INTRASPECIFIC SPERM LENGTH VARIATION AND TWO SECONDARY SEXUAL CHARACTERS IN THE PIED FLYCATCHER (FICEDULA HYPOLEUCA)

INTRASPESIFIKK VARIASJON I SPERMIELENGDE OG TO SEKUNDÆRE KJØNNSKARAKTERER HOS SVARTHVIT FLUESNAPPER (FICEDULA HYPOLEUCA)

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Abstract

Spermatozoa are amongst the most variable of all animal cells, varying enormously in size and shape both between and within different species. While interspecific variation in sperm traits over the last decades has been well documented for several taxa, including birds, surprisingly little is known about intraspecific variation in avian sperm traits. In this thesis I aimed to study and quantify the level of intraspecific variation (both between and within males) in sperm length in the pied flycatcher (*Ficedula hypoleuca*), a migratory passerine. I attempted to repeat the study of Calhim et al. (2009), who argued that their findings of significant negative associations between mean total sperm length and two secondary sexually selected traits in the pied flycatcher indicated that pre-copulatory sexual selection seems to be operational in this species, favouring longer sperm cells. I also investigated whether intra-male sperm length and its variation changes over a breeding season.

Substantial variation in mean total sperm length was found both between and within males in this study, which was consistent with the fact that the level of sperm competition in the pied flycatcher is low. My results gave no evidence for any significant associations between gametic morphology and two secondary sexually selected traits in this species, and were thus in contrast with the findings of Calhim et al. (2009), but in accordance with all previous studies on passerines as well as with sexual selection theory. As blacker and earlier arriving males seem to be chosen by females in order to obtain direct benefits from parental care and good territories rather than indirect genetic benefits, I conclude that pre-copulatory sexual selection on sperm quality traits seems unlikely in the pied flycatcher. As the sample sizes in my analyses regarding change in mean total sperm length and intra-male variation over a breeding season were very small, I found it somewhat difficult to discuss these results, since it is unclear whether my findings are valid at the population level. Future studies investigating larger datasets are therefore required in order to clarify whether or not mean total sperm length and intra-male variation changes in any specific direction over the pied flycatcher breeding season. Additional studies concerning distribution and skewness values in avian sperm samples might also help us understand more about intraspecific sperm length variation in this and other species.

Sammendrag

Spermier er, med sin enorme variasjon i størrelse og form både innen og mellom arter, blant de mest variable cellene i dyreriket. I løpet av de siste tiårene har det blitt publisert mange arbeider som dokumenterer interspesifikk variasjon i spermiekomponenter hos ulike arter, også blant fugler, men kunnskapen vår om intraspesifikk spermievariasjon er fortsatt overraskende liten. I denne masteroppgaven har jeg derfor sett nærmere på graden av intraspesifikk variasjon i spermielengde (både mellom hanner og innen enkeltindivider) hos svarthvit fluesnapper (*Ficedula hypoleuca*). Jeg har forsøkt å gjenta en nylig publisert artikkel skrevet av Calhim et al. (2009), der forfatterne påviste en signifikant negativ sammenheng mellom gjennomsnittlig spermielengde og to ulike sekundære kjønnskarakterer hos denne arten. Videre har jeg undersøkt om gjennomsnittlig spermielengde og variasjon hos enkelthanner endrer seg i løpet av fluesnapperens hekkesesong.

Resultatene mine i denne oppgaven påviste en betydelig variasjon i gjennomsnittlig spermielengde både mellom og innen hanner hos svarthvit fluesnapper, hvilket er logisk med tanke på at graden av spermiekonkurranse hos denne arten er lav. I motsetning til Calhim et al. (2009) fant jeg ingen bevis for en signifikant negativ sammenheng mellom gjennomsnittlig spermielengde og de to ulike sekundære kjønnskarakterene som ble vurdert. Mine resultater sto derfor i kontrast til resultatene fra Calhim et al. (2009), men stemte godt overens med alle øvrige arbeider på spurvefugler og også med gjeldende teori på dette feltet. Siden hunnene hos svarthvit fluesnapper ser ut til å foretrekke svarte og tidlig ankommende hanner hovedsakelig på grunn av foreldreomsorg og gode territorier, og ikke på grunn av indirekte fordeler, konkluderer jeg med at pre-kopulativ seksuell seleksjon på spermielengde hos denne arten virker lite sannsynlig. Da antallet individer som kunne inkluderes i testene vedrørende endring i gjennomsnittlig spermielengde og innenhannsvariasjon i denne oppgaven var veldig lavt, fant jeg det noe vanskelig å diskutere disse resultatene. Jeg anbefaler derfor at flere studier med større datasett bør gjennomføres før generelle konklusjoner vedrørende endringer i gjennomsnittlig spermielengde og innenhannsvariasjon kan presenteres. Videre kan også nye studier som fokuserer på spermienes normalfordeling og skjevhetsverdier være til nytte for å hjelpe oss til å forstå enda mer angående intraspesifikk variasjon i spermielengde både hos svarthvit fluesnapper og hos andre spurvefugler.

Introduction

In the animal kingdom, spermatozoa are amongst the most variable of cells (Briskie et al. 1997, Malo et al. 2006, Calhim et al. 2007, Immler and Birkhead 2007, Laskemoen et al. 2007, Kleven et al. 2008 etc.). In fact, some claim that they are by far the most diverse, varying enormously in size and shape both between and within different species. Considerable research effort has focused on the variation in sperm morphology between species, and such interspecific variation in sperm traits is well documented for several taxa, including birds (Ward 1998, Snook 2005, Laskemoen et al. 2007). However, as pointed out by Ward (1998) and later by Laskemoen et al. (2007) and Immler et al. (2008), surprisingly little is known about the intraspecific variation in avian sperm traits.

Significant intraspecific variation in sperm traits has been documented in several different taxa in previous studies, but the causes of the observed variation remain poorly understood (Ward 1998, Kleven et al. 2008). This is unfortunate, as intraspecific variation is an important statistical parameter in studies of differences in sperm traits (e.g. sperm length) between species. Furthermore, the level of intraspecific variation in a species may in itself be an important feature that could be shaped by evolutionary forces such as selection (Birkhead et al. 2005, Laskemoen et al. 2007). The main aim of this thesis is therefore to study and quantify the level of intraspecific variation (both between and within males) in sperm length in the pied flycatcher (*Ficedula hypoleuca*), a migratory passerine bird that has been the subject of study in a large number of research articles published over the last few decades.

