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## **Sperm dimensions differ between two coal tit *Periparus ater* populations**

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## **Abstract**

The evolutionary forces that shape and maintain variation in sperm traits across species in birds have recently attracted increased attention. Much less is known about the patterns, the causes and the consequences of variation in sperm traits within and, in particular, between populations of the same species. We here analyse intraspecific variation in sperm dimensions within and between two populations of the coal tit *Periparus ater*, a socially monogamous passerine with a high frequency of extra-pair paternity. Spermatozoa from a Norwegian population had a greater mean total length compared to those from a German population, mainly as a result of longer sperm heads. Sperm head length in the Norwegian population also accounted for a larger percentage of sperm total length suggesting differences in sperm proportions between populations. Furthermore, spermatozoa from the Norwegian population showed lower mean within-male variation in sperm length and there was significant between-male variation in sperm total length within each of the two populations. We discuss these results in the light of recent comparative evidence suggesting relationships between sperm length, and variation in sperm length, and the frequency of extra-pair paternity across passerine birds.

## **Keywords**

Sperm morphology, sperm proportions, sperm competition, population differences, intraspecific variation, extra-pair paternity

## Introduction

The staggering diversity in form and function of spermatozoa across taxa and the evolutionary forces that have shaped and do maintain this variation have attracted a lot of attention (reviewed in Birkhead et al. 2009). In socially monogamous bird species, extra-pair fertilisations are the rule rather than an exception (Griffith et al. 2002) and constitute a potentially powerful source of postcopulatory sexual selection on sperm traits. This idea is supported by recent comparative evidence suggesting relationships between sperm total length as well as sperm swimming speed and extra-pair paternity: Both traits were positively – though independently – related to the frequency of extra-pair paternity across passerine bird species (Kleven et al. 2009a; see also Briskie et al. 1997). Furthermore, *within-male* variation in sperm dimensions (Immler et al. 2008; see also Kleven et al. 2008) and *between-male* variation in sperm length (Calhim et al. 2007; Kleven et al. 2008) were negatively related to the frequency of extra-pair paternity across passerines, indicating that postcopulatory sexual selection may not only favour longer and faster spermatozoa, but also sperm of less variable length with increasing risk of sperm competition. While sexual selection through extra-pair fertilisations can explain substantial variation in sperm traits between species, little is known about the intraspecific variation in sperm morphology, its causes and its consequences in natural bird populations (Laskemoen et al. 2007). This is true for variation *within* populations, but in particular for variation *between* populations of the same species.

Here we analyse intraspecific variation in sperm dimensions in the coal tit *Parus ater*, a socially monogamous passerine with a high frequency of extra-pair paternity (Lubjuhn et al. 1999; Dietrich et al. 2004). We address variation within and between individual males in a Norwegian and a German population of coal tits and compare sperm dimensions, and variation in sperm dimensions, between these populations.

## **Methods**

### **Study populations and field methods**

We studied territorial male coal tits during the breeding season in Norway and Germany (for details see Table 1). A total of 20 sperm samples were obtained non-invasively by gently massaging the cloacal protuberance (Wolfson 1952) of birds that had been mistnetted while singing and/or responding to playback (Norway: ten males and Germany: six males) or that were captured while feeding nestlings ten to 14 days old (Germany only: four males). Sperm samples were fixed and stored in 5% formalin solution until further use. If not already ringed, the birds were banded with uniquely numbered metal rings and released immediately after sampling.

### **Sperm morphometry**

A droplet of the formalin solution was placed on a microslide and air-dried. Sperm were then examined by digital light microscopy using a Leica DM6000 B microscope at  $\times 320$  magnification and photographed with a digital camera (Leica DC500). Sperm head, midpiece and tail length (for delimitation of sections see Figure 1 in Kleven et al. 2009b) were measured to a precision of 0.1  $\mu\text{m}$  using Leica IM1000 analysis software (Leica Microsystems, Heerbrugg, Switzerland) and sperm total length was calculated as the sum of these components. Ten randomly selected spermatozoa of each of ten males per population were measured by the same person (O. K.) within a 28 day period in autumn 2009. Sampling effort of a similar magnitude has been shown to give representative estimates for the mean and the variation in sperm dimensions in populations of two other passerine birds (Laskemoen et al. 2007). Measurement repeatabilities (*sensu* Lessels and Boag 1987), based on 10 spermatozoa measured blindly twice, were high for all sperm dimensions (sperm total length:  $r = 0.95$ ,  $F_{9,10} = 36.7$ ,  $p < 0.001$ ; head length:  $r = 0.68$ ,  $F_{9,10} = 5.2$ ,  $p = 0.008$ ; midpiece length:  $r = 0.97$ ,  $F_{9,10} = 74.3$ ,  $p < 0.001$ ; tail length:  $r = 0.96$ ,  $F_{9,10} = 54.0$ ,  $p < 0.001$ ).

