Intraspecific variation in sperm length in two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus trochilus*

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Sperm cells are highly diversified in birds and considerable research effort has focused on variation in sperm morphology between species. However, surprisingly little is known about intraspecific variation in sperm morphology in birds. We analyzed between- and within-male variation in total sperm length in two passerine species, the Bluethroat (*Luscinia svecica*) and the Willow Warbler (*Phylloscopus trochilus*). In both species, the variance in sperm length was nearly twice as high between as within males, resulting in high repeatability of sperm length for individual males (Bluethroat: r = 0.73 and Willow Warbler: r = 0.79). These results suggest that sperm traits are more variable among than within males. With a resampling approach, we illustrate how the spread in estimated mean sperm length and coefficient of variation (CV) is affected by increasing the number of males measured. Further, we illustrate how the CV of sperm length for individual males change with the number of spermatozoa measured. For the two species in our analyses, it seems that measuring 10 males and 10 spermatozoa per male gives adequate estimates of both between- and within-male sperm length and CV.

1. Introduction

Spermatozoa are by far the most diverse cells in the animal kingdom (Cohen 1977). Although spermatozoa generally are numerous and tiny, there is enormous variation in sperm length across animal taxa, ranging from 15.5 µm in the cichlid *Asprotilapia leptura* (Perciformes: Cichlidae) (Balshine *et al.* 2001) to 58,290 µm in the fruit fly *Drosophila bifurca* (Diptera: Drosophilidae) (Pitnick *et al.* 1995). In birds, the variation is lower, but still ranges almost seven-fold, from 42.7 µm in the Red-Backed Shrike (*Lanius collurio*) (Briskie *et al.* 1997) to 291 µm in the Reed Bunting (*Emberiza schoeniclus*) (Dixon & Birkhead 1997). This huge interspecific variation in sperm length is well described in many taxa, including birds (e.g. Koehler 1995, Briskie & Montgomerie 2007).

Table 1. Recent publications (1992–present) addressing sperm size in birds. Indicating number of species, number of males per species and how many spermatozoa measured per male.

Species	Number of species	Males per species	Spermatozoa measured	Reference
Passerines	20	1–5	10	(Briskie & Montgomerie 1992)
Zebra Finch	1	10	50	(Birkhead & Fletcher 1995)
Passerines	13	NA[1]	NA	(Koehler 1995)
Fairy Wrens	3	17–52	NA	(Tuttle et al. 1996)
Sedge Warbler	1	14	30	(Birkhead et al. 1997)
Passerines	21	1–5	10	(Briskie et al. 1997)
Reed Bunting	1	4	30	(Dixon & Birkhead 1997)
Shorebirds	16	1–3	5–10	(Johnson & Briskie 1999)
Zebra Finch	1	913	5	(Birkhead et al. 2005)
Passerines[2]	37	1–33	10	(Immler & Birkhead 2005)
Passerines	21	1–11	5,15 and 30	(Birkhead et al. 2006)
Passerines	18	10–236	5	(Calhim et al. 2007)
Passerines	73	1–10	5	(Immler & Birkhead 2007)
Pheasants	24	1	15	(Immler et al. 2007)

1. NA = data not available.
2. One non-passerine species.

However, Ward (1998) pointed out that far less is known about the level of intraspecific variation in various sperm traits. This is unfortunate for two main reasons. First, intraspecific variation is an important statistical parameter when examining differences in sperm length between species, as for example, in comparative analyses. Second, the degree of intraspecific variation may be an important feature itself which may be shaped by selection or other evolutionary forces (Birkhead et al. 2005).

Much of the variation in sperm length can be attributed to phylogenetic relatedness, but some comparative studies, controlling for phylogeny, have indicated that there is a positive association between the risk of sperm competition and sperm length in insects (Gage 1994), fish (Balshine et al. 2001), mammals (Gomendio & Roldan 1991) and birds (Briskie & Montgomerie 1992, Briskie et al. 1997, Johnson & Briskie 1999). However, more recent studies of mammals (Gage & Freckleton 2003) and birds (Immler & Birkhead 2007) found no support for the positive relationship between risk of sperm competition and sperm length. In this context, especially when comparing closely related taxa, it is important to have adequate estimates of means and their variances.

