated the function of sperm-related genes or helped to purge deleterious mutations. Against the expectation from mutation accumulation, sperm of Independents did not seem to be of highest quality, i.e., they were not the fastest nor the least variable, and they had the shortest tail and midpiece. Although the midpiece contains the energy-producing mitochondria, sperm midpiece length was not associated with sperm swimming speed. Instead, two of three velocity metrics were weakly positively associated with head length (absolute and relative). These results may relate to our observation that although ruff sperm morphologically resemble those of passerines, they generally appear to move differently, vibrating from side to side while slowly moving forward, rather than rotating while moving forwards. To conclude, the three genetically determined morphs showed only minor differences in sperm traits, and these differences were not clearly associated with variation in sperm velocity, indicating a low potential for the evolution of morph-specific sperm adaptations.

Introduction

In many animal species, males use different behavioral strategies to obtain fertilizations (Dougherty et al., 2022; Kustra & Alonzo, 2020; Mank, 2023). For example, dominant males may display, while other males "steal" copulations (Gross, 1996) by pretending to be a female or by positioning themselves in between the dominant male and the female that is about to copulate, a tactic referred to as "sneaking" (Jukema & Piersma, 2006; Shuster & Wade, 1991). Consequently, males using alternative mating tactics often face different levels of sperm competition, i.e., competition between the sperm from different males to fertilize one or more eggs (Parker, 1970). Selection could then favor different sperm traits between males that differ in mating behavior (Dougherty et al., 2022; Kustra & Alonzo, 2020). Despite the general expectation that sneaker males should produce more or more competitive sperm (higher quality or performance), existing theory and data do not make clear predictions about how sperm traits of sneaker males should differ from those of other males (Dougherty et al., 2022; Kustra & Alonzo, 2020). Importantly, if sneaker males are rare in the population, the evolutionary potential for them to evolve superior sperm traits may be limited.

In the ruff *Calidris pugnax,* a lekking shorebird, males occur in three distinct morphs with striking differences in body size, ornaments, endocrinology and reproductive behavior (Hogan-Warburg, 1966; Höglund & Lundberg, 1989; Jukema & Piersma, 2006; Küpper et al., 2016; Loveland et al., 2021a; van Rhijn, 1991; Widemo, 1998). (1) Aggressive Independents make up 80-90% of all males and show a spectacular diversity of predominantly dark ornamental plumages. The locally resident Independents hold small display courts on a lek. Among these, the dominant male obtains the majority of matings (Vervoort & Kempenaers, 2019; Widemo, 1997). (2) Submissive and slightly smaller Satellites make up 10-20% of males, show predominantly white ornamental plumage, and co-display with court-holders on leks (Hogan-Warburg, 1966; Höglund & Lundberg, 1989; van Rhijn, 1991; Widemo, 1998). Although Satellites may "steal" some copulations, Independents may benefit from having Satellites on the lek because their presence helps attract females to the display court of that male (Hugie & Lank, 1997; Tolliver et al., 2023). (3) Faeders are rare (~1%), mimic females in appearance (plumage and smaller body size), and attempt to sneak copulations when a female solicits an ornamented displaying male (Jukema & Piersma, 2006). Despite striking differences in body size, the three morphs have similar testes size (Figure 1; Jukema & Piersma, 2006; Küpper et al., 2016), and hence presumably can produce similar numbers of sperm. However, the morphs differ strikingly in their reproductive endocrinology. During the lekking season, Independent males have high levels of circulating testosterone, whereas Satellites and Faeders have higher levels of androstenedione, a testosterone precursor (Küpper et al., 2016; Loveland et al., 2021a).

The phenotypic differences between the morphs are entirely genetically determined by a 4.5 Mb autosomal inversion region (Küpper et al., 2016; Lamichhaney et al., 2016). Faeders and Satellites carry unique inversion haplotypes. The Faeder haplotype arose first and the Satellite haplotype originated from a rare recombination event between the Independent and Faeder genotypes several hundred thousand generations later (Lamichhaney et al., 2016). Since one inversion breakpoint is homozygous lethal, the inversion haplotypes only occur in hemizygous state (always combined with one Independent haplotype) and they hence cannot recombine and are expected to accumulate mutations. Although the genetic differences between the morphs affect numerous phenotypic traits, it is unknown whether they also affect sperm traits.

Sperm traits and testicular gene expression may differ between morphs from the very moment the morphs originated. (i) Two genes regulating spermatogenesis reside within the autosomal inversion that drives the Satellite and Faeder phenotypes (Küpper et al., 2016). For example, the inversion contains the gene *GAS8*, which has been linked to sperm motility in mice, and the gene *SPATA2L*, a paralog of *SPATA2*, which is involved in spermatogenesis (Graziotto et al., 1999; Küpper et al., 2016; Onisto et al., 2000; Yeh et al., 2002). Interestingly, thus far we know that expression of *SPATA2L* in testes of both Satellites and Faeders is biased toward the inversion allele (Loveland et al., 2021b). (ii) The inversion also influences expression of genes located outside of the inversion (Maney & Küpper, 2022). For example, *STAR,* a gene responsible for providing the cholesterol substrate required for sex hormone synthesis, is located outside of the inversion, and is overexpressed in the testes of Satellites and Faeders (Loveland et al., 2021a). (iii) Some genes or regulatory elements within the inversion region that have strong pleiotropic effects may also affect spermatogenesis.

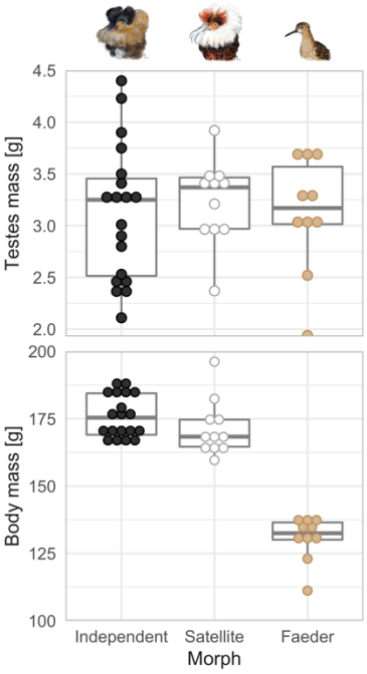


Figure 1 | Testes mass and body mass in relation to morph in the ruff. Each dot represents an individual, dot color highlights the morph (black: Independent, white: Satellite, beige: Faeder). Boxplots depict the median (horizontal line inside the box), the 25th and 75th percentiles (box) and the 25th and 75th percentiles ±1.5 times the interquartile range or the minimum/maximum value, whichever is smaller (bars). Data from (Loveland et al., 2021b) and from an additional 5 males collected under the same protocol in 2022 (3 Independents, 1 Satellite and 1 Faeder). Dots stacked using the 'geom_dotplot' ggplot2 R-function (Wickham, 2016). Ruff morph illustrations by Yifan Pei under Creative Commons Attribution (CC BY 4.0).

