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Individual phenotypic plasticity explains seasonal variation in sperm morphology in a passerine bird

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ABSTRACT

Background: Spermatozoa display impressive variation in size and form among and within animal species. In birds, comparative evidence suggests that post-copulatory sexual selection resulting from extra-pair copulations is a major driver of interspecific sperm trait variation. However little is known about the extent, determinants and dynamics of intraspecific variation in avian sperm traits.

Goal: Characterize and analyze variation in sperm morphology within and among two natural populations of great tits (*Parus major*) — a socially monogamous passerine with frequent extra-pair matings.

Methods: We studied both a German and a Norwegian population of *P. major*. In the German population we sampled spermatozoa during both the first clutch egg-laying and the nestling period (partly from the same individual males). In the Norwegian population we sampled spermatozoa during the pre-laying/egg-laying period. We measured the length of spermatozoa with separate measurements of sperm head, midpiece and tail length.

Results: In the German population, spermatozoa were significantly shorter during the nestling period than during the egg-laying period. Individual phenotypic plasticity was responsible for the seasonal dynamics in sperm morphology. Changes in flagellum length (sum of midpiece and tail length) rather than changes in head length accounted for the change observed in total length. We found that changes in flagellum length were attributable to both midpiece and, in particular, tail shortening. Consequently the ratio, 'midpiece/total length,' increased over the breeding cycle. Controlling statistically for seasonal variation, sperm total length was significantly repeatable across sperm samples from the same males. Furthermore, spermatozoa sampled in a Norwegian population early in the season differed from those obtained from the German population during egg-laying, but not from those obtained from the German population during the nestling period.

Conclusions: Individual phenotypic plasticity across the breeding season may contribute to intraspecific variation in avian sperm morphology. Our comparison across populations illustrates that seasonal variation in sperm dimensions within populations may confound between-population comparisons unless one controls for sampling date in relation to reproductive phenology.

INTRODUCTION

Spermatozoa share a function across all animal taxa, i.e., fertilizing eggs. Despite this common task, spermatozoa display enormous variation in size, form and motility at all taxonomic levels (Pitnick *et al.*, 2009). Comparative and experimental evolution studies in birds, fishes, insects and mammals suggest that post-copulatory sexual selection constitutes a powerful evolutionary force contributing to this variation (e.g. Fitzpatrick *et al.*, 2009; Pitnick *et al.*, 2009; Tourmente *et al.*, 2011; Higginson *et al.*, 2012; Rowe *et al.*, 2015; Godwin, 2017).

In socially monogamous bird species, female extra-pair mating behaviour is widespread (Griffith *et al.*, 2002; Westneat & Stewart, 2003), increases the opportunity for sexual selection (e.g. Webster *et al.*, 1995; Vedder *et al.*, 2011) and represents a major source of post-copulatory sexual selection. For example, both sperm length and sperm velocity have been shown to be positively correlated with the frequency of extra-pair paternity across passerine birds (Briskie *et al.*, 1997; Kleven *et al.*, 2009). Thus progress has recently been made in understanding the selective pressures in the evolutionary past that have contributed to the tremendous interspecific variation of avian sperm traits that we see today (Jamieson, 2007).

In a within-species context, sperm trait variation in general and in birds in particular has received much less attention. This is in remarkable contrast to other reproductive traits like for example secondary sexual characters, although analyzing the ecological and evolutionary dynamics of intraspecific sperm trait variation in natural populations is of major importance for understanding the adaptive function and microevolution of sperm traits under post-copulatory sexual selection. Only few studies, for example, have addressed such fundamental topics as geographical (e.g. Lüpold *et al.*, 2011; Schmoll & Kleven, 2011; Lifjeld *et al.*, 2012; Hogner *et al.*, 2013; Laskemoen *et al.*, 2013a; Støstad *et al.*, 2016) or seasonal (Lüpold *et al.*, 2012; Cramer *et al.*, 2013; Laskemoen *et al.*, 2013a; Edme *et al.*, revision under review) variation in avian sperm morphology. Furthermore, the potential of individual phenotypic plasticity to contribute to intraspecific variation in sperm traits has been largely neglected and evidence for gamete plasticity is scarce in general (Marshall, 2015). In birds, less than a handful of studies have established individual phenotypic plasticity in sperm morphology in relation to e.g. sperm competition risk (Immler *et al.*, 2010), season and harem size (Lüpold *et al.*, 2012) or social dominance rank (Rojas Mora *et al.*, 2017).