## Statistical analysis

We used R 2.9.0 (R Development Core Team 2009) for all computations and linear mixed effects models (LME, R function *lme* in library *nlme*, Pinheiro et al. 2006) to test for differences between populations in sperm traits. Male identity was included as a random effect to account for the non-independence of measurements obtained from the same individual and to estimate between-male random variation in sperm dimensions. P values in the context of LME analysis refer to the increase in model deviance (compared against a  $\chi^2$  distribution) when a term is removed from the current model and the significance of a random effect was tested by removing it from the minimal adequate model. Sperm head, midpiece and tail length relative to sperm total length was compared between populations by testing for differences in sperm head, midpiece and tail length, respectively, while simultaneously controlling for sperm total length in the LME. The coefficient of variation (CV) for a given sample was calculated as  $CV = \text{standard deviation (SD) of the sample} / \text{mean of the sample} * 100\%$ . The *within-male* CVs for sperm total length were compared between populations with a t-test. 95% confidence intervals for *between-male* CVs were obtained by non-parametric bootstrapping (N = 10000 replicates per population). Intraclass correlation coefficients, or repeatability, for sperm total length were calculated according to Lessels & Boag (1987) and the calculation of standard errors for repeatability estimates followed Becker (1992). All statistical tests were two-tailed and the null hypothesis was rejected at  $p < 0.05$ .

## Results

Spermatozoa from the Norwegian population were on average 3.2% longer compared to those from the German population (Table 2). This difference resulted mainly from a difference in sperm head length: Spermatozoa from the Norwegian population had on average 7.3% longer heads (Table 2), while no significant differences between populations were detectable in midpiece and tail size (Table 2). Sperm head length in the Norwegian population accounted

for a slightly larger percentage of sperm total length compared to the German population (Table 2), while no significant differences in relative length were evident for midpiece and tail length (Table 2). While the *between-male* coefficient of variation in sperm total length was 2.57 (95% confidence interval: 1.19-3.17) for the Norwegian but only 1.77 (0.95-2.31) for the German population, the mean *within-male* coefficient of variation was nearly 40% higher in the German population (CV  $\pm$  SE: Norway:  $1.44 \pm 0.35$ ; Germany:  $2.00 \pm 0.53$ ;  $t = -2.78$ ,  $df = 18$ ,  $p = 0.01$ ; see also Figure 1). Repeatability analysis within each of the two populations revealed that 74.4% ( $\pm$  SE: 9.8%;  $F_{9,90} = 30.1$ ,  $p < 0.001$ ) and 38.9% ( $\pm$  13.7%;  $F_{9,90} = 7.4$ ,  $p < 0.001$ ) of the total variation in sperm length was explained by variation between males in the Norwegian and the German population, respectively. We found significant between-male random variation within each of the two populations in sperm total length (Norway:  $\chi^2 = 97.4$ ,  $df = 1$ ,  $p < 0.001$ ; Germany:  $\chi^2 = 27.3$ ,  $df = 1$ ,  $p < 0.001$ ) as well as in all other sperm dimensions measured (all  $\chi^2 > 9.29$ , all  $df = 1$ , all  $p < 0.01$ ).

## Discussion

We have shown differences in the mean, the proportion and the variation of sperm dimensions between a Norwegian and a German coal tit population. But four out of the ten German samples were obtained from males while feeding nestlings which might confound these findings, for example if sperm traits of individual males (and/or variation in these traits) change during the breeding season. However, none of the traits showing significant between-population differences differed between the four males sampled while feeding nestlings *versus* males that were sampled earlier in the season in the German population (LME: all  $p > 0.1$ ). Furthermore, excluding the four males from analysis does not affect conclusions (LME: all  $p < 0.02$ ) except for proportion head length (LME:  $p = 0.06$ ) suggesting a fair degree of robustness for these analyses. Finally, although poorly investigated in birds, sperm size traits seem to be highly repeatable across ejaculates for individual males (Birkhead et al.