Also, sperm trait differences between the morphs could have arisen by *de novo* mutation after the origin of the three morphs, but such evolutionary scenario is far from clear. The entire genetic background outside of the inversion evolved to function well in the context of the most abundant morph, i.e., the Independents (Giraldo-Deck et al., 2022). In contrast, Faeders, and to a lesser extent Satellites, have limited evolutionary potential due to (i) small population size, (ii) a small part (0.3%) of the genome within which a beneficial mutation would have to arise and (iii) only two of the ~125 genes identified within the inversion have known effects on sperm performance or morphology (Küpper et al., 2016). However, because the ruff inversions do not recombine, Faeders and Satellites can accumulate deleterious mutations inside their inversion haplotypes (Berdan et al., 2021). Thus, it can be predicted that the sperm of Faeders and Satellites would be inferior (in quality and performance) to that of Independents. Furthermore, Faeders might fare worse than Satellites, (i) since a larger number of genes have accumulated potentially harmful mutations in the Faeder haplotype than in the Satellite haplotype (Küpper et al., 2016; Lamichhaney et al., 2016) and (ii) if the recombination through which Satellites arose re-instated "good" copies of a gene that is indirectly related to a sperm-related trait.

Here, we use sperm sampled from a captive breeding population of ruffs to address three aims. First, we assess differences in sperm swimming speed and morphology between the three morphs. Comparative studies have shown that species experiencing higher levels of sperm competition typically produce sperm that swim faster, are longer (including longer sperm components), and less variable in length as a result of intense postcopulatory selection on optimal sperm length (Laskemoen et al., 2012; Lifjeld et al., 2010; Lipshutz et al., 2022; Lüpold et al., 2020; Simmons & Fitzpatrick, 2012). Within species, sperm fertilization ability has been linked to sperm viability (Gage et al., 2004; Garcia-Gonzalez & Simmons, 2005), swimming speed (Birkhead et al., 1999; Gage et al., 2004), and length (Bennison et al., 2016; Bennison et al., 2014; Lüpold et al., 2012). In most species, longer sperm is better at fertilization (see references above), but in others, shorter sperm seem to do better (Simmons & Fitzpatrick, 2012). Thus, if the sperm of inversion morphs has been disadvantaged due to mutation accumulation, we expect the sperm of Independents to be (1) faster swimming, (2) longer, and (3) less variable in length. We would also expect the sperm of Independents to have (4) a longer midpiece (energetic component; Cardullo & Baltz, 1991; Knief et al., 2017; Míčková et al., 2023), (5) a longer tail

and flagellum (midpiece + tail, kinetic component; Cardullo & Baltz, 1991), and possibly (6) a longer midpiece and flagellum length relative to total sperm length (Humphries et al., 2008).

Second, as some within-species studies, including those in birds, found correlations between sperm swimming speed and morphology (reviewed in Bennison et al., 2016; Cramer et al., 2021; Fitzpatrick & Lupold, 2014; Kim et al., 2017; Knief et al., 2017; Míčková et al., 2023; Støstad et al., 2018), we test whether variation in ruff sperm morphology predicts variation in sperm swimming speed.

Third, sperm motility has never been described in scolopacids and only rarely in other species of shorebirds, and sperm shape has only been sketchily described in a few species in this group (Retzius, 1909). Furthermore, scolopacid sperm superficially resemble the sperm of passerines, but completely differ from those of all other (so far evaluated) avian lineages (Retzius, 1909). We therefore describe the motility and shape of ruff sperm in comparison with those of other birds.

Methods

We collected and analyzed the data based on *a priori* designed protocols, following the principles outlined in the Helsinki Declaration and national guide for the care and use of laboratory animals.

Captive population

A population of ~300 ruffs is housed at the Max Planck Institute for Biological Intelligence, Seewiesen, Germany (for details see Supplement Methods S1; Bulla et al., 2023). The population was founded in 2018 with 23 ruffs obtained from Dutch breeders, 5 ruffs from German breeders and 194 ruffs from Simon Fraser University, Vancouver, Canada. The latter were individuals from a captive-bred population founded with 110 ruffs hatched from wild eggs collected in Finland in 1985, 1989 and 1990, plus two Faeder males brought from the Netherlands in 2006 (Lank et al., 2013; Lank et al., 1995). Each individual of the population has been genotyped for its morph using a set of six single-nucleotide polymorphism markers located in the inversion region (Giraldo-Deck et al., 2020).

Sperm sampling

In May and June 2021, we collected sperm by abdominal massage (for a detailed protocol of sperm collection – including video - and sample preparation see https://s.gwdg.de/UlejLot) or by electro-stimulation (Lierz et al., 2013). The length (15 mm) and diameter (4 mm) of the electro-stimulation probe, as well as the electric current and the number of electric impulses was adapted to the sampled individuals. The probe was inserted into the urodeum of the cloaca to stimulate the ampullae ductus deferens using three 1 s electric current impulses. The voltage was increased gradually from 0.09 V to a maximum of 1.0 V until contractions of the cloaca and the muscles of the tail were observed; each impulse was followed by a 2-3 s break. In June, we only sampled sperm using abdominal massage. The morphs were sampled in a haphazard order to avoid a potential confounding effect of sampling order. We obtained at least one ejaculate sample from 92 males (59 Independents, 25 Satellites and 8 Faeders).

Ejaculates (~0.5–3µl) were pipetted from the cloaca and immediately diluted and gently mixed in 50µl of preheated (40°C) Dulbecco's Modified Eagle's Medium (Advanced D-MEM, Invitrogen™). To record sperm swimming speed, an aliquot of 2.5µl was then immediately pipetted onto a standard 20µm two-chamber count slide (Leja, The Netherlands) placed on a thermal plate (Tokai Hit, Tokai Hit Co., LtD.) kept at 40 °C. The slide was inspected. When sperm densities were too high, a new aliquot was taken and further diluted. Sperm movements were then recorded. For morphology measurements, an aliquot of 20µl was pipetted into 50µl of a phosphate buffered saline solution (PBS; Sigma P-4417) containing 1% formalin.

Measurement of sperm swimming speed

For each sperm sample we recorded sperm swimming speed at 25 frames per second for approximately 45s in eight different fields of the Leja slide under a 100x magnification, using phase contrast and a digital camera (UI-1540-C, Olympus) mounted on a microscope (CX41, Olympus) fitted with a thermal plate (Tokai Hit, Tokai Hit Co., LtD) kept at a constant temperature of 40°C. A single person (JA) analysed each recorded field using the CEROS computer-assisted sperm analysis system (Hamilton Thorne Inc.), visually inspecting the tracked objects and excluding non-sperm objects and static spermatozoa from the analysis (Cramer et al., 2016; Laskemoen et al., 2010; Opatova et al., 2016), and noted the quality of the recording (e.g., presence of faeces). We recorded a median of 192 sperm cells per sample (mean = 206, range: 5 – 562; N = 134 recordings from a total of 92 males, 46 recorded in May and 88 in June) and measured curvilinear, straight-line, and average-path velocities (for details see Supplement Methods S2 and Figure S1 and S2, for within male repeatability Figure S3 and S4, Table S1).

Measurements of sperm morphology

Within 5 days of sample collection, we pipetted (i) 20µl of the PBS-1% formalin fixed sample into 50µl of 5% formalin for long-term storage and (ii) smeared 10µl onto a microscope slide, and let it dry at room temperature or on a heating block set at 37°C. The next day, each slide was rinsed gently with deionised water to remove salt crystals and dried at room temperature.