Here we analyze variation in sperm morphology within and among two natural populations of great tits (*Parus major*), a socially monogamous passerine bird with frequent extra-pair mating behaviour (see overview in Table 1 in Lubjuhn *et al.*, 2007). We first focus on sperm total length but also explore contributions of the sperm sections (head, midpiece and tail) *posthoc*. Furthermore, we include into our analysis two sperm proportions, which showed seasonal variation in other species (flagellum/head length ratio, Lüpold *et al.*, 2012; Cramer *et al.*, 2013) or were shown to predict competitive fertilization success in a passerine bird species (midpiece/total length ratio, Laskemoen *et al.*, 2010). We demonstrate individual phenotypic plasticity (sensu Nussey *et al.*, 2007) in sperm morphology in relation to the reproductive cycle and discuss possible non-adaptive and adaptive explanations for this in a sperm competition context and its implications for comparisons of sperm traits across populations.

METHODS

Study populations and field methods

We sampled sperm of territorial male great tits between April 17th and May 20th 2010 in NW Germany (near Lingen/Ems, 52°27' N, 7°15' E) during egg-laying of the focal males' social females (N = 20) and later during the nestling feeding period (N = 24); and between April 17th and April 30th 2010 in S Norway (near Kråkstad, 59°41' N, 10°55' E) during the pre-laying and/or egg-laying period (N = 10; no detailed information on reproductive phenology is available, but egg-laying in a nearby nestbox area started April 25th). We sampled ten males from the German population during both periods, resulting in a total of 54 sperm samples from 44 males. Bird were caught with mist-nets or by hand and sampled non-invasively by cloacal massage (Wolfson, 1952; Laskemoen *et al.*, 2013b). This typically took between just a few and approximately 30 seconds with birds showing no visible signs of stress. Sperm samples were diluted and mixed well in approximately 3 µl standard phosphate buffered saline and immediately transferred into 250 µl of an approximately 5% formaldehyde solution (equivalent to an approximately 12.5% formalin solution assuming a stock solution of 40% formaldehyde). Samples were stored at room temperature until sperm morphometric analysis in the laboratory (differential storage duration has been shown not to affect avian sperm length, Schmoll *et al.*, 2016).

Sperm morphometry

Sperm morphometric analysis was conducted by a single observer (MR). A droplet of approximately 3.6 µl sperm solution was transferred onto a microscope slide, covered with a coverslip and examined immediately by digital light microscopy at × 400 magnification under bright-field conditions using an Olympus BX50 microscope. A micrometre scale was pictured for each sperm sample before slides were screened for intact spermatozoa with no obviously abnormal morphology. Pictures of approximately 30 spermatozoa with clearly addressable sperm sections (head, midpiece, tail) were taken per sample with a Canon PowerShot A95 AiAF digital camera, of which 20 were selected for further analysis. MR measured sperm head, midpiece and tail length of 19.8 ± 0.5 SD spermatozoa per sample to a precision of 0.01 µm during a continuous measuring period using ImageJ 1.43 (Rasband, 1997-2012). We calculated sperm total length as the sum of these components and flagellum length as the sum of midpiece and tail length. To enforce blind measurement with respect to sperm sample identity, TS anonymized all samples before analysis, including an additional make-believe sample containing an identical number of pictures, but composed of spermatozoa from three different males to be measured twice for assessing measurement error via repeatability analysis. Repeatability of measurements was ≥ 0.91 for all components (all $F_{19,20} \geq 23.7$, all $p < 0.001$; see additional Table 1 in additional file 1).