In taxa other than birds, quite a few studies have followed Ward's (1998) recommendations and addressed intraspecific variation in sperm length (e.g. Morrow & Gage 2001, Joly et al. 2004, Schulte-Hostedde & Millar 2004, Bernasconi & Hellriegel 2005, Hettyey & Roberts 2006, Malo et al. 2006, Minoretti & Baur 2006, Schulte-Hostedde & Montgomerie 2006, Harris et al. 2007, Locatello et al. 2007), but such studies are still missing in birds. With a few notable exceptions (Tuttle et al. 1996, Dixon & Birkhead 1997, Birkhead et al. 2005, Birkhead et al. 2006), the variance or standard error of the means are usually not reported. These shortcomings call for an evaluation of intraspecific variation in sperm length in birds, especially since birds to a great extent are subject to evolutionary and ecological studies of mating systems, in which various aspects of sperm biology might play an important role.

As part of ongoing studies of infertility and testis size variation in the Bluethroat (Luscinia svecica) and the Willow Warbler (Phylloscopus trochilus) in Southern Norway (Lifjeld et al. 2007, Laskemoen et al. in press), we also collected sperm samples from breeding males of both species. In the present study, we quantify the between- and within-male variation in sperm length in these species. As sample sizes (number of males sampled) vary considerably between studies (see Table 1), we wanted to examine how the spread in estimated mean sperm length and coefficient of variation (CV) is influenced by increasing the number

of males measured. In order to do so we apply resampling procedures on mean sperm length (1–30 males) and CV of sperm length (2–30 males). Further, the numbers of spermatozoa measured per male vary to a great extent in recently published studies (Table 1). Therefore, we present resampling estimates on within-male CV of sperm length (2–30 spermatozoa) as a function of the number of spermatozoa measured.

2. Material and methods

2.1. Study area and species

Field work was carried out in the valley Øvre Heimdalen (61º25'N, 8º52'E), Øystre Slidre municipality, Oppland county, Norway, during two field seasons (2002 and 2004). The study area is located at an altitude of about 1,100 meters above sea level.

The Bluethroat population in Øvre Heimdalen has been thoroughly studied since 1991. It is a common migrant breeding in the study area, with an estimated breeding density of 38 pairs per km^2 (Anthonisen *et al.* 1997). The Willow Warbler is a very common migrant that breeds quite densely in the study area, approximately 140 pairs pr km^2 (Bjørnstad & Lifjeld 1996).

2.2. Field procedures

Adult males of both species were caught in their territories using mist nets and playback, and transported into a lab building. The Bluethroat males were caught during the breeding season of 2004 (between 1 and 21 June), and the Willow Warblers during the breeding season of 2002 (between 12 and 29 May). In the lab, the birds were measured, blood sampled and sacrificed by cervical dislocation. Blood was sampled by brachial venipuncture for inclusion in the tissue collection at the Natural History Museum, University of Oslo. After dissection, the seminal glomera from both species were stored in 3% glutaraldehyde and squeezed in the solution so that sperm could rapidly be diluted and fixed in the medium for later morphometric analyses.

2.3. Sperm morphometry measurements

A droplet (approximately 3 µl) of fixed sperm was applied on a microscope slide. We used a Leica DC500 camera mounted on a Leica DM6000 B light microscope to take digitalized photographs of spermatozoa at a magnification of 320 ×. Abnormal spermatozoa (broken tail, damaged or missing acrosome) were not used. Using a line-chain tool in the Leica IM1000 software, we measured the total sperm length from the anterior tip of the acrosome to the end of the flagellum on the digital images. We measured 30 spermatozoa per individual in the Bluethroat and 20 spermatozoa per individual in the Willow Warbler. For a single Willow Warbler male, the same 20 spermatozoa were measured blindly twice, and the measurements were highly repeatable (r = 0.99, $F_{19,20}$ = 152.72, P < 0.001; Lessells & Boag 1987). All sperm measurements were conducted by T.L.