To each dried slide we added a drop of Bisbenzimide (Hoechst 33342; Molecular Probes) to stain the sperm nucleus, and a drop of MitoTracker™ Green FM Dye (Invitrogen™ M7514) to stain the sperm midpiece (mitochondria; Picture 1). Within 48h of staining, we inspected the microscope slides under 200x magnification with a Zeiss Axio Imager.M2 light microscope fitted with a Zeiss Axiocam 512 colour camera (12 megapixel; 4250 × 2838, pixel size of 3.1µm × 3.1µm) and DAPI 465nm and green 519nm filters using Zeiss ZEN blue 3.1 imaging software. For each male we used a single sample (30 from May, 62 from June) and photographed at least 10 intact, normal-looking spermatozoa under 400x magnification (objective size 40, ocular size 10). We then selected the 10 best, single-sperm images per male for measurements. Previous studies also measured ten sperm per male to estimate the coefficient of variation in sperm traits (Kleven et al., 2008; Lifjeld et al., 2010). We then randomized and renamed all pictures, such that we measured sperm blind to the morph and the identity of the individuals.

Picture 1 | Stained ruff sperm. From left to right, faint greenish tail, green midpiece (mitochondria) stained by MitoTracker™ Green FM Dye, turquois nucleus stained by Hoechst 33342 and greenish acrosome.

For each sperm, we measured the length of the acrosome, the nucleus, the midpiece and the tail to the nearest 0.1µm using the open software Sperm Sizer 1.6.6 (McDiarmid et al., 2021; https://github.com/wyrli/sperm-sizer; for our Sperm Sizer protocol see https://s.gwdg.de/iGFz4F). To assess within- and between-observer repeatability of Sperm Sizer measurements, we selected 40 single-sperm pictures and remeasured them, once by the same, once by a different person. The measurements were highly repeatable, both within- and between persons (mean = 97%; Table S2). Nevertheless, all measurements used in the analyses were taken by the same person (KT). Each measured sperm and sperm part were numbered and referenced in the database and hence can be linked to the original picture as well as to the pictures of each part generated by Sperm Sizer, which contain the lines and measurement values. This ensures transparency and allows re-measurement of the same sperm by the same or a different person.

We calculated (i) total sperm length as the sum of all parts, (ii) head length as the sum of acrosome and nucleus length, (iii) flagellum length as the sum of midpiece and tail length, (iv) relative midpiece length as midpiece length divided by total length and (v) relative flagellum length as flagellum length divided

by total length (for relationships between sperm components see Methods S3 and Figure S5, for within male repeatability see Figure S3 and Table S1). We then computed coefficients of variation within each male for each absolute trait as the standard deviation divided by the mean.

Statistical analyses

All analyses were performed in R (R-Core-Team, 2022) using the 'lm' function to fit linear models and the 'lmer' function of the 'lme4' R-package to fit linear mixed-effects models (Gelman & Hill, 2007; Gelman & Su, 2021). We used the 'sim' function from the 'arm' R-package and a non-informative prior distribution (Gelman & Hill, 2007; Gelman & Su, 2021) to create a sample of 5,000 simulated values for each model parameter (i.e., posterior distribution). We report effect sizes and model predictions as medians, and the uncertainty of the estimates and predictions as Bayesian 95% credible intervals represented by the 2.5 and 97.5 percentiles (95% CI) from the posterior distribution of 5,000 simulated or predicted values. Unless stated differently, plots were created with 'ggplot' function from the 'ggplo2' R-package (Wickham, 2016).

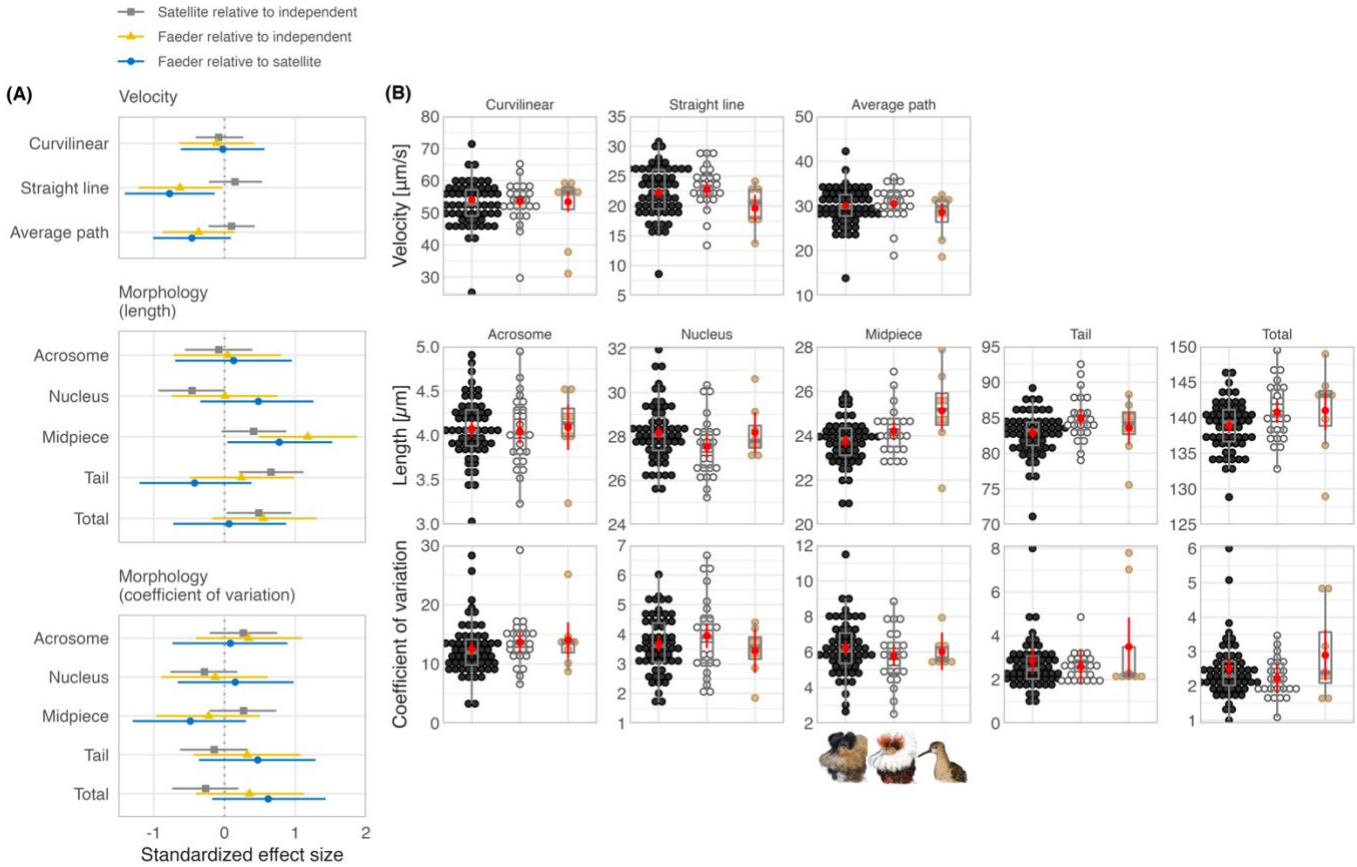
To investigate whether sperm traits differ between the morphs we fitted linear models with each sperm trait as a response variable, and with male morph as a predictor (three-level factor). For analyses of velocity, we used June values because the breeding season was at its peak in June and we obtained data from all but four males (for those four we used May values). We added the number of tracked sperm cells per sample (ln-transformed) as a covariate to the model to control for potential effects of sperm density or sample quality. For analyses of morphology, we used average male values. Alternative models using all velocity recordings or using individual sperm measurements gave similar results (Figure S6). Moreover, correlations between sperm traits and inbreeding were weak (mean r = -0.09, range: -0.26 to 0.07; Figure S7, Methods S4) and the results were not confounded by relatedness of the sampled individuals (Methods S5, Table S3, Figure S8).