Statistical analysis

We used R 3.1.1 (R Development Core Team, 2014) and linear mixed effects models (LME) to test for seasonal effects and for differences between populations in sperm traits. We included sperm sample identity (for German samples only) and male identity as nested random effects to account for the non-independence of measurements obtained from the same sperm sample and the same individual and to estimate between-male variance in sperm total length. We determined the significance of fixed effects by removing the focal term from a maximum likelihood (ML) fit of the model and the significance of random intercept effects by removing the focal term from a restricted maximum likelihood (REML) re-fit of our model. Thus *P*-values in the context of LME analysis refer to the increase in model deviance (compared against a χ^2 distribution) when a term is removed from the model. We used the R package *rptR* (Stoffel *et al.*, 2017) to calculate between-sperm-sample repeatability of sperm total length with 95% confidence intervals (parametric bootstrapping, N = 10000) based on mixed effects models. We report both repeatability of sperm length based on measurements of

individual spermatozoa and repeatability of mean sperm length per sperm sample (the latter to allow direct comparisons with other studies).

For the German samples, we used sperm sampling date relative to the day the first egg was laid during the first brood period by a focal male's social female (hereafter laying date) to analyze seasonal effects on sperm traits in reference to individual reproductive phenology. We used within-subject centring of this covariate in additional regression models to tease apart within- from between-individual effects (van de Pol & Wright, 2009). Detailed data on reproductive phenology were unavailable for the Norwegian population (see above) and we therefore used a three-level factorial predictor variable (followed by Tukey posthoc tests) to compare sperm traits between the Norwegian population, the egg-laying and the nestling feeding period in the German population, respectively.

RESULTS

In the German population, sperm total length decreased with increasing time interval between the first brood laying date of a focal male's social female and sperm sampling date (LME with sperm sample identity and male identity as random effects: $\chi^2 = 14.9$, $df = 1$, $p < 0.001$, slope (± SE): −0.09 ± 0.02; see Fig. 1a). Slopes of the within- (−0.11 ± 0.02) and between- (−0.07 ± 0.03) individual effect were statistically indistinguishable ($\chi^2 = 0.82$, $df = 1$, $p = 0.37$) and very similar to the population-level slope (see above), indicating that longitudinal changes within individuals (i.e. individual phenotypic plasticity) and not selective (dis-) appearance of individuals with particularly short or long spermatozoa produced the seasonal dynamics in sperm total length. This was confirmed by restricting the population-level analysis to ten males with paired samples during both the egg-laying and the nestling period ($\chi^2 = 10.9$, $df = 1$, $p = 0.001$, slope ± SE: −0.10 ± 0.02; data points linked by lines in Fig. 1a).

Based on our data set with multiply sampled males and controlling for the fixed effect of time of the season and the random effect of sperm sample identity, we found a significant ($\chi^2 = 5.67$, $df = 1$, $p = 0.009$) repeatability of 0.31 (95% CI: 0.04 – 0.54) of sperm total length within males. Furthermore, controlling for the effect of time of the season, we found a significant ($\chi^2 = 6.29$, $df = 1$, $p = 0.006$) repeatability of 0.68 (95% CI: 0.18 – 0.92) of mean sperm total length per sperm sample within males.

Analyses of sperm components revealed that changes in flagellum length (with contributions of both midpiece but particularly tail), rather than changes in head length, explained the patterns observed for sperm total length (Table 1, see also additional Fig. 1 in

additional file 2). As a consequence, the flagellum/head length ratio decreased (Table 1, Fig. 1b) and the midpiece/total length ratio tended to increase over the breeding cycle (Table 1, Fig. 1c).