2.4. Statistical methods

We used a resampling procedure to illustrate how the accuracy of mean sperm length is influenced by the number of males measured. From our samples of 46 males in each species we randomly selected a given number of males, calculated their mean sperm length and repeated the procedure 1,000 times. This was done for all sample sizes between 1 and 30 males. Further, we applied similar resampling procedures, on the between-male level, to illustrate how the spread in estimated CV is influenced by the number of males sampled, and on the within-male level, to illustrate how the spread in CV is influenced by the number of spermatozoa measured. This was done for sample sizes between 2 and 30 males and 2 and 30 spermatozoa respectively. All resampling was done with replacement and were conducted using Resampling Stats for Excel 3.2 (Resampling Stats, Inc.) and Microsoft® Office Excel 2003 (Microsoft® Corporation). All other statistical analyses were performed using STATISTICA version 7.1 (StatSoft, Inc). Graphs were constructed using Origin® v7.0300 (OriginLab Corporation).

Table 2. Descriptive statistics for sperm length (µm) in Bluethroats and Willow Warblers. Data derived from 1380 spermatozoa from 46 Bluethroat males and 920 spermatozoa from 46 Willow Warbler males (30 and 20 spermatozoa per individual, Bluethroat and Willow Warbler respectively).

Species	Mean sperm length ± SD (CV[1])	Range (min–max)	Mean intra-male CV
Bluethroat	216.43 ± 6.09 (2.81)	197.40–232.83	1.43
Willow Warbler	94.48 ± 2.35 (2.49)	89.08–99.60	1.46

1. CV = coefficient of variation calculated as SD/mean*100

3. Results

The Bluethroat sperm were more than twice as long as the Willow Warbler sperm (Table 2). The mean CV of within-male sperm length was considerably lower than between-male CV of sperm length in both species (Table 2). Hence, there was a significant variation in mean sperm length among males, and a high repeatability of sperm length for individual males (Bluethroat: $r = 0.79$, $F_{45,1334} = 112.49$, $P < 0.001$; Willow Warbler: $r = 0.73$, $F_{45,874} = 54.84$, $P < 0.001$). This is also apparent when plotting mean sperm length ± SD for each of the males (Fig. 1). Individual male mean sperm lengths were normally distributed in both species (Shapiro-Wilks W tests: Bluethroat: $W = 0.98$, $P = 0.43$, n = 46 males; Willow Warbler: $W = 0.99$, $P = 0.98$, n = 46 males). A resampling procedure illustrates how the spread in estimates of mean sperm length is reduced with the number of males measured (Fig. 2).

We ran a similar resampling procedure on the spread in between-male CV, illustrating that CV can well be under- or overestimated when few males are sampled (Fig. 3). Further, we obtained resampling estimates of within-male CV for two individuals of each species, the one with the lowest and the one with the highest CV, respectively (Fig. 4).