In his review, Ward (1998) ended by stating that "male fertilization success is highly variable and this variation could be due, at least partly, to differences amongst males in their sperm morphologies". As sperm from several different males may compete within a single female's reproductive system in order to successfully fertilize her eggs, it has been hypothesized that sperm competition may put a powerful selection pressure on intraspecific sperm variation, enforcing stabilizing selection on sperm variation through selection against extreme sperm sizes (Ward 1998, Birkhead et al. 2005, Calhim et al. 2007, Immler et al. 2008, Kleven et al. 2008). Furthermore, directional selection might operate; favouring longer sperm, as these are thought to swim faster and have a reproductive advantage (Gomendio and Roldan 1991, Laskemoen et al. 2008). Recent studies have indeed demonstrated a negative relationship

between intraspecific sperm length variation and indices of sperm competition risk (Calhim et. al. 2007, Immler et al. 2008, Kleven et al. 2008). Thus, post-copulatory sexual selection mediated by sperm competition may lead to a considerable selection pressure on sperm cells in species with a higher competition risk, reducing the intraspecific variation in sperm length.

Also, in a study on the genetic effects on sperm design in the zebra finch (*Taeniopygia guttata*), Birkhead et al. (2005) found a high level of additive genetic variance, implying a high selection potential. The authors concluded that the high inter-male variation found in sperm size traits in this study could be attributed to the low level of sperm competition in the zebra finch (Birkhead et al. 2005). Inter-male variation in sperm traits is generally predicted to be greater in species where post-copulatory selection is relaxed (Calhim et al. 2007). In these species, where there is little or no sperm competition, little is known about the effect of pre-copulatory sexual selection on sperm traits. It is therefore of interest to examine whether male traits subject to pre-copulatory sexual selection, e.g. male ornaments preferred by females in social mate choice, are associated with sperm traits.

In a recent study of selection on sperm morphology in the pied flycatcher, Calhim et al. (2009) examined a study population under relaxed levels of sperm competition. Assuming that longer sperm length indicates gametic quality (as hypothesized in other studies), so that males with longer sperm have a greater fertilization success, Calhim et al. (2009) tested whether there exists an association between gametic morphology and two major female preference traits in this species; namely male plumage blackness and breeding date (as a proxy for arrival date). The results gave evidence for such an association between sperm total length and each of the two traits (Calhim et al. 2009). Thus, it seemed that blacker and earlier arriving males, which are preferred by females in pair formation, also had the longest total sperm length. Calhim et al. (2009) concluded that out of several theories regarding the role of sexual selection on sperm traits, their results were most consistent with the sexually selected sperm hypothesis (reviewed by Pizzari and Birkhead 2002). Furthermore, they suggested that pre-copulatory sexual selection seems to favour longer sperm in this species. However, the results of Calhim et al. (2009) are associated with some uncertainty, as the analyzed dataset was very small, consisting of data from 17 and 13 pied flycatchers for the analyses on plumage blackness and breeding date, respectively. When examining Fig.1 a) and b) in the published article, it becomes clear that the results are strongly affected by a few single data

points (to the right in both figures). Thus, one might speculate whether any associations would have been found in the study had these points been excluded from the dataset.

Moreover, Calhim et al. (2009) did only measure the sperm length of five sperm per individual male. Laskemoen et al. (2007) had previously recommended that, based on studies on the bluethroat (*Luscinia svecica*) and the willow warbler (*Phylloscopus trochilus*), a minimum of ten males per species and ten sperm cells per individual male should be measured in order to get an adequate estimate of mean sperm length and its variation. Because of these and other uncertainties, I have aimed to repeat the analysis of Calhim et al. (2009). By analyzing a new and extended data set from the same study population of pied flycatchers as was studied by Calhim et al. (2009), I have tested whether the associations between mean total sperm length and the two secondary sexually selected traits are robust when sample sizes are increased, and thus also whether or not pre-copulatory sexual selection on sperm length may be invoked for this species.

In their study, Calhim et al. (2009) "used breeding date as a proxy of arrival date since early breeding directly reflects male quality", because arrival date (and consequently the territory quality) is the main predictor of female settlement in several populations of pied flycatchers (Alatalo et al. 1986, Calhim et al. 2009). While breeding date (the day the first egg is laid by the social female) may indeed be used as an indicator of arrival date in studies like this, it would be better to use the actual arrival date directly in a test when such data are available. This is because several males in the field might be unsuccessful in finding a mate, and as a result, no breeding date will recorded for these males, as there are no eggs laid by a social female. Males without a registered breeding date may then be excluded from analyses, giving a smaller dataset. Furthermore, breeding date may not directly reflect arrival date, as some early arriving males may breed relatively late, and late males sometimes succeed in breeding relatively quickly. Apart from giving a more precise measure of male quality, as arrival date is the actual trait preferred by female pied flycatchers, arrival date may, in contrast to breeding date, ideally be obtained from all males. I have therefore used actual arrival date rather than breeding date as a measure of male quality in the analyses of this thesis. In order to fully compare my results to those of Calhim et al. (2009), however, I have also tested for an association between mean total sperm length and breeding date.

Sperm production is a highly complex process (Immler et al. 2008). In the breeding season, the reproductive organs in both sexes of the pied flycatcher first increase in size before the relatively short period of copulations immediately before egg laying, before decreasing again around the time of egg hatching. Birkhead et al. (1997) showed that female pied flycatchers seem to store sperm in their sperm-storage tubules (SSTs) for only a very short period in each breeding attempt; meaning that males should time their copulation activity to coincide with this limited period. Testis size in birds have been used in several studies as an index of sperm competition, as there is a positive relationship between the risk of sperm competition and testis size (Laskemoen et al. 2008). The common explanation for this relationship is that sperm competition drives evolution of testis size through a higher fertilization success for males having higher sperm production rates or better-quality sperm (Laskemoen et al. 2008). As the testes of pied flycatcher males develop both simultaneously and rapidly in a brief period before decreasing shortly afterwards (Jan T. Lifjeld pers. comm.), it might be interesting to study the intra-male variation in sperm length to find out whether the mean sperm lengths and variation changes throughout a breeding season. The testes may produce more sperm of an "optimal" phenotype when they are at the largest, so that the sperm cells are less variable in morphology at this period, when competitive sperm cells are most needed. When sperm is no longer needed for reproduction and the testes are decreasing in size, it is imaginable that sperm cells of less optimal phenotypes may also be produced.

In this thesis I have therefore also aimed to test the prediction that sperm cells within males are more homogeneous when the testes are fully developed, and more heterogeneous when the testes are increasing or decreasing in growth. In order to test this prediction, each individual male has been sampled twice during the breeding season; once around the start of egg laying and again around the time of hatching (see Materials and Methods). Furthermore, I have tested whether or not data on sperm lengths from different males fit a normal distribution. The sperm cells of pied flycatcher males are formed in the seminiferous tubules located in the testes. As these tubules may vary in diameter, it is thought that different-sized tubules could cause intra-male variation in sperm length as well as more variable sperm cells at the beginning and end of the breeding season, when the testes are increasing or decreasing in size. Unpublished data on house wrens in the United States have indeed shown that males sampled early in the breeding season may have more shorter sperm cells than what is predicted from a normal distribution (Jan T. Lifjeld pers. comm.). Such sperm samples will have a negative skewness value, i.e. a negative skew in the distribution of sperm lengths. By

plotting skewness values from different males against the date of sperm sampling, I have attempted to find out if males sampled earlier in the breeding season have a higher skewness value, thereby indicating more shorter sperm than expected from a normal distribution compared to males sampled later on. In sum, my study on sperm size variation is divided into two parts, both of which are looking deeper into the level of intraspecific variation in avian sperm length.