Replacing relative sampling date by a two-level factorial predictor reflecting the two distinct sampling periods led to very similar results and identical conclusions (Table 1b, see also Table 2).

Sperm total length differed between German samples from the egg-laying period, German samples from the nestling period and Norwegian samples ($\chi^2 = 18.4$, $df = 2$, $p < 0.001$). Tukey posthoc analysis revealed that the Norwegian samples differed significantly only from the German samples obtained during egg-laying ($z = 3.52$, $p = 0.001$), but not from those obtained during the nestling period ($z = 0.57$, $p = 0.83$; Fig. 2, see also Table 2).

DISCUSSION

Across animal taxa, evidence for gamete plasticity is scarce in general (see compilation of relevant work in Table 1 in Marshall, 2015). Examples include age-dependent effects on sperm size in insects (Green, 2003) and fishes (Gasparini *et al.*, 2010, Mehlis & Bakker 2013) or temperature-dependent effects on sperm size in insects (Blanckenhorn & Hellriegel, 2002), fishes (Adriaenssens *et al.*, 2012) and molluscs (Minoretti *et al.*, 2013). Only four studies, however, all using bird model systems, were able to demonstrate seasonal variation in sperm morphology (Lüpold *et al.*, 2012; Cramer *et al.*, 2013; Laskemoen *et al.*, 2013a; Edme *et al.*, revision under review). In contrast to our results in great tits, Lüpold et al. (2012) found absolute and relative flagellum length in red-winged blackbirds *Agelaius phoeniceus* to increase within individual males across the breeding season and Cramer et al. (2013) likewise reported a seasonal increase in the flagellum/head length ratio in house wrens *Troglodytes aedon* (note, however, that the latter study could not distinguish effects within individual from selective (dis-) appearance). Laskemoen et al. (2013a) found no evidence for seasonal variation in sperm morphology in barn swallows *Hirundo rustica*. Finally, Edme et al. (revision under review) demonstrated an increase in sperm total length over the breeding season in the collared flycatcher *Ficedula albicollis*. Thus our study is one of the first to demonstrate that individual phenotypic plasticity underlies seasonal variation in sperm morphology in any taxon. Clearly more studies are required to address why responses appear to differ by species though.

One possible non-adaptive explanation for the observed seasonal decrease in sperm length in our study could be that males have trimmed back investment in reproductive organs

during the first brood nestling period, possibly mediated by corresponding seasonal trajectories of hormonal profiles (e.g. Wingfield & Farner, 1978; Pinxten *et al.*, 2007; Kempenaers *et al.*, 2008). In this case shrinking reproductive tissues may constrain sperm design and result in the production of shorter spermatozoa (see Lüpold *et al.*, 2009). The seasonal decrease in sperm total length was mainly due to a decrease in tail length and it is conceivable that this morphologically least complex part of the sperm cell, which is produced last during spermatogenesis in the elongation phase, is affected most by shrinkage of the testes and their seminiferous tubules. However, when we were collecting sperm samples of great tits as well as coal tits *Periparus ater* and blue tits *Cyanistes caeruleus* during the nestling period of the first brood in our study area, males demonstrated a well-developed cloacal protuberance, and provided large experimental ejaculates with highly motile sperm. (Appendix has representative sample videos of three great tit males included in our study. The three come from the German population. Video equipment was available only during the nestling period of that population hence we could not include sperm motility in our analyses.) In the year of our study, 64% out of 22 identified females whose males were sperm-sampled during the first brood nestling period were found initiating second clutches. Laying dates for these clutches (calculated back from either incomplete clutches or a combination of clutch size and hatching date while assuming a 12-day incubation period) were on average (± SD) 10.9 ± 4.1 (range: 3 – 17) days separated from the date of sperm sampling. Given the potential of extended sperm storage in female passerines (e.g. Birkhead & Møller, 1992) and probable male uncertainty over when exactly social and potential extra-pair mating partners initiate second clutches and become fertile again, we expect the sperm phenotypes observed during the first brood nestling period to be fully functional and well comparable to those sampled earlier (see also below).