To investigate whether sperm morphology explained variation in sperm swimming speed, we fitted a set of linear models with each velocity measure as the dependent variable (using June values), and with the male average of each sperm morphology trait as a predictor. All models were controlled for the number of tracked sperm (ln-transformed) and morph (three-level factor). As the relationship between sperm swimming speed and morphology might be non-linear (Bennison et al., 2016), we also fitted a set of linear models that included the quadratic term of each morphology trait (2nd order polynomial). We then tested whether the quadratic term improved the model fit, using Akaike's Information Criterion corrected for sample size (Anderson, 2008), with the 'AICc' function from the 'MuMIn' R-package (Bartoń, 2022). Because this was not the case for any trait (Table S4), we report results from the simple model without the quadratic term. To investigate whether the effect of one sperm component (head, midpiece and tail length) was influenced by the other components, we also ran a multivariate model that contained the three sperm components as predictors. Correlations between the three predictors were low (Figure S5). The effect sizes and uncertainties for head, midpiece and tail length were similar in the multivariate and the univariate models (Figure S9) and we report the results from the univariate models in the main text.

Results and Discussion

Between-morph differences in sperm traits

Sperm swimming speed in vitro in a standard medium, measured as curvilinear velocity, did not differ between the three morphs (Figure 2 and Supplement Figure S6 and Table S5; Bulla et al., 2023). However, when sperm swimming speed was measured as straight-line or average-path velocity, according to expectations based on mutation accumulation, Faeder sperm moved more slowly than the sperm of both Independents and Satellites, whereby the latter two morphs did not differ in sperm velocity (Figure 3 and S6, Table S5). If true, the slower sperm of Faeders appeared either with the origin of the Faeder genotype (i.e., the inversion) or later due to accumulation of deleterious mutations. In the latter case, recombination through which Satellites arose may have re-instated a "good" copy of a gene related to sperm velocity.



294
295 **Figure 2 | Differences in sperm traits between ruff morphs.** (**a**) Model predictions of between-morph differences with their
296 95% CI (Table S5). (**b**) Morph-specific sperm traits, shown as individual data points with dot color highlighting morph (black:
297 Independents, white: Satellites, beige: Faeders) and summarized as boxplots (see Fig. 1 for definition). To aid visualization, the
298 outlier coefficients of variation for tail (18.7) and total length (9.8) in Independents are depicted as 8 and 6, respectively. The red
299 dots with bars show model predictions and their 95% CI (Table S5). Dots were stacked using the 'geom_dotplot' ggplot2 R-
300 function (Wickham, 2016). (**a, b**) Velocity represents June values (with the exception of four males with May values only).
301 Morphology represents average trait length and coefficients of variation are based on measurements of 10 sperm cells per male.
302 For illustrations of head, flagellum (absolute and relative) and relative midpiece length, see Fig. S6 and S8. Ruff morph illustrations
303 by Yifan Pei under Creative Commons Attribution (CC BY 4.0).
304
305 Sperm morphology differed weakly between the three morphs, mostly by less than one standard
306 deviation (Figure 2 and S6, Table S5). Sperm of Independents were the shortest with the shortest
307 midpiece and tail, and hence flagellum (midpiece and tail length combined). Nucleus and head length
308 did not differ between Faeders and Independents. Satellite sperm had the shortest nucleus and head,
309 but the longest tail. The midpiece was longest in Faeders and 1.19 standard deviations longer than the
310 midpiece from Independents (95%CI: 0.46 - 1.88; Figure 2, Table S5). Flagellum and total sperm length
311 were similar for Faeders and Satellites (Figure 2 and S6). Consequently, midpiece length relative to
312 total sperm length was largest for Faeders, but similar for Satellites and Independents; flagellum length
313 relative to total sperm length was largest in Satellites and similar in the other two morphs (Figure S6).
314 The coefficients of variation in sperm traits were noisy with unclear trends and sperm of Independents
315 was not less variable than that of the Faeders and Satellites (Figure 2 and S8). These findings go against
316 the expectation of lower quality sperm in Satellites and Faeders. Specifically, when compared to
317 Independents, the sperm of the two derived morphs were not shorter or more variable in terms of
318 midpiece, tail, and flagellum, in contrast to predictions from mutation accumulation. Thus, sperm of
319 Independents seemed of similar or lower (not better) quality than sperm of the derived morphs (Figure
320 2), although what constitutes a high quality sperm, especially within species, is still debated (Dougherty
321 et al., 2022).

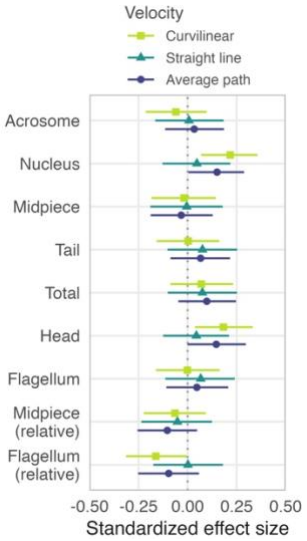


Figure 3 | Sperm swimming speed in relation to sperm morphology. Shapes with horizontal bars represent estimated standardized effect sizes with their 95%CIs based on the joint posterior distribution of 5,000 simulated values generated from linear models, controlled for number of tracked sperm (ln-transformed) and morph (Table S6). The results are based on single June-values for velocity (with exception of four males with May-values only) and average trait lengths of 10 sperm cells per male. For raw data and predicted relationships see Figure S10.

The lack of clear differences in sperm morphology among the three male morphs suggests that the two genes related to spermatogenesis that reside within the inversion have little impact on the measured sperm traits. Such finding might be unsurprising given that multiple (tens to hundreds of) genes influence spermatogenesis (Linn et al., 2021). Furthermore, the lack of major differences in sperm traits accords with previous studies showing no clear and consistent differences in sperm traits between males that use alternative mating tactics (Dougherty et al., 2022; Kustra & Alonzo, 2020). Although one expects a steeper selection gradient for males that have a lower probability to copulate (reviewed in Dougherty et al., 2022; Kustra & Alonzo, 2020), selection will act on all males to optimize sperm traits and the strength of selection might thus be limited. Specifically, in ruffs sexual selection and sperm competition might already have been intense before the alternative morphs arose. More than half of female ruffs have their clutch fertilized by more than one male (Lank et al., 2002; Thuman & Griffith, 2005), implying that most females mate with multiple males during a single reproductive event Also, consistent with intense sperm competition, ruffs have the longest sperm measured among shorebirds (Johnson & Briskie, 1999), based on samples taken from Independent males (Jim Briskie, personal communication). It is thus unclear how much unrealized potential is left for further sperm improvement.