Materials and methods

Study area and study species

The field work for this thesis was carried out at the Sinober study area located in Sørkedalen (60°01'N, 10°37'E), Oslo, Norway. All sperm samples from pied flycatcher males were collected during the breeding season of 2010 (from 22 May to 25 June). The Sinober area is the same area as was used by Calhim et al. (2009). In this area, birds under study are individually marked for easy recognition, and breed in nest boxes of similar shape and size.

The pied flycatcher is a small, migratory passerine that breeds in the Palearctic region during late spring and early summer, and overwinters in tropical West Africa. It is cavity-nesting, naturally breeding in holes in trees, but is easily attracted to nest boxes, and suitable nest boxes are strongly preferred over natural cavities (Lundberg and Alatalo 1992). During the breeding season, the pied flycatcher is dichromatic, with males being very variable in plumage colour; ranging continuously from individuals being similar to (and sometimes almost indistinguishable from) the brown females to individuals exhibiting a jet black dorsal plumage (Lundberg and Alatalo 1992, Svensson and Grant 2004, Calhim et al. 2009).

Field procedures

Males were caught in their territories by use of mist nets and nest box traps together with playback and caged males. Sperm samples from each of the males were collected by gently massaging the cloacal protuberance of the birds, as described by Wolfson (1952). Freshly collected sperm samples were diluted in a droplet of Phosphate Buffered Saline (PBS) and then stored in a 5 % formalin solution, in order to preserve the sperm before later analyses in the laboratory. When possible, each individual male was sampled twice during the breeding season; once around the start of egg laying and again around the time of hatching.

All males that were captured and subsequently sperm sampled were given a score between 1 and 7, depending on their plumage colour, using the Drost seven-point scale (Drost 1936). On this scale, a score of 1 indicates a conspicuous black and white plumage, while 7 indicates a female-like, brown male. The scoring (range=1.75-7.00, median=4.00) was performed by an experienced researcher, Helene M. Lampe, who also scored the plumage of the birds in the

analyses of Calhim et al. (2009), using the same scale. Furthermore, arrival date (i.e. the date when a male was first observed in an area/territory) and breeding date (i.e. the date when the first egg of a male's social female was laid) was noted for each of the pied flycatcher males.

Sample analyses

The analyses of the sperm samples took place at the Natural History Museum, Oslo, Norway, during autumn 2010. In order to obtain sperm morphometric data, the sample tubes were first put in a centrifuge and spun for about half a minute, until most of the sperm could be found at the bottom of the tubes. After this, approximately 15 µl of fixed sperm from each sample were applied on a microscope slide using a pipette. The microscope slides were allowed to dry over night, before they were "washed" with a small amount of distilled water to remove any salt crystals. When the slides had been allowed to dry once again, they were ready for examination under the microscope.

The equipment used in the analyses of sperm variation was a Leica DFC420 camera mounted on a Leica DM6000 B light microscope, which enabled me to take digitalized photographs of sperm cells at a total magnitude of 160 X. For each sperm sample that was studied under the microscope, up to 30 cells were photographed and subsequently measured using a line-chain tool in the Leica Application Suite (version 2.6.0, Leica Microsystems, Switzerland) software. For each sperm cell, the length of the head, midpiece and tail was measured separately. Total sperm length was then calculated by adding the lengths of these three components. Sperm cells that were abnormal or broken were not measured.

After having studied all microscope slides, the mean total sperm length of an individual was calculated for each of the samples where five or more sperm cells had been measured. These samples were later included in the experiments testing for significant associations between mean total sperm length and plumage blackness score and between mean total sperm length and arrival date. Several males had been sampled twice in the field, and were thus represented in two samples rather than one. However, mean total sperm length for an individual male was included only once in the analyses (meaning that each individual male is represented only once in each of the figures in the Results section). Samples where less than five sperm cells had been measured were not included in any of the experiments, because the mean total sperm length was not calculated. For each sample where 30 sperm cells had been successfully

measured, the intra-male coefficient of variance; CV (SD/mean * 100), was calculated in addition to mean total sperm length. Lastly, I calculated the mean intra-male CV (CVwm) and inter-male CV (CVbm) value for the same samples.

Repeatability testing and statistical analyses

In order to test the repeatability and robustness of my measurements, I calculated the measurement repeatability following Lessells and Boag (1987). To do this, the lengths (head, midpiece, tail and total sperm length) of 18 randomly selected spermatozoa from one of the sperm samples were measured twice. The measurements were highly repeatable (head: r=0,920, $F_{17,18}$=24,016, p=<0.0001; midpiece: r=0,960, $F_{17,18}$=48,486, p=<0.0001; tail: r=0,978, $F_{17,18}$=88,718, p=<0.0001 (ANOVA test calculating the coefficient of repeatability)). I therefore felt confident that my measurements were both robust and accurate. The statistical analyses in this thesis were performed using STATISTICA version 6.1 (StatSoft, Inc., Tulsa, USA). Graphs were constructed using Origin® version 8.1 (OriginLab, Corporation, Northampton, USA) and Microsoft Office Excel 2007.

Results

Sperm characteristics and intraspecific variation in sperm length

Mean total sperm length for all the males included in my analyses was 104.2 µm (n=32, SD=2.55), where the mean head, midpiece and tail lengths were 12.3, 73.3 and 18.6 µm (SD=0.48, 1.91 and 1.93), respectively, and constituted 11.8%, 70.4% and 17.8% of the total length, respectively. Thus, the midpiece component of pied flycatcher sperm cells contributes most to the total length of the spermatozoa. While there was no correlation between mean head length and mean total sperm length (Fig. 1), I found that both the mean midpiece lengths and mean tail lengths were positively related to mean total sperm length (Fig. 2 and 3).

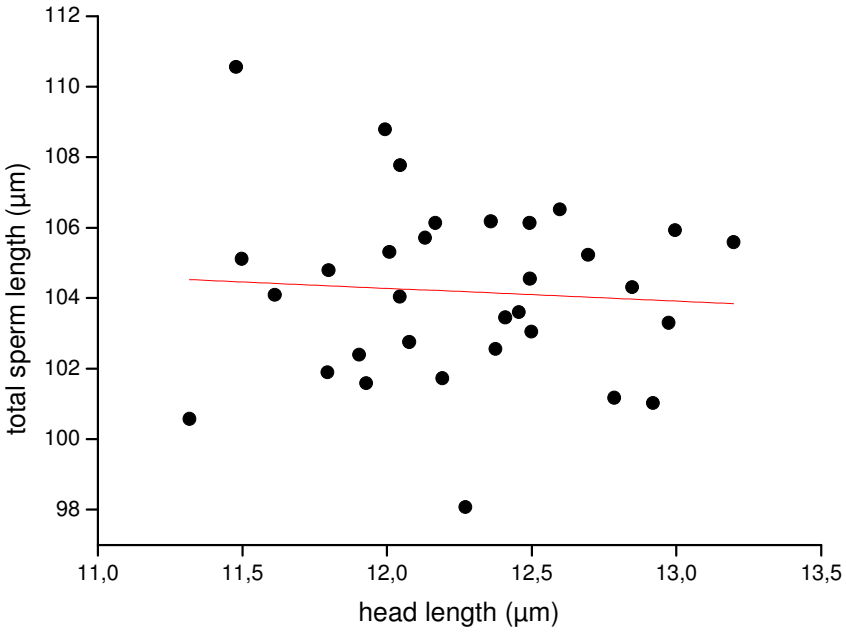

Figure 1. Relationship between mean total sperm length and mean head length in the pied flycatcher (n=32, r=-0.07, p=0.71).