The observed seasonal dynamics in sperm size could potentially also represent an adaptive response to changes in the level of sperm competition (Crean & Marshall, 2008; Immler *et al.*, 2010; Marshall, 2015) assuming the latter varies with season. This appears to be the case in our study population, where the probability that a nestling in a second brood was sired by an extra-pair male was four times that of first broods (binomial generalised linear mixed model, $p < 0.001$, own unpublished data from the year 2012, see also Lubjuhn *et al.*, 2001). There is, however, no clear picture as to which sperm morphological traits, or trait values, promote competitive fertilization success in general (Pitnick *et al.*, 2009) and in passerine birds in particular (cf. Table 6 in Saetre *et al.*, 2018). In passerine birds, for example, results from tree swallows *Tachycineta bicolor* showing a positive correlation

between the ratio midpiece/total sperm length and competitive fertilization success (Laskemoen *et al.*, 2010) would support an adaptive explanation, while evidence from the zebra finch *Taeniopygia guttata* demonstrating that longer (and thus faster, Mossman *et al.*, 2009) sperm are more successful (Bennison *et al.*, 2015) would not. In order to conclusively test the hypothesis that individual phenotypic plasticity in avian sperm morphology represents an adaptive response to variation in levels of sperm competition, an experimental approach is needed that not only creates experimental social environments predictive of differential levels of sperm competition (Immler *et al.*, 2010), but also includes fitness assays to probe the competitive fertilization success of different sperm phenotypes under a match/mismatch paradigm (Groothuis & Taborsky, 2015).

Conditional on the detected seasonal effects, we have documented significant between-ejaculate repeatability in both sperm total length (0.31) and mean sperm length per sample (0.68) within males (see also Birkhead & Fletcher, 1995; Morrow & Gage, 2001; Lüpold *et al.*, 2012; Laskemoen *et al.*, 2013b). This demonstrates a high degree of within-individual consistency in sperm length despite the observed phenotypically plastic changes across the breeding season and likely reflects the substantial heritability of sperm morphological traits (Birkhead *et al.*, 2005; Edme *et al.*, revision under review). In fact, our repeatabilities, reflecting estimates of upper bounds of expected realised heritabilities in ecologically relevant settings, align well with the only available heritability estimates from a natural bird population (0.21 ± 0.07 SE for sperm total length or 0.44 ± 0.14 SE for mean sperm total length per sample in the collard flycatcher, Edme *et al.*, revision under review).

Heritabilities for sperm morphological traits from natural bird populations have yet to be estimated but our repeatability estimates may serve to indicate an upper bound of the expected realised heritabilities in ecologically relevant settings.

Another important consequence of the observed seasonal variation in sperm dimensions is that whether or not populations appeared to differ in sperm morphology in our study depended on which of the German sub-samples was used. Our analysis thus tellingly illustrates that seasonal variation in sperm dimensions within populations has the potential to confound between-population comparisons unless sampling date in relation to the reproductive cycle is controlled for. As the Norwegian samples had been collected during the pre-laying and/or egg-laying period, we propose that both populations might indeed differ in sperm total length and thus our study appends to the few studies suggesting population differentiation in avian sperm traits (Lüpold *et al.*, 2011; Schmoll & Kleven, 2011; Hogner *et al.*, 2012; but see Lifjeld *et al.*, 2012; Laskemoen *et al.*, 2013a; Gohli *et al.*, 2015). More

generally – and applicable well beyond avian study systems and seasonality – accounting for potentially widespread environmental effects on sperm phenotype will advance our understanding of sources of sperm morphological variation in a within-species context.

APPENDIX online at evolutionary-ecology.com/data/3131Appendix.pdf

It contains:

1- A table 1 recording repeatability for repeated measurements of sperm total length and the sperm sections (head, midpiece and tail length) of the same individual spermatozoa.