The simulations reveal a general pattern of rapid decline in the spread of estimates for small sample sizes. Beyond a sample size of 10 spermatozoa, there is only a marginal decrease in the spread of estimated CV values. The simulations
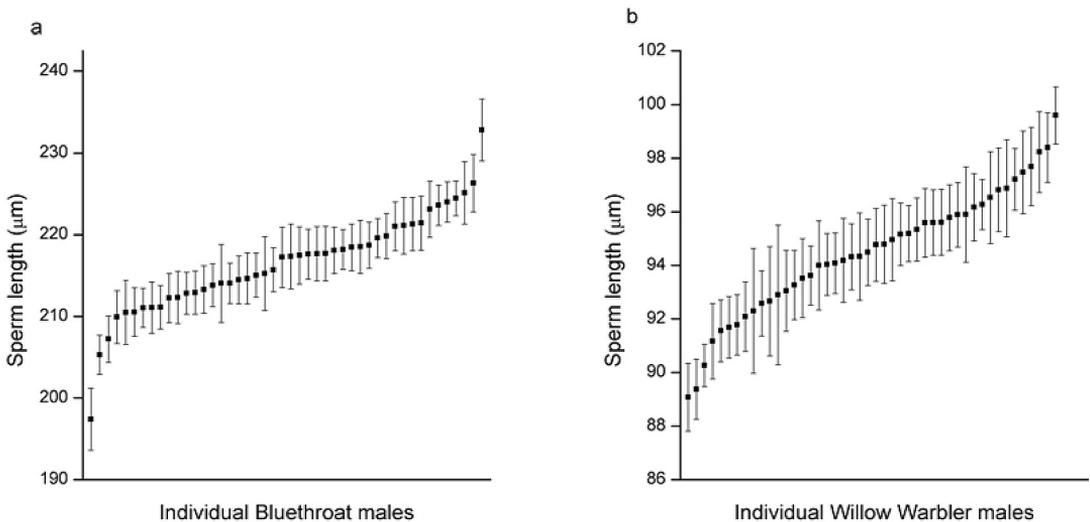
Fig. 1. Estimates of mean sperm length ± SD (µm) of individual Bluethroat (a) and Willow Warbler (b) males, sorted by mean length. Calculations were based on 30 spermatozoa per individual in the Bluethroat and 20 spermatozoa per individual in the Willow Warbler.
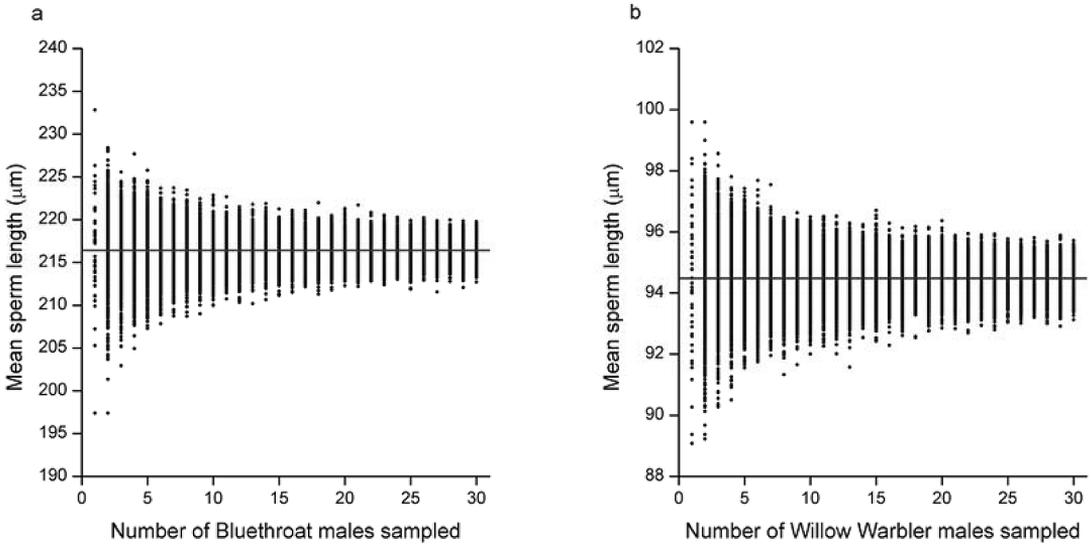