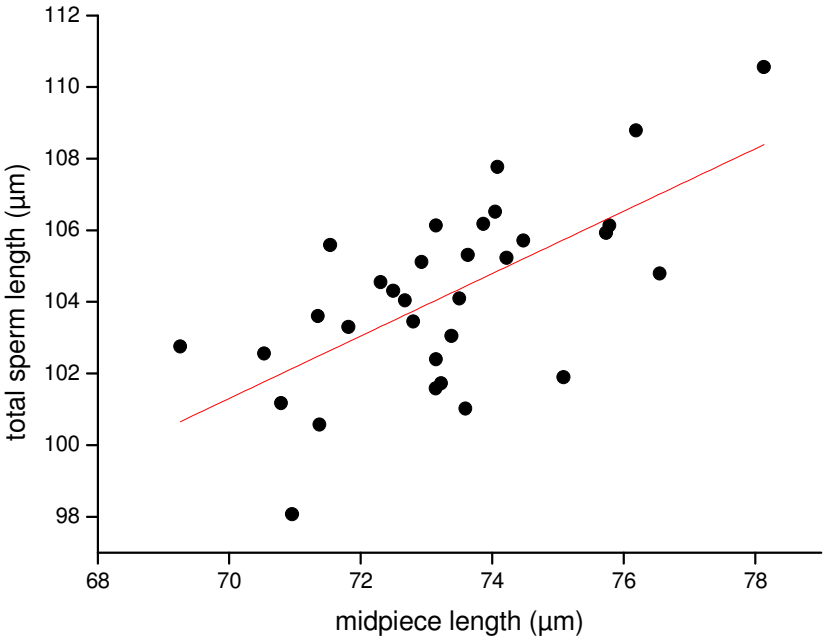

Figure 2. Relationship between mean total sperm length and mean midpiece length in the pied flycatcher (n=32, r=0.65, p<0.0001).

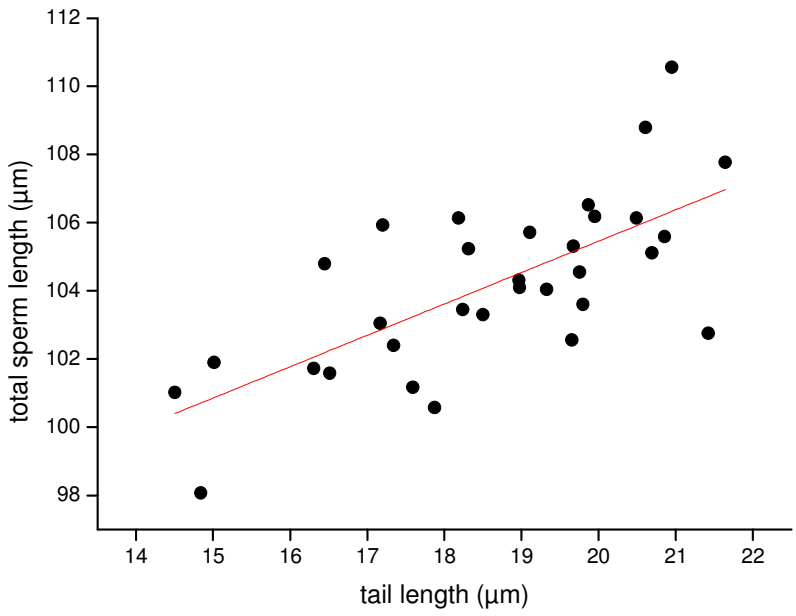

Figure 3. Relationship between mean total sperm length and mean tail length in the pied flycatcher (n=32, r=0.70, p<0.0001).

Substantial variation was found in mean sperm length between the different males, with mean values ranging from 98.1 μm to 110.6 μm (Fig. 4, n=32). The dataset on sperm length was tested using a Shapiro- Wilk's test and did not deviate from a normal distribution (W=0.99, p=0.96). For the 19 males where 30 sperm cells had been measured and within-male coefficients of variation (CVwm) had been calculated, I found significant differences in mean sperm length between males (one-way ANOVA $F_{18,551}$=29.0, p<0.0001), as well as a moderate repeatability (Lessells and Boag 1987) of sperm length for individual males (r=0.48). The CV for mean sperm length among males (CVbm) was 2.25; while the mean intra-male CV (mean CVwm) was 2.26.

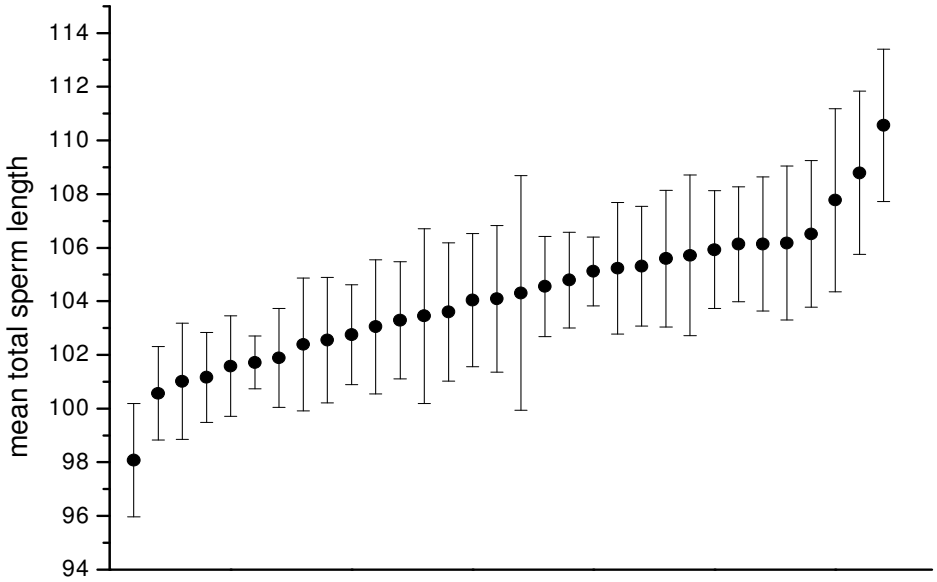

Figure 4. Estimates of mean total sperm length ± SD (μm) of individual pied flycatcher males (n=32), sorted by mean length. A minimum of five sperm cells were measured per male.