It remains unclear how the Faeder males compensate for the lower reproductive success of Faeder females (Giraldo-Deck et al., 2022). The sperm traits we measured seem to give the Faeder males little advantage over Independent males. However, there is a strong mating skew with only few Independents obtaining most copulations on a given lek (Tolliver et al., 2023; Vervoort & Kempenaers, 2019; Widemo & Owens, 1995), such that the average reproductive success of an Independent will be low. Thus, the rare Faeder males might only need moderate mating success through sneaking, facilitated by their female mimicking appearance and behavior (Jukema & Piersma, 2006) to compensate. Possibly, Faeders do better, because morphs differ in other fitness-relevant sperm traits. For example, the longer midpiece of Faeder males might correlate with faster swimming (but see below) or with longer survival of their sperm inside the female reproductive tract. However, this would need further study.

Relationship between sperm swimming speed and morphology

Sperm morphology had only weak and unclear effects on variation in sperm swimming speed (Figure 3, S9 and S10, Table S6). Contrary to expectations (reviewed in Bennison et al., 2016; Cramer et al., 2021; Fitzpatrick & Lupold, 2014; Kim et al., 2017; Knief et al., 2017; Míčková et al., 2023; Støstad et al., 2018), longer sperm, sperm with a longer midpiece (absolute and relative) or sperm with a longer flagellum did not swim faster (r varied between 0.05 and 0.18; Figure S10). The lack of an association between sperm midpiece length and swimming speed may have several explanations. First, our method to measure sperm velocity might not reflect the swimming speed of the sperm within the seminal fluid

or within the female reproductive tract. Note, however, that the way the sperm moved did not change after we placed the sperm into a solution that contained cloacal fluid of a ruff female (details not shown). Although our method is the same as the one used to measure swimming speed in passerine sperm and worked well for zebra finch sperm (Figure S0), it may be suboptimal for scolopacid sperm. Second, although the midpiece contains the mitochondria, midpiece volume rather than length might be relevant for sperm swimming speed (Bennison et al., 2016; Cramer et al., 2021; Rowe et al., 2013) or midpiece length might be associated with other sperm performance parameters such as longevity (which we did not measure).

Against predictions (Humphries et al., 2008), sperm with longer heads (nucleus) or smaller relative flagellum length swam faster, when measured as curvilinear and average-path velocity (but not straight-line velocity), although these effects were small (< 0.25 of standard deviation, maximum r = 0.22; Figure 4). Theory predicts that sperm with a shorter head relative to flagellum should swim faster because a relatively shorter head reduces drag (Humphries et al., 2008). To our knowledge this pattern has not been tested within species, but has been confirmed in a between-species study of mammals (Tourmente et al., 2011) and of passerine birds (Lüpold et al., 2009). However, a recent study on passerines showed that sperm with a more pronounced helical form, i.e., a long acrosome, a short nucleus, a wide helical membrane, and a more pronounced waveform along the sperm head "core" swam faster (Støstad et al., 2018).

Sperm motility and sperm shape in ruffs compared to other taxa

We observed that ruff sperm move in a different manner to that previously described for passerine and likely also for non-passerine sperm. Specifically, ruff sperm 'vibrate' from side to side while slowly moving forward (video example), whereas passerine sperm typically swim straight (video example) by rapidly rotating around their longitudinal axis (Ballowitz, 1888; Humphreys, 1972; Vernon & Woolley, 1999). This obvious difference in movement is not clearly due to differences in morphology, because ruff sperm have a helical, screw-like head (Picture 1), reminiscent of that observed in passerine sperm (Retzius, 1909). A studies have described that non-passerine sperm moves with regular helical (dextral) waves (Ballowitz, 1888; Humphreys, 1972; Vernon & Woolley, 1999) or via counter-clockwise turning of the entire sperm body (Bird & Laguë, 1977). Given the few descriptions and the lack of video recordings of non-passerine sperm movements (e.g., Cheng et al., 2002; Denk et al., 2005; Dogliero et al., 2017; Fischer et al., 2014; Gloria et al., 2014), it remains to be seen whether the way ruff sperm propel is unique to this species, or typical for other Scolopacids, shorebirds or Charadriiformes in general. Clearly, the propelling of ruff sperm is distinct from the smooth, snake-like, propelling seen in sperm of non-avian taxa (e.g., human or sea-urchin *Psammechinus miliaris* (Cosson et al., 2015; Gillies et al., 2009; Gray, 1955; Saggiorato et al., 2017; Smith et al., 2009), but see squid sperm (Bishop, 1958)). We can exclude that the unusual head movements of ruff sperm were caused by a lack of depth in the standard 20µm two-chamber count Leja-slide, because using a deeper slide (100µm) resulted in similar movements.

The screw-like head of ruff sperm is similar to the head shape reported for other Scolopaci (sandpipers), but differs from the straight heads observed in other Charadriiformes, e.g., genus *Larus* or *Vanellus* (Retzius, 1909). We confirm that ruff sperm is remarkably long (median and mean = 139.5 µm, range: 105.7 - 150 µm) compared to other shorebirds, with species means ranging between 57 and 100 µm (Johnson & Briskie, 1999).

Conclusions

Using sperm collected from a population of captive ruffs, we found mostly weak between-morph differences in sperm traits. As expected from mutation accumulation in the inversion, sperm of Faeders were the slowest, but – against such expectation – sperm of Independents were not the fastest, longest or least variable. Furthermore, we found that sperm of ruffs propelled differently than sperm of passerines, despite having a similar head shape (Ballowitz, 1888; Humphreys, 1972; Retzius, 1909; Vernon & Woolley, 1999). Our study shows at best weak associations between sperm swimming speed and morphology that are inconsistent with general expectations. Our results corroborate other comparative work that revealed a lack of clear and consistent differences in sperm traits of males using alternative mating tactics (Dougherty et al., 2022; Kustra & Alonzo, 2020). Moreover, our study leaves open the question about how Faeder males can achieve higher reproductive success to compensate for the lower reproductive output of Faeder females (Giraldo-Deck et al., 2022), but emphasizes little potential for the evolution of strong morph-specific sperm adaptations in this system.

Data and code

All data, including sperm recordings and pictures, and computer code used to generate the results, and Supplement (figures, tables and documents) of this study are freely available at https://github.com/MartinBulla/ruff_sperm_v2 (Bulla et al., 2023).

Funding

Max Planck Society (to BK and CK), the Czech Science Foundation project 19-22538S (to TA and JA).

Acknowledgements

We thank the many people that cared for the captive ruffs, Melanie Schneider for genotyping, Callum S. McDiarmid for helping with his Sperm Sizer software, Esteban Botero-Delgadillo for the script on homozygosity by locus, Jarrod Hadfield for advice on genomic relationship matrix, Mihai Valcu for advice on statistics, and Yifan Pei for ruff illustrations.

Author contributions

MB initiated the project with support of BK and DL. BK secured funding. MB, BK, TA, DL, CK, JL conceptualized the project. JL collected and measured the testes. MB, KT, TA, ML collected the sperm samples. JA measured velocity. KT and MB with support of TA optimized the photographing of the sperm. MB, MC and KT developed and tested the sperm measuring protocol. KT and MC prepared sperm samples, took sperm pictures and KT measured sperm morphology. MB curated and analyzed the data with input from BK, WF and TA. MB wrote the first draft and revised it with the help of BK. All authors commented and MB with BK, WF, CK and DL finalized the paper.