1995; Møller et al. 2008). We therefore consider it unlikely that the sampling at partly different stages during the reproductive cycle in the two study populations has affected our results. However, to elucidate potential within-seasonal variation in sperm traits, a longitudinal approach that tracks and compares sperm size traits within individual males is required (see Immler et al. 2010 for a recent example of individual phenotypic plasticity in sperm size traits). Furthermore, storage duration differed for samples from the two populations (two and a half vs. half a year). If storage duration in formalin affects sperm morphology we would assume that spermatozoa tend to shrink with time. In our case, however, the on average shorter spermatozoa (from the German population) are the ones with the shorter storage duration (see methods). We therefore argue that our results were not confounded by a storage duration effect.

Between-population comparisons in sperm morphology are scarce in general and a recent review compiled a few case studies from insect, mollusc and anuran species, but none from birds (Pitnick et al. 2009). Three other studies have shown differences in sperm morphology between populations in birds. Møller et al. (2008) compared sperm morphology and sperm motility of barn swallows *Hirundo rustica* from the Chernobyl region with two less radioactively contaminated control areas further away. Swallow males breeding near Chernobyl showed a higher proportion of sperm with abnormal morphology and had, on average, shorter tails compared to those breeding in control areas (note that "tail" here refers to the sum of midpiece and tail in our terminology, see Møller et al. 2008). Kleven and co-workers (2009b) compared published data on sperm length in the sand martin *Riparia riparia* (Helfenstein et al. 2008) with data obtained from two other populations sampled by themselves. Apparent differences in mean sperm total length between their samples and the data of Helfenstein et al. (2008), however, were likely due to methodological problems in the latter study (discussed in Kleven et al. 2009b). Finally, Lüpold et al. (2010) recently reported significant between-population variation in sperm morphology in the red-winged blackbird



*Agelaius phoeniceus*. Thus the present study is among the first to demonstrate differences in sperm dimensions between undisturbed populations of the same bird species.

Recent comparative evidence indicates a positive relationship between sperm length and extra-pair paternity (Kleven et al. 2009a; see also Lüpold et al. 2009a) and a negative relationship between within-male variation (Immler et al. 2008; see also Kleven et al. 2008) as well as between-male variation (Calhim et al. 2007; Kleven et al. 2008) in sperm length and extra-pair paternity across passerine birds. In our study, the Norwegian population showed longer spermatozoa and lower within-male variation in sperm length than the German population, while between-male variation was statistically indistinguishable (although the Norwegian population shows a lower degree of variation here, too, and the limited sample size may have restricted the power of this analysis). Based on this, we would predict that the frequency of extra-pair paternity in the Norwegian is higher compared to the German population. However, while the frequency of extra-pair paternity in the German study population is quite high (around 28% extra-pair offspring in first and up to nearly 50% extra-pair offspring in second broods, Dietrich et al. 2004), no information is available on the frequency of extra-pair paternity in the Norwegian population. The difference in sperm total length resulted mainly from a difference in sperm head length and sperm heads were proportionally larger in the Norwegian population. The sperm head contains the acrosome and the nucleus and it is not clear why postcopulatory sexual selection should favour longer sperm heads. Rather it may be expected that the midpiece (containing the mitochondria) and/or the tail should be under postcopulatory sexual selection for greater length through extra-pair fertilisations (Lüpold et al. 2009b; see also Laskemoen et al. 2010). Furthermore, interspecific associations between traits as revealed by comparative methods are not necessarily found also on intraspecific levels. Thus the observed differences between the two populations may well not be related at all to (the contemporary degree of) sperm competition in these populations. For example, a different phylogenetic history (involving genetic drift or different correlated

selection pressures) may be responsible for the differential patterns of sperm morphology in these two populations as they are likely to belong to different phylogeographic lineages (Martens et al. 2006; see also Lüpold et al. 2010, who report a lack of an association between mating system and sperm morphology across populations in red-winged blackbirds).

We conclude that at present it is unclear whether the detected between-population differences in sperm morphology represent the rule or an exception and whether and how they may reflect adaptations to sperm competition and/or to postcopulatory mechanisms of female choice. Thus an important goal for future research is to test for differences in sperm traits across many different populations in this and in other species and to investigate the relation to the degree of sperm competition and possibly other traits.