Associations between mean sperm size and two secondary sexually selected traits in the pied flycatcher

No significant associations were found between mean total sperm length and male plumage blackness and arrival date; the two sexually selected male traits in the pied flycatcher (Fig. 5 and 6). As the midpiece component of passerine sperm cells makes up a large part of the total length, I tested for an association between mean sperm midpiece length and male plumage score and arrival date. No significant associations were found in these analyses (linear regressions; n=32, r=-0.27, p=0.13 between mean midpiece length and male plumage

blackness, n=29, r=-0.10, p=0.61 between mean midpiece length and arrival date), nor when testing mean head length and mean tail length against the two secondary sexually selected traits (linear regressions; n=32, r=-0.04, p=0.84 between mean head length and male plumage blackness, n=29, r=0.25, p=0.19 between mean head length and arrival date, n=32, r=0.19, p=0.31 between mean tail length and male plumage blackness, n=29, r=-0.08, p=0.68 between mean tail length and arrival date). Furthermore, no significant association was found between mean total sperm length and breeding date (Fig. 7), and no significant associations could be found between intra-male CV and plumage blackness score (Fig. 8) nor between intra-male CV and arrival date (Fig. 9) in the present study.

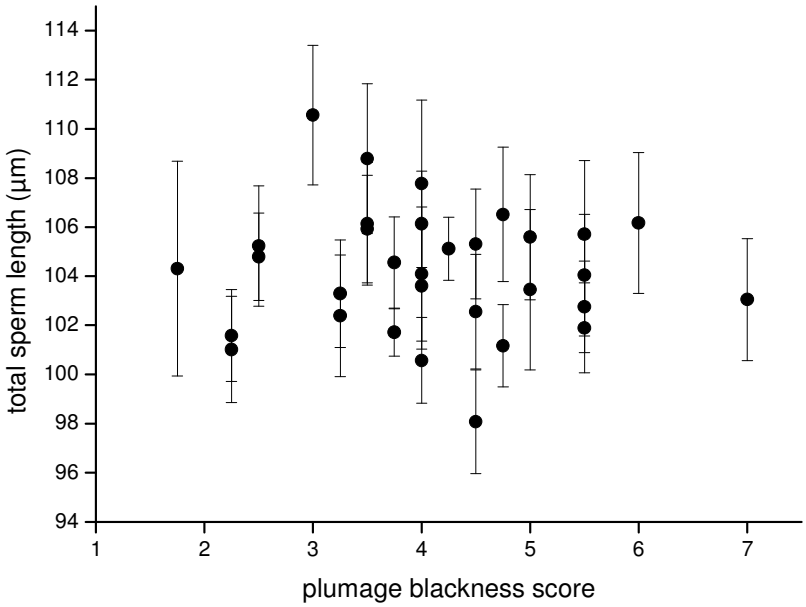

Figure 5. Relationship between total sperm length (mean ± standard deviation) and male plumage blackness in the pied flycatcher (n=32, r=-0.07, p=0.71). Preferred males have lower plumage scores.

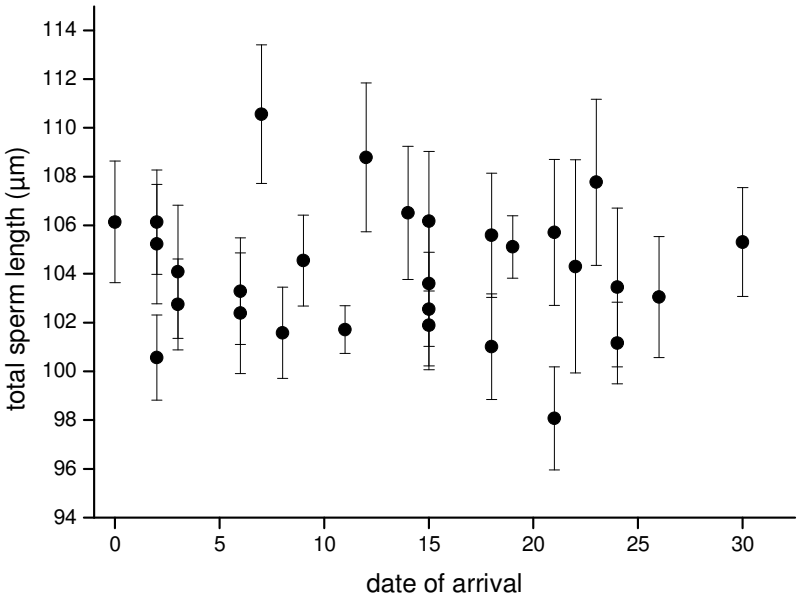

Figure 6. Relationship between total sperm length (mean ± standard deviation) and date of arrival (given as calendar date in May) in the pied flycatcher (n=29, r=-0.08, p=0.67). 0=30 April, 1=1 May etc. Preferred males arrive earlier in the breeding season.

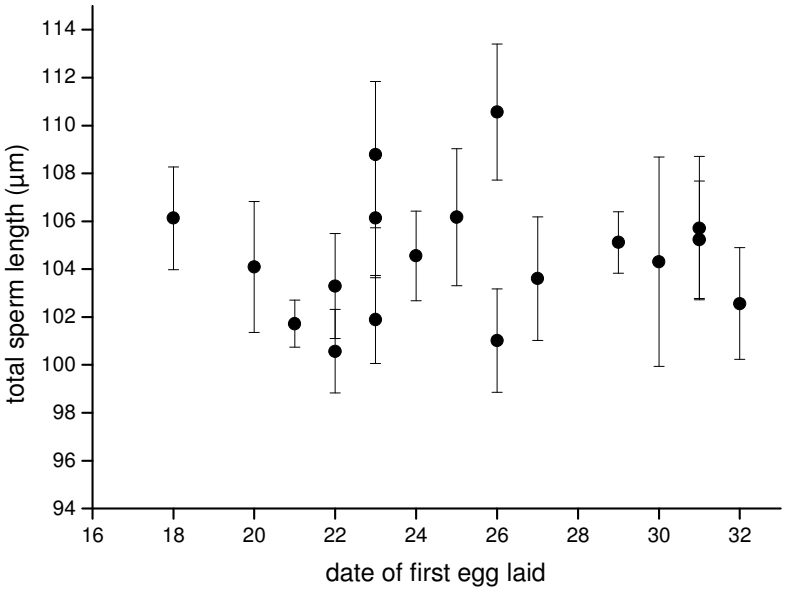

Figure 7. Relationship between total sperm length (mean ± standard deviation) and breeding date (given as calendar date in May) in the pied flycatcher (n=18, r=0.06, p=0.81). 1=1 May, 32=1 June. Preferred males breed earlier in the breeding season.