Conflict of interest

The authors have no competing interests.

References

Ballowitz, E. (1888). Untersuchungen über die Struktur der Spermatozoën, zugleich ein Beitrag zur Lehre vom feineren Bau der contraktilen Elemente. *Archiv für Mikroskopische Anatomie,* **32**(1), 401-473.

Bartoń, K. (2022). MuMIn: Multi-Model Inference. *R package version 1.46.0*. doi:https://CRAN.R-project.org/package=MuMIn

Bennison, C., Hemmings, N., Brookes, L., Slate, J., & Birkhead, T. (2016). Sperm morphology, adenosine triphosphate (ATP) concentration and swimming velocity: unexpected relationships in a passerine bird. *Proc Biol Sci,* **283**(1837). doi:https://doi.org/10.1098/rspb.2016.1558

Bennison, C., Hemmings, N., Slate, J., & Birkhead, T. (2014). Long sperm fertilize more eggs in a bird. *Proceedings of the Royal Society B: Biological Sciences,* **282**(1799), 20141897-20141897. doi:https://doi.org/10.1098/rspb.2014.1897

Berdan, E. L., Blanckaert, A., Butlin, R. K., & Bank, C. (2021). Deleterious mutation accumulation and the long-term fate of chromosomal inversions. *PLoS Genetics,* **17**(3), e1009411. doi:https://doi.org/10.1371/journal.pgen.1009411

Bird, D. M., & Laguë, P. C. (1977). Semen production of the American kestrel. *Canadian Journal of Zoology,* **55**(8), 1351-1358. doi:https://doi.org/10.1139/z77-175

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

Bishop, D. W. (1958). Motility of the sperm flagellum. *Nature,* **182**, 1638-1640. doi:https://www.nature.com/articles/1821638a0

Bulla, M., Küpper, C., Lank, D. B., Albrechtová, J., Loveland, J. L., Martin, K., . . . Kempenaers, B. (2023). Supporting information for "'Sperm traits of the three genetic morphs in the ruff sandpiper ": GitHub. Retrieved from https://github.com/MartinBulla/ruff_sperm_v2

Cardullo, R. A., & Baltz, J. M. (1991). Metabolic Regulation in Mammalian Sperm: Mitochondrial Volume Determines Sperm Length and Flagellar Beat Frequency. *Cell Motility and the Cytoskeleton,* **19**, 180-188. doi: https://doi.org/10.1002/cm.970190306

Cheng, F. P., Guo, T. J., Wu, J. T., Lin, T. E., Ursem, P. J., Colenbrander, B., & Fung, H. P. (2002). Annual variation in semen characteristics of pigeons (*Columba livia*). *Poultry Science,* **81**(7), 1050-1056. doi:https://doi.org/10.1093/ps/81.7.1050

Cosson, J. J., Prokopchuk, G., & Inaba, K. (2015). The Flagellar Mechanics of Spermatozoa and its Regulation. In J. J. Cosson (Ed.), *Flagellar Mechanics and Sperm Guidance* (pp. 3-134). doi:https://doi.org/10.2174/9781681081281115010 1

Cramer, E. R., Alund, M., McFarlane, S. E., Johnsen, A., & Qvarnstrom, A. (2016). Females discriminate against heterospecific sperm in a natural hybrid zone. *Evolution*. doi:https://doi.org/10.1111/evo.12986

Cramer, E. R. A., Garcia-Del-Rey, E., Johannessen, L. E., Laskemoen, T., Marthinsen, G., Johnsen, A., & Lifjeld, J. T. (2021). Longer Sperm Swim More Slowly in the Canary Islands Chiffchaff. *Cells,* **10**(6). doi:https://doi.org/10.3390/cells10061358

Denk, A. G., Holzmann, A., Peters, A., Vermeirssen, E. L. M., & Kempenaers, B. (2005). Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. *Behavioral Ecology,* **16**(5), 825-833. doi:https://doi.org/10.1093/beheco/ari065

Dogliero, A., Rota, A., Lofiego, R., von Degerfeld, M. M., & Quaranta, G. (2017). Semen Collection and Spermatozoa Characteristics in the Kea Parrot (*Nestor notabilis*). *Journal of Avian Medicine and Surgery,* **31**(1), 24-28. doi:https://doi.org/10.1647/2015-132

Dougherty, L. R., Skirrow, M. J. A., Jennions, M. D., & Simmons, L. W. (2022). Male alternative reproductive tactics and sperm competition: a meta-analysis. *Biol Rev Camb Philos Soc*. doi:https://doi.org/10.1111/brv.12846

Fischer, D., Neumann, D., Wehrend, A., & Lierz, M. (2014). Comparison of conventional and computer-assisted semen analysis in cockatiels (*Nymphicus hollandicus*) and evaluation of different insemination dosages for artificial insemination. *Theriogenology,* **82**(4), 613-620. doi:https://doi.org/10.1016/j.theriogenology.2014.05.023

Fitzpatrick, J. L., & Lupold, S. (2014). Sexual selection and the evolution of sperm quality. *Mol Hum Reprod,* **20**(12), 1180-1189. doi:https://doi.org/10.1093/molehr/gau067

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

Garcia-Gonzalez, F., & Simmons, L. W. (2005). Sperm viability matters in insect sperm competition. *Current Biology,* **15**(3), 271-275. doi:https://doi.org/10.1016/j.cub.2005.01.032

Gelman, A., & Hill, J. (2007). *Data analysis using regression and multilevel/hierarchical models*. Cambridge: Cambridge University Press.

Gelman, A., & Su, Y.-S. (2021). arm: Data Analysis Using Regression and Multilevel/Hierarchical Models. R package version 1.12-2. (Version R package version 1.12-2.). Retrieved from http://CRAN.R-project.org/package=arm

Gillies, E. A., Cannon, R. M., Green, R. B., & Pacey, A. A. (2009). Hydrodynamic propulsion of human sperm. *Journal of Fluid Mechanics,* **625**, 445-474. doi:https://doi.org/10.1017/s0022112008005685

Giraldo-Deck, L. M., Loveland, J. L., Goymann, W., Tschirren, B., Burke, T., Kempenaers, B., . . . Küpper, C. (2022). Intralocus conflicts associated with a supergene. *Nat Commun,* **13**(1), 1384. doi:https://doi.org/10.1038/s41467-022-29033-w

Giraldo-Deck, L. M., Goymann, W., Safari, I., Dawson, D. A., Stocks, M., Burke, T., . . . Küpper, C. (2020). Development of intraspecific size variation in black coucals, white-browed coucals and ruffs from hatching to fledging. *Journal of Avian Biology,* **51**(8). doi:https://doi.org/10.1111/jav.02440

Gloria, A., Contri, A., Carluccio, A., Parrillo, S., Cicconi, M., & Robbe, D. (2014). The breeding management affects fresh and cryopreserved semen characteristics in *Melopsittacus undulatus*. *Anim Reprod Scis,* **144**(1-2), 48-53. doi:https://doi.org/10.1016/j.anireprosci.2013.11.009

Gray, J. (1955). The Movement of Sea-Urchin Spermatozoa. *Journal of Experimental Biology,* **32**(4), 775–801. doi:https://doi.org/10.1242/jeb.32.4.775