2- A figure showing seasonal variation and individual phenotypic plasticity in the length of sperm sections (head, flagellum, midpiece, tail).

3- Three video files (MP4) that illustrate typical patterns of sperm motility of three individual great tit (*Parus major*) males sampled in Germany during the first brood-nestling period and included in this study.

AUTHORS' CONTRIBUTIONS

TS conceived the study, participated in the design of the study, supervised and participated in field work, performed the statistical analysis and wrote the manuscript. OK participated in the design of the study and in field work. MR participated in field work and developed and executed the microscopy protocol. All authors proofread earlier versions of the manuscript and approved its final version.

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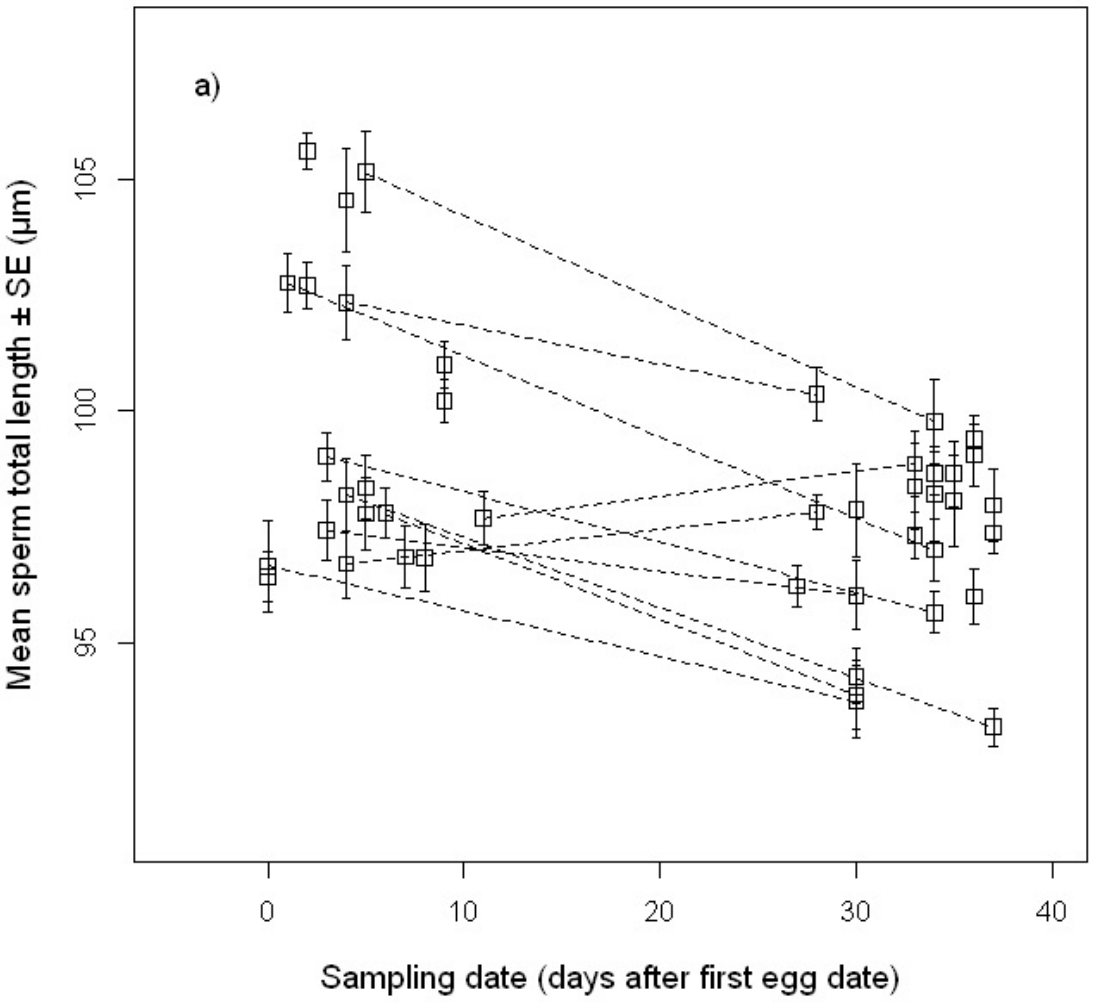
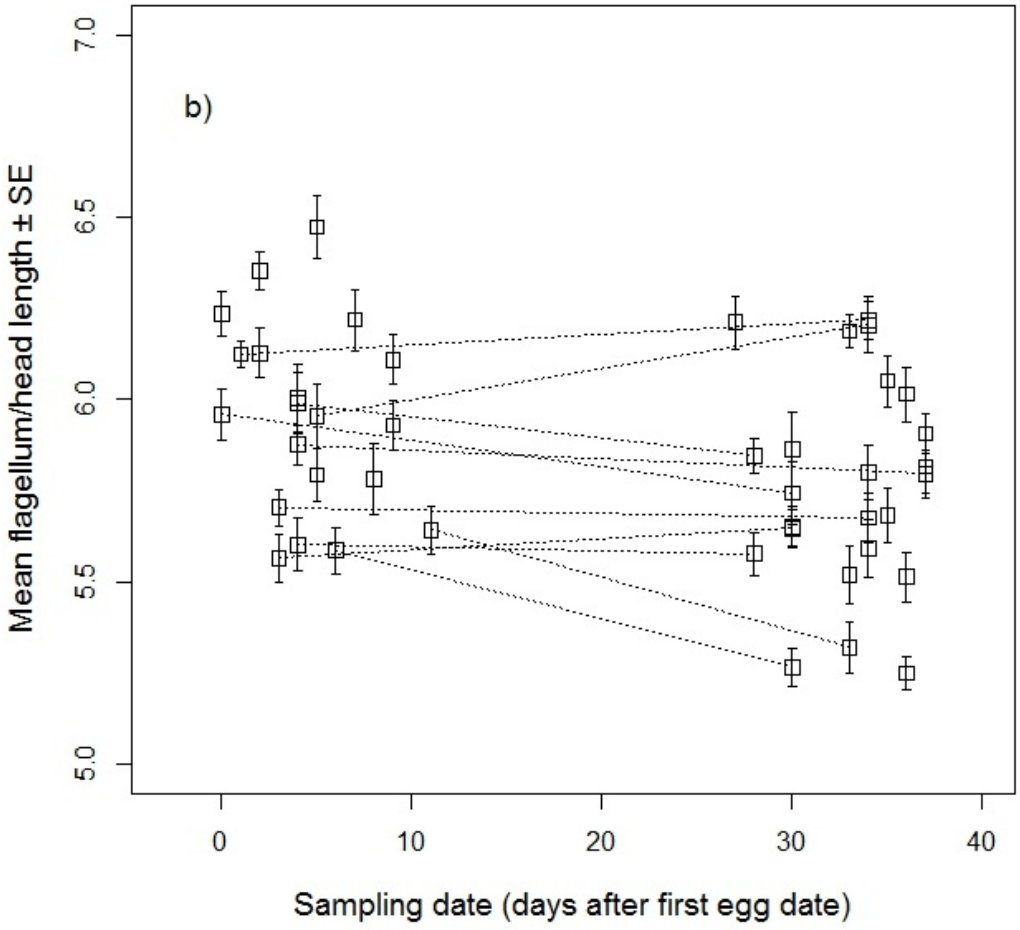
FIGURES

Fig. 1 Seasonal variation and individual phenotypic plasticity in a) sperm total length, b) flagellum/head length ratio and c) midpiece/total length ratio in a German great tit population (N = 44 sperm samples from 34 males). Flagellum length is the sum of sperm midpiece and tail length. Lines connect estimates (± SE) for the same males sampled during both the first brood egg-laying period of their social females and the respective nestling feeding period.