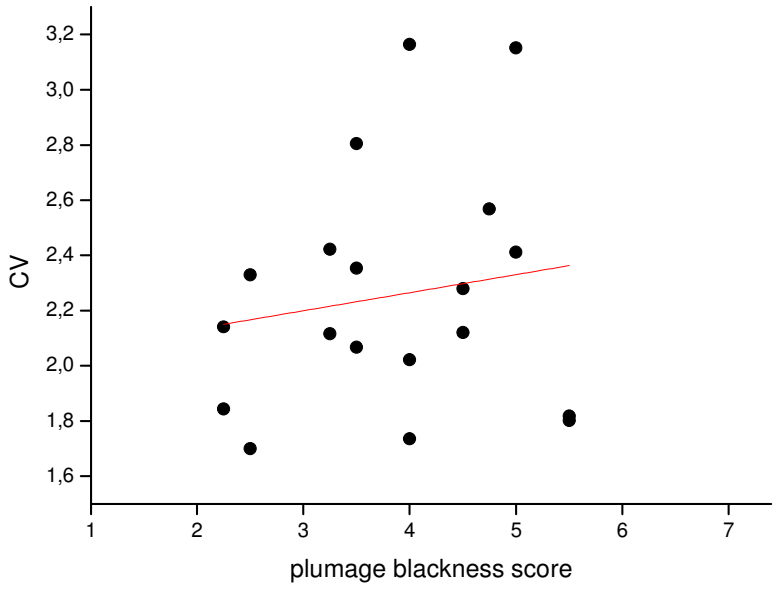

Figure 8. Relationship between intra-male CV and male plumage blackness in the pied flycatcher (n=19, r=0.16, p=0.52).

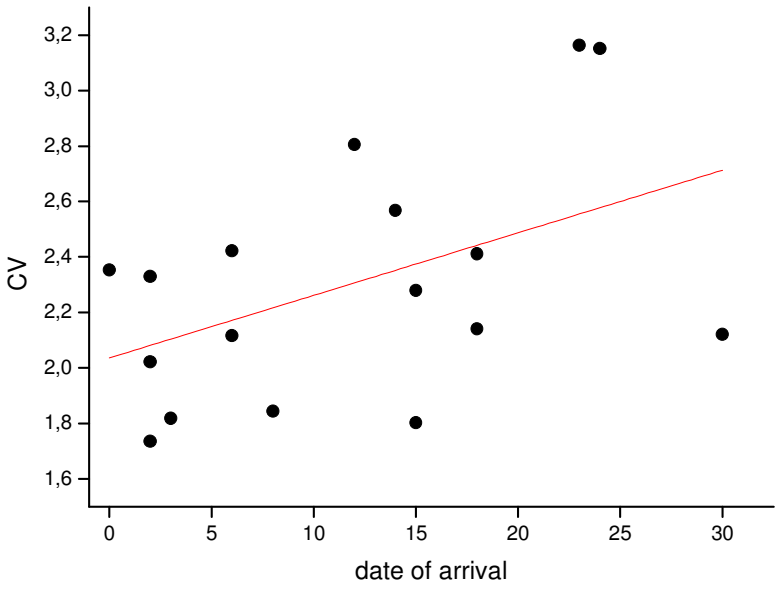

Figure 9. Relationship between intra-male CV and date of arrival (given as calendar date in May) in the pied flycatcher (n=17, r=0.47, p=0.06). 0=30 April, 1=1 May etc.

Change in intra-male sperm length and its variation throughout the breeding season

When testing whether the mean sperm length and the CVwm of individual pied flycatcher males would change throughout a breeding season, a paired T-test was employed. First, I tested for a change in mean total sperm length. As I was only able to include individuals for which a minimum of five sperm cells had been measured in both the early and late sperm samples in this test, the sample size was very small (n=6). The results nevertheless seemed to give no evidence of an increasing or decreasing trend in sperm length throughout the breeding season (paired T-test; mean diff.=-0.27, n=6, t=-0.37, df=5, p=0.72).

In order to test for a change in intra-male CV (CVwm) for the same six individuals, I first had to calculate the CV values for the samples where less than 30 sperm cells had been measured. Having done this, both early and late CVwm values for each of the individuals were adjusted for the number of sperm cells that had been used to calculate the CV, using the formula $CV_{adj.} = ((1+ 1/4n) * CV)$, where n is the number of sperm cells present in the sample (following Sokal and Rohlf 1995). The adjustments were made in order to correct for a possible sample-size bias, as CV tends to be underestimated in small samples. The change in intra-male CV over the breeding season is shown in Fig. 10. While all but one male seemed to show a reduction in CVwm from the time of egg laying to the time of hatching, I found no overall significant change in intra-male CV in this test (paired T-test; mean diff.=0.39, n=6, t=1.00, df=5, p=0.36). The male that showed an increase in intra-male CV over the breeding season was examined more closely to find out if a single sperm cell length might be the cause of the high CVwm measured in the late sample of this individual. However, this did not seem to be the case. Furthermore, the skewness value was only slightly negative for the late sample.

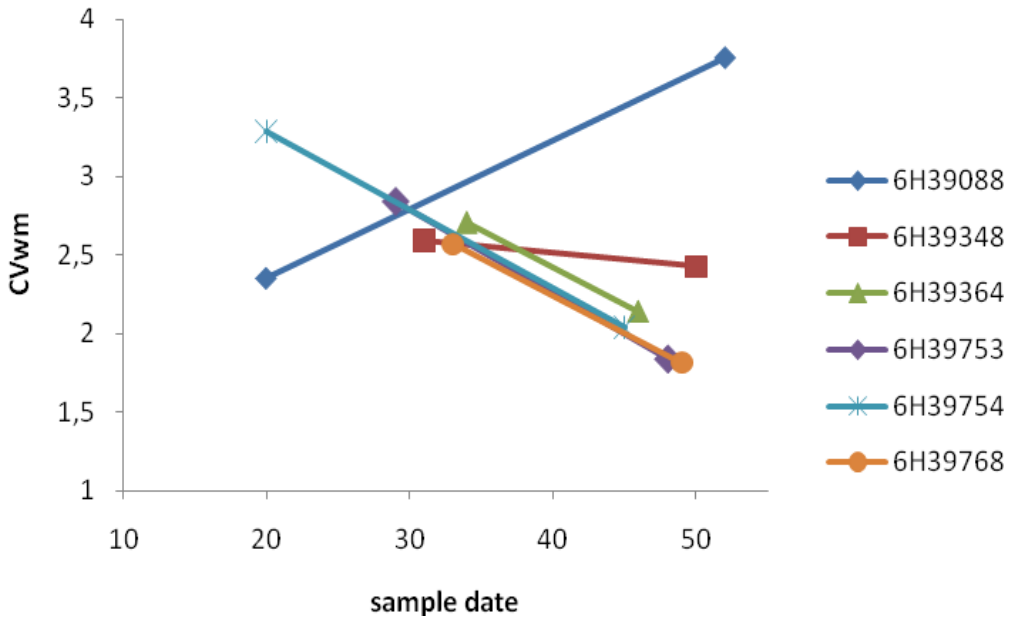

Figure 10. Change in intra-male CV in pied flycatcher males from the time of egg laying to the time of hatching during the breeding season of 2010 (n=6). Sample dates are given as calendar date in May. 20=20 May, 30=30 May, 40=9 June etc.

