Variation in testis size and sperm morphology in the bluethroat, *Luscinia svecica svecica*

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Forord

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Abstract

In this study I examined intra-specific variation in primary sex traits in male bluethroats (*Luscinia svecica svecica*), a passerine species with a high intensity of sperm competition, The single most important predictor of the size of testes and seminal glomera was male age (yearlings versus older). This finding suggests that older males have higher sperm production rates, which may allow for higher copulation rates and/or larger ejaculates than in younger males. Previous findings of older males having a higher extra-pair fertilization success, and similar paternity loss in own nest as younger males despite less intense mate guarding, contribute to a general pattern of age-dependent sperm competition investments in male bluethroats. None of the measured conditional variables (body mass, haemoglobin, haematocrit), nor the body size variables (wing length, tarsus length, scull length) correlated significantly with testis size. The bluethroat sperm were relatively long (216.4 µm, n=46, SD=3.1µm) as expected for a species with intense sperm competition. Between-male variation in average sperm length was considerably larger than the within-male variation in individual sperm length. However, the between-male coefficient of variation in mean sperm length was considerably lower than that reported recently for another passerine with low intensities of sperm competition. More species should be examined to test for a possible relationship between inter-male variation in sperm length and the intensity of sperm competition.

Introduction

Sperm competition can be defined as the competition between the sperm of two or more males for a given set of ova (Birkhead & Møller 1998; Parker 1970; 1984). This definition accounts for the fact that sperm competition also occurs in externally fertilizing species, such as sea urchins and various species of fish. Birds usually exhibit high levels of sperm competition despite having a socially monogamous mating system (Birkhead & Møller 1992). Normally, sperm competition in birds occurs through extra-pair copulation (EPC), but sperm competition can also arise through rapid mate switching (Birkhead *et al.* 1998; Birkhead & Møller 1992). Passerine birds are favoured models of sperm competition due to the high frequency of EPC behaviour (Griffith *et al.* 2002). As a matter of fact, only 14% of the species reviewed in Griffith *et al*. (2002) are truly monogamous, and genetic polyandry occurs regularly in the remaining 86% species.

Sperm competition will generate a selection pressure on the males' behaviour, anatomy and morphology to ensure fertilization and paternity. Relatively large testes, large sperm stores, long spermatozoa, mate guarding and frequent copulations are all male adaptations to intense sperm competition (Birkhead *et al.* 1998). Testicular size in birds is likely to affect the individual bird's success in sperm competition as large testes are thought to produce high sperm numbers and thus facilitate frequent copulations and large ejaculates (Birkhead & Møller 1992; Briskie 1993; Møller 1988; 1991; 1994).

In many bird species older males (second breeding season or older) tend to have larger testes (mass and volume) than yearling males (first breeding season) (Birkhead *et al.* 1997; Deviche *et al.* 2000; Evans & Goldsmith 2000; Graves 2004; Merilä & Sheldon 1999). The implications of this fact are still unknown, but it has been hypothesized that

the reproductive capacities of yearling passerines may not be equivalent to those of older males (Graves 2004).

Another testicular trait documented in many bird species is that the left testis often is larger than the right one (Birkhead *et al.* 1997; Graves 2004; Møller 1994). Møller (1994) found that this asymmetry was positively correlated with expression of secondary sexual characters. He hypothesized that the right testis increases in size to compensate for reduced function of the left one and suggested further that the degree of directional asymmetry in testicular size reflects male quality.

Sperm morphology, and especially sperm length, can have important impact on sperm competition (Birkhead *et al.* 1998). Although no studies have actually shown that longer sperm swim faster than shorter sperm within species, comparative studies show that sperm length increases with level of EPC and extra-pair paternity (EPP) across species (Briskie *et al.* 1997; Johnson & Briskie 1999). This correlation could imply that long sperm is selected through sperm competition. One should thus expect relatively low variation in sperm length between different males of species and similarly low variation between sperm within individual males. To this date surprisingly few studies have been conducted on intra-specific variation in sperm length. In a recent study, Birkhead *et al*. (2005) showed that the zebra finch (*Taeniopygia guttata*) exhibits a considerable variation in both sperm length and sperm phenotype (head-, mid-piece- and tail length). Sperm competition is not very intense in the zebra finch (Birkhead *et al.* 1990), and thus the species may have been subject to weak selection on sperm length (Birkhead *et al.* 2005). Birkhead *et al*. (2005) called for more work on sperm design in more promiscuous passerine birds.

The bluethroat (*Luscinia svecica svecica*) is a monogamous species with strong tendencies to EPC behaviour, with 19-32% of the chicks being sired by extra-pair males (Johnsen *et al.* 2001; Krokene *et al.* 1996). Hence sperm competition is intense. Previous studies have documented that mate guarding is a well-developed paternity guard in the species (Johnsen *et al.* 2003; Krokene *et al.* 1996). Little is known about copulation behaviour and copulation frequency, but intense sperm competition should select for increased sperm production and competitive spermatozoa. Analyses of male fertilization success have revealed that there are few if any morphological traits that co-vary with fertilization success, except that male age is a strong predictor of male extra-pair fertilization (EPF) success (Johnsen *et al.* 2001). Thus, the bluethroat is a good candidate for studying variation in testis size and sperm length, and how the variation in these traits relates to male age, as well as morphological characters.