With respect to within-population variation, it is surprising that only a single study up to date has related individual variation in sperm traits to individual variation in male fertilisation success in a natural bird population. Laskemoen et al. (2010) showed that relative midpiece size was positively correlated to male fertilization success in tree swallows *Tachycineta bicolor* and suggested that this effect is mediated via increased sperm swimming speed due to higher mitochondrial loading. In the German coal tit study population, no consistent predictors of male extra-pair fertilisation success were detectable (T. Schmoll, W. Winkel, T. Lubjuhn, unpublished data) with the notable exception of male age (Schmoll et al. 2007). Thus the significant between-male variation in sperm morphology suggests a potential for sperm traits to explain individual differences in (extra-pair) fertilisation success in this and possibly in other natural populations of birds. Establishing that variation in sperm traits relates to variation in extra-pair paternity not only between, but also within species may represent a major step towards understanding sexual selection in birds. We therefore propose analysing variation in key sperm traits and their relationship to extra-pair paternity in a within-species context in the future.

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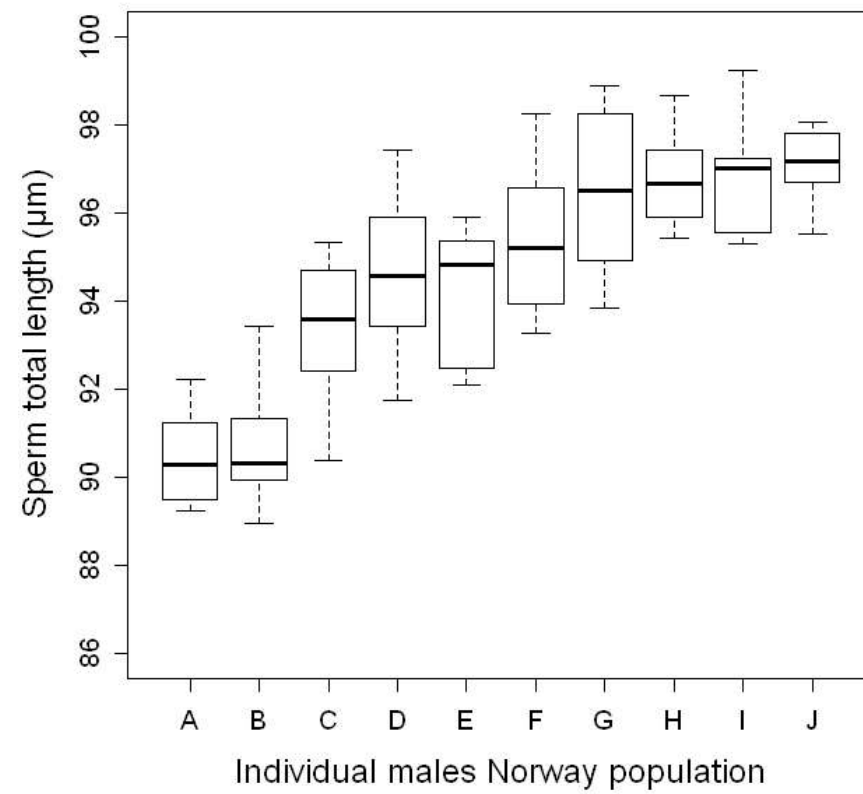
### Figure captions

**Fig. 1** Variation in sperm total length for a) ten males of a Norwegian and b) ten males of a German coal tit population ( $N = 10$  spermatozoa per male). Plots show medians, interquartile range (box) and data within 1.5 times the interquartile range (whiskers)