Lastly, I tested whether the sperm length data from each of the 19 males where 30 sperm cells had been measured were normally distributed. Using the Shapiro-Wilk test for normality, I found that data from all but three males were normally distributed. For the three males where the data did not fit a normal distribution, I found that the skewness values were strongly negative (values of -0.82, -0.74 and -1.36), indicating that more short sperm cells were present in these samples than what is expected from a normal distribution (where the skewness value is equal to 0). The three males had been sampled at quite different dates (30 May, 15 June and 23 May, respectively), thus; based on this, it did not seem like males that were sampled early in the breeding season were more likely to have more shorter sperm than expected compared to other males.

Having calculated a skewness value for all of the 19 males, I plotted the values against the date when the sperm samples had been collected. While I was unable to find significant evidence for the prediction that earlier sampled males had more shorter sperm than predicted compared to other males also in this analysis, the results showed that the skewness values in fact were negative for the majority of the males, indicating that these males all seemed to have more shorter sperm than would be expected from a normal distribution (Fig. 11).

(Figure 11 shows the results from a linear regression analysis. While I also performed a polynomial regression analysis on this dataset, the results were nevertheless the same; no significance was found (n=19, r=0.19, p=0.75).)

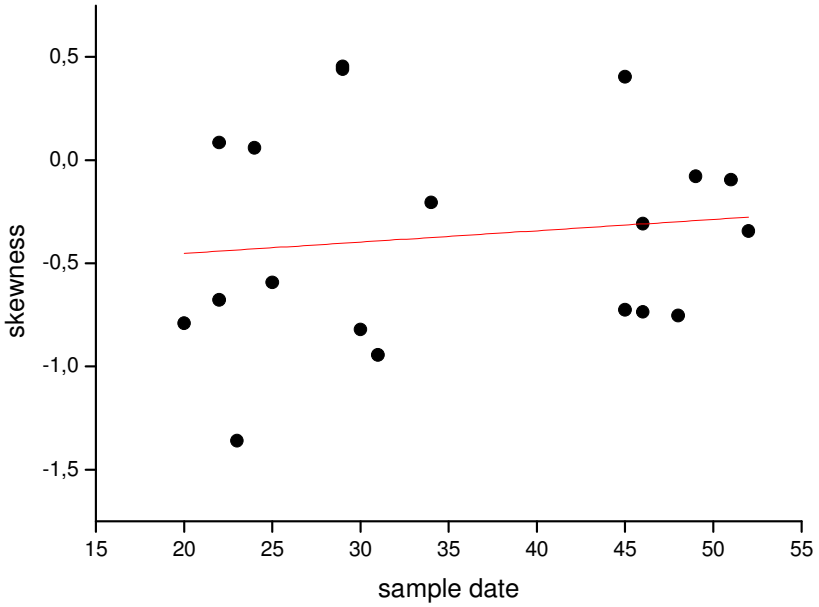

Figure 11. Relationship between skewness value and date of sperm sampling in the pied flycatcher (given as calendar date in May) (n=19, r=0.12, p=0.62). 20=20 May, 30=30 May, 40=9 June etc.

Discussion

Unlike the results presented by Calhim et al. (2009), my results revealed no significant association between mean sperm length and two sexually selected male traits (i.e. plumage colour and arrival date) in the pied flycatcher. Thus, a new and extended dataset from the same study population as that examined in the former study failed to support the previous finding of a negative association between gametic morphology and two female preference traits in this species, therefore making it reasonable to question the generality of the results and conclusions reported by Calhim et al. (2009). (Also, I found no significant associations between intra-male CV and the two traits, although the relationship between CVwm and arrival date was close to significance.) There are several possible reasons as to why the results in the present study differed from those of Calhim et al. (2009). In this section, I will present some of these reasons, and furthermore argue why pre-copulatory sexual selection on sperm length in the pied flycatcher seems unlikely, not only based on my results, but also from a theoretical perspective. I will then discuss my findings on intraspecific sperm length variation and its change over the breeding season.

As pointed out in the Introduction, the dataset analyzed by Calhim et al. (2009) was relatively small, and the results were strongly affected by a few single data points having a high plumage score and late breeding date. Hence, their results may have been subject to a statistical Type I error. Another striking pattern is that the mean total sperm lengths reported in the article are remarkably short. While the mean total sperm length for the males in my analyses was 104.2 µm, only a small fraction of the males in Calhim et al. (2009) had mean sperm lengths at this value or higher. In fact, the male having the lowest mean (approx. 93 µm) had a mean total sperm length that was shorter than any single sperm cell measured in my analyses. The mean total sperm lengths reported by Calhim et al. (2009) were also shorter than those found in two recent studies on sperm length in pied flycatchers from Røros, Norway and Lingen, Germany (Lifjeld et al. unpublished data). Mean total sperm lengths in these populations were measured to 102.9 µm (n=34 males) and 103.2 µm (n=14), respectively, which is quite similar to the value I found, but larger than that found by Calhim et al. (2009). This is intriguing, because while most pied flycatcher males in Norway have a black dorsal plumage, the majority of pied flycatcher males in Germany (as well as in other Central European countries) are brown, and brown morphs in countries such as Norway may

thus be attributable to gene flow from southern countries. If there truly exists a negative association between sperm length and plumage blackness score and between sperm length and breeding date in the pied flycatcher, then brown males should generally have shorter sperm cells than black males. What was seen in these two studies, however, was that despite the difference in plumage colour in Norwegian and German males, the mean total sperm length in pied flycatchers did not differ between the two populations. Hence, there was no indication that the browner males had shorter sperm.

In contrast to the field procedures of my and the two above-mentioned studies from Røros and Lingen, Calhim et al. (2009) collected sperm from the faeces of individual males in order to obtain sperm samples from pied flycatcher males in the field. While this is considered a non-invasive sampling method, involving little disturbance of the birds in study, it may be a less suitable method than that of Wolfson (1952; where sperm samples are obtained by massaging the cloacal protuberance of the birds) for obtaining good samples for morphometric analysis. This is because it may be difficult to retrieve and measure spermatozoa from faeces, and also because parts of the fragile sperm cells (such as the tail component) may break off, so that broken cells are included in analyses of sperm length (Terje Laskemoen pers. comm.). As seen in the Results section, the tail component of a sperm cell may make up nearly one fifth (17.8% on average) of the total sperm length. If a loss of sperm components occurs, and the number of sperm cells measured per male in addition is small, such as in the study of Calhim et al. (2009), it is easy to imagine how mean total sperm length values may be underestimated from only one or two damaged sperm cells. A possible explanation for the shorter mean total sperm length values reported by these authors may therefore be that parts of several sperm cells may have been lost or measured erroneously, giving shorter measures of mean total sperm length than what is “normal” for pied flycatcher males.