Graziotto, R., Foresta, C., Scannapieco, P., Zeilante, P., Russo, A., Negro, A., . . . Onisto, M. (1999). cDNA Cloning and Characterization of PD1: A Novel Human Testicular Protein with Different Expressions in Various Testiculopathies. *Experimental Cell Research,* **248**(2), 620-626. doi:https://doi.org/10.1006/excr.1999.4449

Gross, M. R. (1996). Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology & Evolution,* **11**(2), 92-98. doi:https://doi.org/10.1016/0169-5347(96)81050-0

Hogan-Warburg, A. J. (1966). Social Behavior of the Ruff, *Philomachus Pugnax* (L.). *Ardea,* **54**(3-4), 109-229. doi:https://doi.org/10.5253/arde.v54.p109

Höglund, J., & Lundberg, A. (1989). Plumage Color Correlates with Body Size in the Ruff *The Auk,* **106**(2), 336–338. doi:https://doi.org/10.1093/auk/106.2.336

Hugie, D. M., & Lank, D. B. (1997). The resident's dilemma: a female choice model for the evolution of alternative mating strategies in lekking male ruffs (*Philomachus pugnax*). *Behavioral Ecology,* **8**(2), 218-225. doi:https://doi.org/10.1093/beheco/8.2.218

Humphreys, P. (1972). Brief observations on the semen and spermatozoa of certain passerine and non-passerine birds. *Journal of Reproduction and Fertility,* **29**(3), 327-336. doi:https://doi.org/10.1530/jrf.0.0290327

Humphries, S., Evans, J. P., & Simmons, L. W. (2008). Sperm competition: linking form to function. *BMC Evolutionary Biology,* **8**, 319. doi:https://doi.org/10.1186/1471-2148-8-319

Johnson, D. D. P., & Briskie, J. V. (1999). Sperm competition and sperm length in shorebirds. *Condor,* **101**(4), 848-854. doi:https://doi.org/10.2307/1370074

Jukema, J., & Piersma, T. (2006). Permanent female mimics in a lekking shorebird. *Biology Letters,* **2**(2), 161-164. doi:https://doi.org/10.1098/rsbl.2005.0416

Kim, K.-W., Bennison, C., Hemmings, N., Brookes, L., Hurley, L. L., Griffith, S. C., . . . Slate, J. (2017). A sex-linked supergene controls sperm morphology and swimming speed in a songbird. *Nature Ecology & Evolution,* **1**(8), 1168-1176. doi:https://doi.org/10.1038/s41559-017-0235-2

Kleven, O., Laskemoen, T., Fossøy, F., Robertson, R. J., & Lifjeld, J. T. (2008). Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution,* **62**(2), 494-499. doi:https://doi.org/10.1111/j.1558-5646.2007.00287.x

Knief, U., Forstmeier, W., Pei, Y., Ihle, M., Wang, D., Martin, K., . . . Kempenaers, B. (2017). A sex-chromosome inversion causes strong overdominance for sperm traits that affect siring success. *Nature Ecology & Evolution,* **1**(8), 1177-1184. doi:https://doi.org/10.1038/s41559-017-0236-1

Küpper, C., Stocks, M., Risse, J. E., Dos Remedios, N., Farrell, L. L., McRae, S. B., . . . Burke, T. (2016). A supergene determines highly divergent male reproductive morphs in the ruff. *Nat Genet,* **48**, 79-83. doi:https://doi.org/10.1038/ng.3443

Kustra, M. C., & Alonzo, S. H. (2020). Sperm and alternative reproductive tactics: a review of existing theory and empirical data. *Philosophical Transactions of the Royal Society of London B Biological Sciences,* **375**(1813), 20200075. doi:https://doi.org/10.1098/rstb.2020.0075

Lamichhaney, S., Fan, G., Widemo, F., Gunnarsson, U., Thalmann, D. S., Hoeppner, M. P., . . . Andersson, L. (2016). Structural genomic changes underlie alternative reproductive strategies in the ruff (*Philomachus pugnax*). *Nat Genet,* **48**, 84-88. doi:https://doi.org/10.1038/ng.3430

Lank, D. B., Farrell, L. L., Burke, T., Piersma, T., & McRae, S. B. (2013). A dominant allele controls development into female mimic male and diminutive female ruffs. *Biology Letters,* **9**(6). doi:https://doi.org/10.1098/rsbl.2013.0653

Lank, D. B., Smith, C. M., Hanotte, O., Burke, T., & Cooke, F. (1995). Genetic polymorphism for alternative mating behaviour in lekking male ruff *Philomachus pugnax*. *Nature,* **378**(6552), 59-62. doi:https://doi.org/10.1038/378059a0

Lank, D. B., Smith, C. M., Hanotte, O., Ohtonen, A., Bailey, S., & Burke, T. (2002). High frequency of polyandry in a lek mating system. *Behavioral Ecology,* **13**(2), 209-215. doi:https://doi.org/10.1093/beheco/13.2.209

Laskemoen, T., Albrecht, T., Bonisoli-Alquati, A., Cepak, J., Lope, F., Hermosell, I., . . . Lifjeld, J. (2012). Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behavioral Ecology and Sociobiology*, 1-9. doi:https://doi.org/10.1007/s00265-012-1450-0

Laskemoen, T., Kleven, O., Fossøy, F., Robertson, R. J., Rudolfsen, G., & Lifjeld, J. T. (2010). Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*. *Behavioral Ecology and Sociobiology*, 1-11. doi: https://doi.org/10.1007/s00265-010-0962-8

Lierz, M., Reinschmidt, M., Muller, H., Wink, M., & Neumann, D. (2013). A novel method for semen collection and artificial insemination in large parrots (Psittaciformes). *Sci Rep,* **3**, 2066. doi:https://doi.org/10.1038/srep02066

Lifjeld, J. T., Laskemoen, T., Kleven, O., Albrecht, T., & Robertson, R. J. (2010). Sperm Length Variation as a Predictor of Extrapair Paternity in Passerine Birds. *PLoS ONE,* **5**(10), e13456. doi:https://doi.org/10.1371/journal.pone.0013456

Linn, E., Ghanem, L., Bhakta, H., Greer, C., & Avella, M. (2021). Genes Regulating Spermatogenesis and Sperm Function Associated With Rare Disorders. *Front Cell Dev Biol,* **9**, 634536. doi:https://doi.org/10.3389/fcell.2021.634536

Lipshutz, S. E., Torneo, S. J., & Rosvall, K. A. (2022). How female-female competition affects male-male competition: insights on post-copulatory sexual selection from socially polyandrous species. *The American Naturalist*. doi:https://doi.org/10.1086/722799

Loveland, J. L., Giraldo-Deck, L. M., Lank, D. B., Goymann, W., Gahr, M., & Küpper, C. (2021a). Functional differences in the hypothalamic-pituitary-gonadal axis are associated with alternative reproductive tactics based on an inversion polymorphism. *Hormones and Behavior,* **127**, 104877. doi:https://doi.org/10.1016/j.yhbeh.2020.104877

Loveland, J. L., Lank, D. B., & Küpper, C. (2021b). Gene Expression Modification by an Autosomal Inversion Associated With Three Male Mating Morphs. *Front Genet,* **12**, 641620. doi:https://doi.org/10.3389/fgene.2021.641620