b)
Mean flagellum/head length ± SE
7.0
6.5
6.0
5.5
5.0
0
10
20
30
40
Sampling date (days after first egg date)

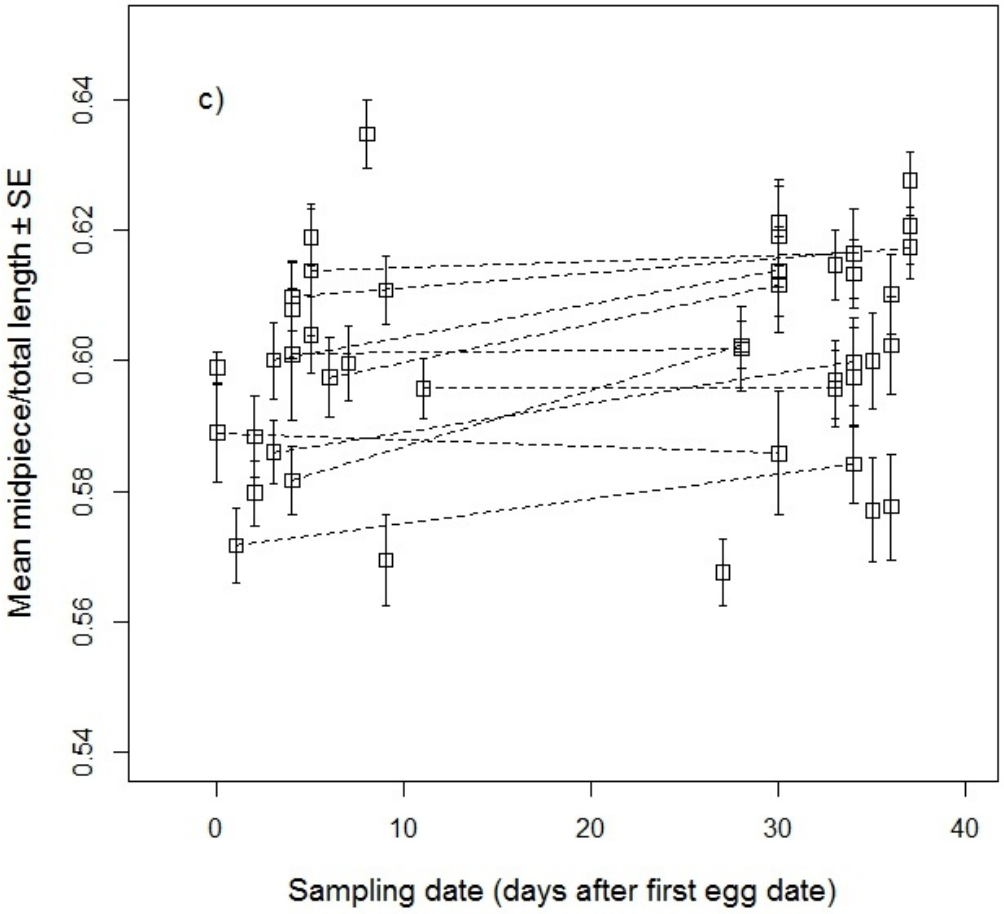

c)
Mean midpiece/total length ± SE
0.54
0.56
0.58
0.60
0.62
0.64
0
10
20
30
40
Sampling date (days after first egg date)

Fig. 2 Tukey's Honestly Significant Differences (± 95% family-wise confidence intervals, CI) between mean sperm total length of spermatozoa sampled in a German great tit population during the egg-laying period of the males' social females (Germany Laying, $N = 20$), the respective nestling feeding period (Germany Feeding, $N = 24$) and a Norwegian population (Norway, $N = 10$). We show results from Tukey posthoc tests for a linear mixed effects model including sperm sample identity nested in male identity as random effects.