It is unclear why Calhim et al. (2009) used breeding date rather than arrival date of pied flycatcher males in their analyses, but one might simply believe that data on actual arrival date was unavailable in this study, making it necessary to use data on breeding date instead. In this thesis, I have used actual arrival date rather than breeding date as one of the two secondary sexually selected traits in the pied flycatcher. No significant associations were found between sperm length and arrival date in my studies. Furthermore, while I did test for an association between mean total sperm length and breeding date, in order to fully compare my results to those of Calhim et al. (2009), my results were nevertheless different from those

in the previous study, as no association was found. Thus, regardless of the variable used in the analyses (breeding date or actual arrival date), the results in the present study failed to support the findings of Calhim et al. (2009).

My results cast doubt upon the findings of Calhim et al. (2009) and suggests that there are no associations between sperm morphology and female preference traits and hence no precopulatory sexual selection on sperm length. In fact, the only other animal where such an association has been found so far is the guppy (*Poecilia reticulata*; see Pitcher et al. 2007), and even in this species, the associations were only found in some populations (Calhim et al. 2009). In addition to my present study, other recent studies have also been unable to replicate the results reported by Calhim et al. (2009), as no significant associations could be found between mean total sperm length and male plumage blackness in either of the two pied flycatcher populations from Røros and Lingen sampled in 2009 and 2010 (Lifjeld et al. unpublished data). Thus, the empirical evidence for pre-copulatory sexual selection on sperm length in pied flycatchers (as well as in other passerines) is rather scarce.

Pre-copulatory sexual selection on passerine sperm length is also difficult to explain from theory. The pied flycatcher is a species with a low frequency of extra-pair matings; an extra-pair paternity (EPP) rate of 4 % has previously been reported for a study population from Sørkedalen, Oslo (Lifjeld et al. 1991). Brommer et al. (2010) similarly reported extra-pair young (EPY) values around 4-5 % for this bird. In species where the EPP is low and little or no extra-pair mating takes place, it is thought that evolution of female preference traits should be mediated primarily through direct benefits obtained from material resources, not through indirect genetic benefits. The reason for this is that if female preference traits were to evolve through indirect genetic benefits, and females were to obtain such benefits by choosing blacker and earlier arriving males, this would lead to selection for extra-pair mating preferences and a higher level of sperm competition. In the pied flycatcher, it has been shown that the EPP rate in reality is low even in experiments where females have the opportunity to copulate with blacker extra-pair males (Slagsvold et al. 2001).

Previous studies on the pied flycatcher have furthermore stated that blacker males are preferred by the females because of their parental care (Sætre et al. 1995), while earlier arriving males are preferred for their good territories (Alatalo et al. 1986). Hence, by mating with blacker and early arriving males, females do gain direct benefits from material resources

provided by the male. If choosing these males also would ensure genetic benefits for the females, then females paired to inferior males should seek to copulate with less inferior males when given the opportunity, making extra-pair paternity common. As we know, this does not seem to be the case with pied flycatchers. Extra-pair paternity should also be common if females seek extra-pair copulations for the sole purpose of fertility insurance, and blacker and early arriving males have more fertile spermatozoa than other males. Blacker and earlier males do not appear to possess such qualities, however; as Lifjeld et al. (1997) reported that blacker males actually seemed to be cuckolded more often than their brown conspecifics. In sum; both theory and empirical evidence suggests that female pied flycatchers do not seek extra-pair matings with blacker and earlier arriving males in order to obtain indirect genetic benefits. Rather, these males are chosen as a social mate in order to obtain direct benefits from parental care and good territories, making pre-copulatory sexual selection on sperm quality traits seem unlikely in this species.

Substantial variation was found in mean total sperm lengths both between and within pied flycatcher males. The CVbm and CVwm values were slightly similar to one another, with values of 2.25 and 2.26, respectively, and were fairly consistent with previous CV measurements from this species (Lifjeld et al. 2010). They were also quite consistent with the fact that the risk of sperm competition is low in the pied flycatcher. In their study on intraspecific variation in sperm length in the bluethroat and willow warbler, Laskemoen et al. (2007) found that the variance in sperm length was almost twice as high between males than within males, and they therefore stated that sperm traits seem more variable between males than within males. However, it is not uncommon to observe a higher CVwm value than CVbm value, as was indeed done in my study (see e.g. Table S1 in Lifjeld et al. 2010) In a species such as the pied flycatcher, where the risk of sperm competition is low, it is easy to imagine how both the variation between and the variation within males in total sperm length may become relatively high, as there is little or no selection pressure acting on the intraspecific sperm length variation.

When testing for a change in mean total sperm length throughout the breeding season, no significant change was detected between early and late samples, and there was no evidence of an increasing or decreasing trend in sperm length. One should be cautious about making any general conclusions regarding this analysis, however, as only six individual males could be included in the test. In order to properly and fully assess the change in sperm length in a

population throughout the breeding season, I recommend more experiments including larger datasets to be carried out. One should furthermore also be careful when discussing the results of the test investigating change in intra-male CV throughout the breeding season. As with the previous experiment, I was only able to include six individuals in this test. While no overall significant change in CVwm was found, I nevertheless found that all but one male seemed to experience a reduction in CV from the early to the late sample. In conclusion, further tests are needed before general conclusions regarding change in mean total sperm length and intra-male CV in the pied flycatcher can be made. While I was unable to find evidence for the prediction that males sampled earlier in the season had more shorter sperm than predicted from a normal distribution compared to later males, I nevertheless found that the majority of the males sampled in this study had negative skewness values, thus indicating that these males all seemed to have more shorter sperm than what would be expected from a normal distribution. The reason for this is not known, but it might be interesting to examine the subject more closely in future studies, in order to increase our knowledge on intraspecific sperm length variation both in pied flycatchers and in passerines in general.

To summarize, the aim of this study has been to study and quantify the level of intraspecific variation in sperm length in the pied flycatcher. Substantial variation in mean total sperm length was found both between and within males, which is consistent with the fact that the level of sperm competition is low in this species. My results gave no evidence for any significant associations between gametic morphology and the two secondary sexually selected traits in the pied flycatcher. Thus, they were in contrast to the findings of Calhim et al. (2009), but in accordance with all previous studies on passerines as well as with sexual selection theory. As blacker and earlier arriving males seem to be chosen by females in order to obtain direct benefits from parental care and good territories rather than indirect genetic benefits, pre-copulatory sexual selection on sperm quality traits seems unlikely in this species. While it is currently somewhat difficult to discuss my results regarding change in mean total sperm length and intra-male variation over a breeding season due to low sample sizes in these analyses, future studies investigating larger datasets might be able to clarify whether or not mean total sperm length and intra-male variation changes in a specific direction over the breeding season of pied flycatchers. Additional studies concerning distribution and skewness values in avian sperm samples could also help us understand more about intraspecific sperm length variation in this and other species.

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