Fig. 2. Estimates of mean sperm length of Bluethroats (a) and Willow Warblers (b) as a function of the number of males sampled. Values are calculated from resampling (1,000 iterations per sample size) of the mean sperm length of 46 males in each species. The straight line indicates the overall mean sperm length (mean of means).
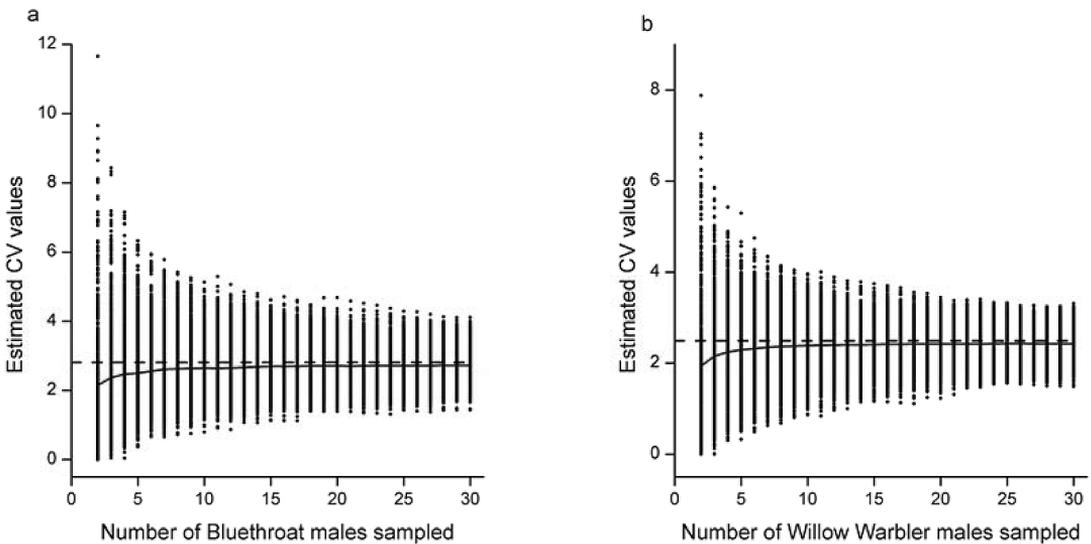
Fig. 3. Estimates of inter-male coefficient of variation (CV) of sperm length in Bluethroats (a) and Willow Warblers (b) as a function of the number of males sampled (2–30 males). Values calculated from resampling (1,000 iterations per sample size). The dashed line indicates the mean inter-male CV, the continuous line indicates the mean CV calculated from the simulations.