Lüpold, S., Calhim, S., Immler, S., & Birkhead, T. R. (2009). Sperm morphology and sperm velocity in passerine birds. *Proc Biol Sci,* **276**(1659), 1175-1181. doi:https://doi.org/10.1098/rspb.2008.1645

Lüpold, S., de Boer, R. A., Evans, J. P., Tomkins, J. L., & Fitzpatrick, J. L. (2020). How sperm competition shapes the evolution of testes and sperm: a meta-analysis. *Philosophical Transactions of the Royal Society of London B Biological Sciences,* **375**(1813), 20200064. doi:https://doi.org/10.1098/rstb.2020.0064

Lüpold, S., Manier, Mollie K., Berben, Kirstin S., Smith, Kyle J., Daley, Bryan D., Buckley, Shannon H., . . . Pitnick, S. (2012). How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in *Drosophila melanogaster*. *Current biology : CB*. doi:https://doi.org/10.1016/j.cub.2012.06.059

Maney, D. L., & Küpper, C. (2022). Supergenes on steroids. *Philosophical Transactions of the Royal Society of London B Biological Sciences,* **377**(1855), 20200507. doi:https://doi.org/10.1016/10.1098/rstb.2020.0507

Mank, J. E. (2023). Sex-specific morphs: the genetics and evolution of intra-sexual variation. *Nature Reviews Genetics,* **24**(1), 44-52. doi:https://doi.org/10.1038/s41576-022-00524-2

McDiarmid, C. S., Li, R., Kahrl, A. F., Rowe, M., & Griffith, S. C. (2021). Sperm Sizer: a program to semi-automate the measurement of sperm length. *Behavioral Ecology and Sociobiology,* **75**(5). doi:https://doi.org/10.1007/s00265-021-03013-4

Míčková, K., Tomášek, O., Jelínek, V., Šulc, M., Pazdera, L., Albrechtová, J., & Albrecht, T. (2023). Age-related changes in sperm traits and evidence for aging costs of sperm production in a sexually promiscuous passerine. *Frontiers in Ecology and Evolution,* **11**. doi:https://doi.org/10.3389/fevo.2023.1105596

Onisto, M., Graziotto, R., Scannapieco, P., Marin, P., Merico, M., Slongo, M. L., & Foresta, C. (2000). A novel gene (PD1) with a potential role on rat spermatogenesis. *Journal of Endocrinological Investigation volume,* **23**, 605–608. doi:https://doi.org/10.1007/BF03343783

Opatova, P., Ihle, M., Albrechtova, J., Tomasek, O., Kempenaers, B., Forstmeier, W., & Albrecht, T. (2016). Inbreeding depression of sperm traits in the zebra finch *Taeniopygia guttata*. *Ecol Evol,* **6**(1), 295-304. doi:https://doi.org/10.1002/ece3.1868

Parker, G. A. (1970). Sperm Competition and Its Evolutionary Consequences in the Insects. *Biological Reviews (Cambridge),* **45**, 525-567. doi: https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

R-Core-Team. (2022). R: A language and environment for statistical computing (Version Version 4.2.0). Vienna (Austria): R Foundation for Statistical Computing. Vienna, Austria. Retrieved from http://www.R-project.org/

Retzius, G. (1909). Die Spermien der Vögel. *Biol. Unters. New Ser,* **14**, 89-122.

Rowe, M., Laskemoen, T., Johnsen, A., & Lifjeld, J. T. (2013). Evolution of sperm structure and energetics in passerine birds. *Proc Biol Sci,* **280**(1753), 20122616. doi:https://doi.org/10.1098/rspb.2012.2616

Saggiorato, G., Alvarez, L., Jikeli, J. F., Kaupp, U. B., Gompper, G., & Elgeti, J. (2017). Human sperm steer with second harmonics of the flagellar beat. *Nat Commun,* **8**(1), 1415. doi:https://doi.org/10.1038/s41467-017-01462-y

Shuster, S. M., & Wade, M. J. (1991). Equal mating success among male reproductive strategies in a marine isopod. *Nature,* **350**, 608–610. doi:https://doi.org/10.1038/350608a0

Simmons, L. W., & Fitzpatrick, J. L. (2012). Sperm wars and the evolution of male fertility. *Reproduction,* **144**(5), 519-534. doi:https://doi.org/10.1530/REP-12-0285

Smith, D. J., Gaffney, E. A., Gadelha, H., Kapur, N., & Kirkman-Brown, J. C. (2009). Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity. *Cell Motility and the Cytoskeleton,* **66**(4), 220-236. doi:https://doi.org/10.1002/cm.20345

Støstad, H. N., Johnsen, A., Lifjeld, J. T., & Rowe, M. (2018). Sperm head morphology is associated with sperm swimming speed: A comparative study of songbirds using electron microscopy. *Evolution,* **72**(9), 1918-1932. doi:https://doi.org/10.1111/evo.13555

Thuman, K. A., & Griffith, S. C. (2005). Genetic similarity and the nonrandom distribution of paternity in a genetically highly polyandrous shorebird. *Animal Behaviour,* **69**(4), 765-770. doi:https://doi.org/10.1016/j.anbehav.2004.10.003

Tolliver, J. D. M., Kupán, K., Lank, D. B., Schindler, S., & Küpper, C. (2023). Fitness benefits from co-display favour subdominant male–male partnerships between phenotypes. *Animal Behaviour,* **197**, 131-154. doi:https://doi.org/10.1016/j.anbehav.2022.12.004

Tourmente, M., Gomendio, M., & Roldan, E. R. (2011). Sperm competition and the evolution of sperm design in mammals. *BMC Evolutionary Biology,* **11**(12). doi:https://doi.org/10.1186/1471-2148-11-12

van Rhijn, J. G. (1991). *The Ruff*: T. & A.D. Poyser, London.

Vernon, G. G., & Woolley, D. M. (1999). Three-Dimensional Motion of Avian Spermatozoa. *Cell Motility and the Cytoskeleton,* **42**, 149-161. doi:https://doi.org/10.1002/(sici)1097-0169(1999)42:2%3C149::aid-cm6%3E3.0.co;2-0

Vervoort, R., & Kempenaers, B. (2019). Variation in lek attendance and copulation success of Independent and Satellite male Ruffs *Calidris pugnax*. *Ardea,* **107**. doi:https://doi.org/10.5253/arde.v107i3.a9

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*: Springer-Verlag New York.

Widemo, F. (1997). The social implications of traditional use of lek sites in the ruff *Philomachus pugnax*. *Behavioral Ecology,* **8**(2), 211-217. doi:https://doi.org/10.1093/beheco/8.2.211

Widemo, F. (1998). Alternative reproductive strategies in the ruff, *Philomachus pugnax*: a mixed ESS? *Animal Behavior,* **56**, 329-336. doi:https://doi.org/10.1006/anbe.1998.0792

Widemo, F., & Owens, I. (1995). Measuring sexual selection. *Nature,* **376**, 471-472. doi:https://doi.org/10.1038/376471b0

Yeh, S. D., Chen, Y. J., Chang, A. C., Ray, R., She, B. R., Lee, W. S., . . . Lin-Chao, S. (2002). Isolation and properties of Gas8, a growth arrest-specific gene regulated during male gametogenesis to produce a protein associated with the sperm motility apparatus. *Journal of Biological Chemistry,* **277**(8), 6311-6317. doi:https://doi.org/10.1074/jbc.M106941200