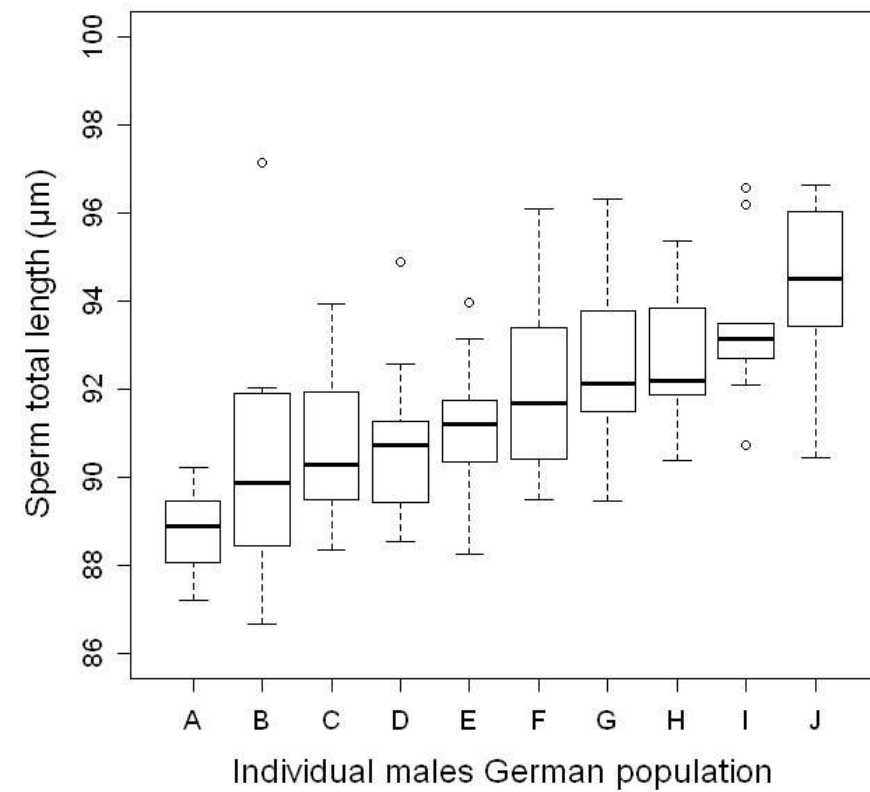
## Figures

Figure 1

a)



b)





## Tables

Table 1 Details of the study sites and coal tit study populations investigated.

	Norwegian population	German population
Locations	Three localities near Oslo	D-48488 Elbergen near Lingen/Ems
Coordinates	Sørkedalen: 59°58' N, 10°37' E, Sørkedalen: 60°01' N, 10°37' E, Skien: 59°20' N, 09°33' E	52°27' N, 7°15' E
Habitat	Lowland mixed coniferous forest	Lowland mixed coniferous forest
Sampling period	2007, April 13 <sup>th</sup> to May 5 <sup>th</sup>	2009, April 18 <sup>th</sup> to June 5 <sup>th</sup>

Table 2 Sperm morphometrics for ten spermatozoa of each of ten males from each of the two coal tit study populations. Descriptive statistics refer to population-wide estimates and significance testing used linear mixed effects models that included male identity as a random effect.

Trait	Norway		Germany		$\chi^2$	df	p
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range			
Total length [ $\mu\text{m}$ ]	94.6 $\pm$ 2.7	89.0-99.2	91.7 $\pm$ 2.4	86.7-97.2	8.67	1	0.003*
Head length [ $\mu\text{m}$ ]	14.6 $\pm$ 0.7	13.1-17.2	13.6 $\pm$ 0.9	11.6-15.8	15.83	1	<0.001*
Midpiece length [ $\mu\text{m}$ ]	56.1 $\pm$ 2.1	52.1-61.1	55.1 $\pm$ 2.1	50.3-60.3	2.08	1	0.15
Tail length [ $\mu\text{m}$ ]	23.9 $\pm$ 2.6	17.6-29.4	23.0 $\pm$ 2.3	17.4-28.8	1.18	1	0.28
Percentage head length	15.4 $\pm$ 0.8%	13.6-18.1%	14.8 $\pm$ 0.9%	12.8-17.4%	7.30 <sup>a</sup>	1	0.007*
Percentage midpiece length	59.3 $\pm$ 2.2%	55.2-64.6%	60.1 $\pm$ 2.1%	54.9-65.5%	0.02 <sup>b</sup>	1	0.90
Percentage tail length	25.2 $\pm$ 2.3%	19.5-29.8%	25.1 $\pm$ 2.2%	19.7-30.5%	1.20 <sup>c</sup>	1	0.27

<sup>a</sup>Effect of population on sperm head length while controlling for sperm total length.

<sup>b</sup>Effect of population on sperm midpiece length while controlling for sperm total length.

<sup>c</sup>Effect of population on sperm tail length while controlling for sperm total length.

\*Significant after sequential Bonferroni-Holm correction.