also revealed a general underestimation of CVs at small samples sizes, as illustrated by the lines for average CV (Fig. 3 and 4). Hence, CV values should be adjusted for sample size, especially if the sample size is low, as suggested by Sokal and Rohlf (1995).

4. Discussion

In the present study we have demonstrated a significant variation in sperm length at the intraspecific level in the Bluethroat and the Willow Warbler, and that sperm are more variable between than
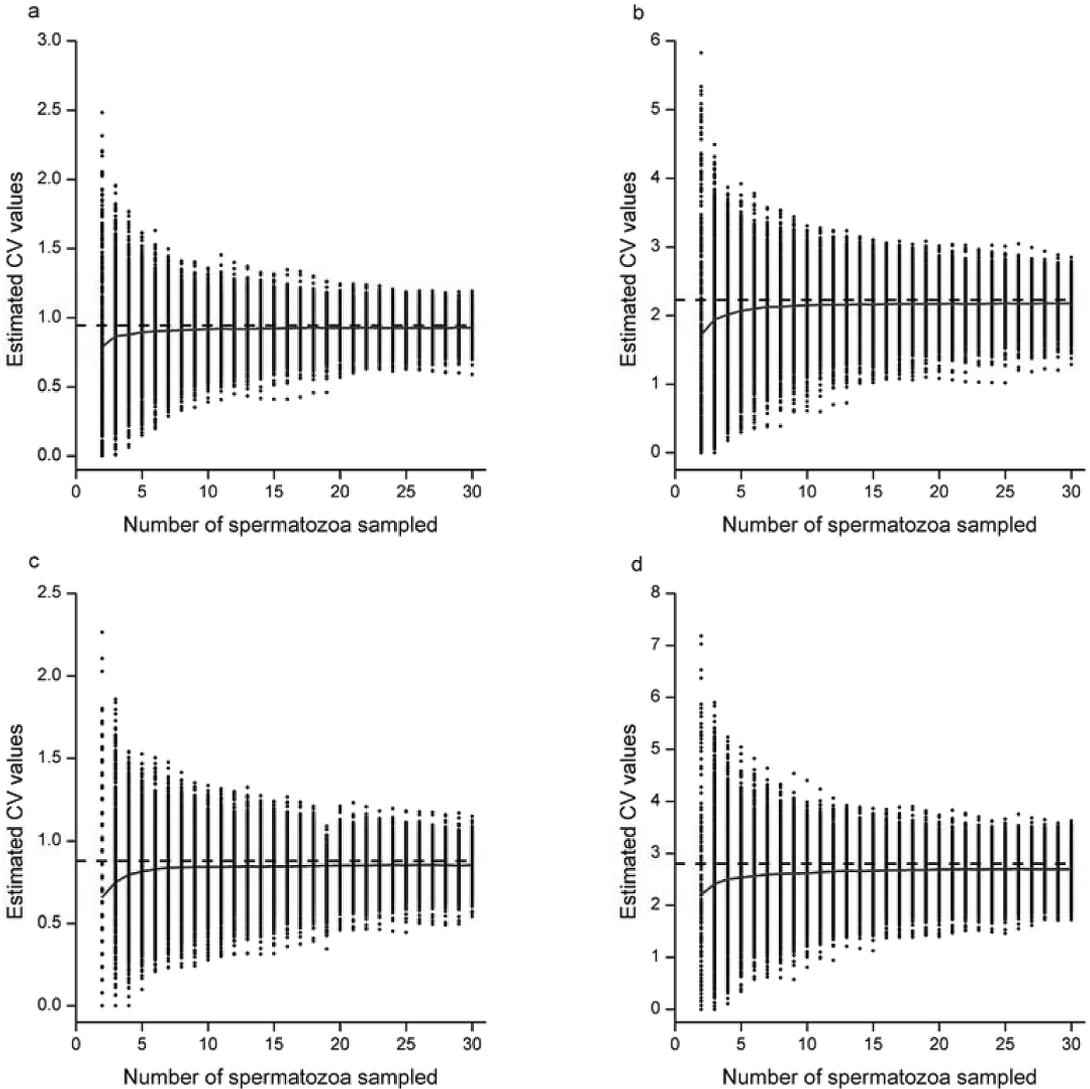
Fig. 4. Estimates of intra-male coefficient of variation (CV) of sperm length in the Bluethroat showing lowest CV (a), the Bluethroat showing highest CV (b), the Willow Warbler showing lowest CV and the Willow Warbler showing highest CV (d), as a function of the number of spermatozoa sampled (2–30). Values calculated from resampling (1,000 iterations per sample size). The dashed line indicates the intra-male CV, the continuous line indicates the mean CV calculated from the simulations.

within males in both species. Consequently, for adequate estimates of mean sperm length, it seems more important to measure several males per species than several spermatozoa per male. In addition, we illustrate how the spread in estimates of mean sperm length and CV values is reduced when sample size increases. Interestingly, both species show more or less the same pattern with just a mar-

ginal improvement of estimates beyond ten males (Fig. 2 and 3). It is important to note that these simulations are based on the observed sperm lengths in our two study species, and generality can therefore not be claimed.

There are some reports on between-male variation in sperm length in birds (e.g. Allen *et al.* 1968, Birkhead & Fletcher 1995, Tuttle *et al.* 1996, Birk-

head *et al.* 1997, Birkhead *et al.* 2005, Birkhead *et al.* 2006), and published information on sperm length is available for about 120 bird species (Briskie & Montgomerie 2007). However, as far as we know, there is only one previous study that has reported both between-male and within-male variation in sperm lengths in birds. Dixon & Birkhead (1997) measured the length of 30 spermatozoa in each of four Reed Buntings and reported average sperm length ± SD for each male, hence CV could easily be calculated. The mean within-male CV in sperm length amounted to 1.89, whereas the between-male CV was only 0.51. Hence, in that species there was a considerably higher within-male variance than between-male variance in sperm length, that is, a pattern opposite to what we found in Bluethroats and Willow Warblers. However, it is unclear whether the result in Dixon & Birkhead (1997) reflects the actual intraspecific variation, or is an artifact of the low sample size. In fact, our resampling procedures illustrate how CV is likely to be underestimated when the sample size is low (Fig. 3 and 4).

From these examples, it is obvious that both levels of intraspecific variation in sperm length, i.e. between-male and within-male, are relevant and important for characterizing intraspecific variation in sperm length and for sample size assessments (number of males and spermatozoa per male to be measured). We would therefore recommend future studies to report both the within-male and the between-male variance in sperm length, as well as the number of sperm measured per male, the number of males examined and the overall mean sperm length (mean of means). We found high repeatabilities for sperm length within ejaculates, but our data did not allow us to investigate repeatability between ejaculates. However, high between-ejaculate repeatability has been demonstrated in the Zebra Finch (*Taeniopygia guttata*) (Birkhead & Fletcher 1995), hence obtaining one ejaculate should give an adequate estimate of individual sperm length.

Notably, sperm samples can easily be obtained from wild birds either through gently massaging the cloacal protuberance of males in breeding condition (Wolfson 1952) or through fecal sampling (Immler & Birkhead 2005). Indeed, Briskie & Montgomerie (2007) encourages field ornithologists to consider including sperm sampling as a routine procedure when handling male birds during the breeding season.

Birkhead *et al.* (2005) hypothesized that sperm competition may select for lower variation in sperm traits. Indeed, a negative relationship between indices of sperm competition risk and variation in sperm length and other sperm traits has recently been documented in passerines (Calhim *et al.* 2007, own unpublished data). Calhim *et al.* (2007) suggests that sperm competition may enforce stabilizing selection on sperm size variation through selection against the extreme sperm sizes.

In the present study, we have shown that variation in sperm length is considerably lower within males as compared to between males in Bluethroats and Willow Warblers. Furthermore, our resampling simulations suggest that sampling a minimum of 10 males and measuring a minimum of 10 spermatozoa per male will give adequate estimates of both within- and between-male sperm length in these species. Future studies on more species will establish whether our findings in the present study reflect general patterns of intraspecific variation in sperm length in birds.

Lajinsisäistä vaihtelua pajulinnun ja sinirinnan siittiöiden pituudessa

Siittiöiden morfologiaa linnuilla on tutkittu paljon ja niiden muoto vaihtelee huomattavasti eri lajien välillä. Aiemmista tutkimuksista huolimatta ei lajinsisäisestä siittiöiden koon vaihtelusta tiedetä paljoakaan. Analysoimme koiraiden välisen ja yksilöllisen vaihtelun siittiöiden kokonaispituudessa sinirinnoilla ja pajulinnulla. Molemmilla lajeilla koiraiden välinen vaihtelu siittiöiden pituudessa oli lähes kaksi kertaa niin suurta kuin yksilöllinen vaihtelu. Yksittäisten lintujen siittiöiden pituuksien toistettavuus mittausten välillä oli erittäin suur-

ta (sinirinta: r = 0.73, pajulintu: r = 0.79). Näytämme tutkimuksessamme, kuinka mitattujen koiraiden määrä vaikuttaa sukusolujen pituuden arvioituun vaihteluväliin ja muuntelukertoimeen (CV). Lisäksi näytämme kuinka yksilöllinen siittiöiden pituuden CV muuttuu lisääntyvien mittausten mukana. 10 koiraan ja 10 sukusolun mittaaminen koirasta kohden antaa riittävän yksilöllisen ja koiraiden välisen siittiöiden pituuden ja muuntelukertoimen molemmille lajeille.

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