The aim of the present study is to quantify the variation in size of the testes and sperm length in the bluethroat and test for possible correlations with various morphological and conditional traits, age and indicators of genetic quality. Based on patterns reported in other species, three particular predictions could be made; I. Due to the intensity of sperm competition the bluethroat should have relatively long spermatozoa and the intra- and inter-male variation in sperm length should be low, II. Older males should have larger testes than yearling males. III. The left testis should be larger than the right testis, and the directional asymmetry should be correlated with secondary sexual characters.

Materials and methods

Study area and species

The field work was carried out in the valley Øvre Heimdalen (61º25'N, 8º52'E), Øystre Slidre municipality, Oppland county, Norway, from 18 May to 21 June 2004. The study area is located at an altitude of about 1100 m a.s.l. The area is dominated by dwarf birch *Betula nana*, willows *Salix* spp. and juniper *Juniperus communis*. In addition there is a belt consisting of mountain birch *Betula pubescens tortuosa* (Vik 1978).

The bluethroat is a migratory, relatively small passerine belonging to the family Turdidae. The species is sexually dichromatic, with males of this subspecies having a bright blue throat with a chestnut round patch in the middle and a chestnut band below (RB). Females have a paler throat with less blue and the chestnut colour is usually missing. It is a socially monogamous and territorial species. Males arrive on the breeding grounds in the middle of May (about a week prior to females), and initiate territory activities. The population in Øvre Heimdalen has been thoroughly studied since 1991, and the breeding density has been estimated to 23-38 breeding pairs per km^2 (Anthonisen *et al.* 1997; Johnsen *et al.* 2000). Females start nest building soon after pair formation. The female builds the nest alone in dense vegetation on the ground, and incubates 5-7 eggs for 13-15 days without male assistance (Johnsen & Lifjeld 1995). Both parents feed the nestlings, which stay in the nest for 10 to 14 days (Anthonisen *et al.* 1997).

Field procedures

Adult males were caught in their territories using mist nets, caged and transported to the lab. All birds were caught within a three-week period (1-21 June) that corresponds to the period in which egg-laying is initiated and males certainly are sexually mature. Cages were supplied with a Petri dish containing mealworm larvae, *Tenebrio molitor*. In the lab the birds were measured (body mass, wing length, tarsus length and RB), aged (yearling or older; (Svensson 1992)), and a blood sample (approx. 50 µl) was taken by brachial venipuncture. Haemoglobin was measured using HemoCue B-Hemoglobin Photometer (HemoCue AB, Ängelholm, Sweden), and haematocrit was measured using a digital calliper (amount red blood cells/amount total blood) after centrifugation of the micro capillary for approx. 2 minutes. The remaining blood sample was stored in lysis buffer for later genetic analyses. The birds were sacrificed by cervical translocation and then carefully dissected. Right and left seminal glomerus (the coiled distal ends of the vas deferens), right and left testis and liver were weighed to the nearest 0.001 g using a digital scale (Sartorius Talent TE153S Sartorius AG, Goettingen, Germany). The left seminal glomerus was carefully opened using a scalpel and a sample of sperm was fixed in Glutaraldehyd for later analyses of sperm length. A sample of living sperm was taken as well, and sperm motility was analysed (F. Fossøy, G. Rudolfsen, T. Laskemoen & J. T. Lifjeld in progress).

Right and left testis were measured to the nearest 0.1 mm using a digital calliper. Figure 1 illustrates the typical shape of the bluethroat testes, left testis bean-shaped and right testis pea-shaped. Three measures were taken; length, breadth1 and breadth2 (perpendicular to each other), and further calculated to radiuses (r_1, r_2 and r_3 respectively).

Testis volume was calculated according to the equation; (testis volume (mm^3) = 1.33π r_1(mm) r_2(mm) r_3(mm)), (assuming an ellipsoidal testis shape) derived from Møller (1991). Volume of testes = left testis volume + right testis volume. In the analyses of testis size and correlations between testes and other variables, data from testis mass were used due to the more exact value of testis size given by the digital scale. Calculations of testis volume and t-tests of testis volume between yearlings and older males can be found in the appendix (table B). After dissection each bird was frozen for later measurements of plumage traits and skin preservation. During preservation scull length was measured using a calliper.

Plumage measurements

The reflectance from the blue feathers in the throat patch was measured using a spectroradiometer, Ocean Optics USB2000 spectrometer, PX-2 pulsed xenon light source connected by a bifurcated fibre optics cable, (Ocean Optics BV, Duiven, The Netherlands). The methods for these reflectance measurements and the calculation of objective colour parameters (chroma, UV chroma hue and brightness) can be found in Johnsen *et al*. (2001).

Genetic analyses

DNA was extracted from blood using QIAamp® DNA Blood Kit (QIAGEN, Venlo, The Netherlands). A total of 10 microsatellite markers were amplified by polymerase chain reaction (PCR) on an ABI Prism® GeneAmp PCR System 9700 (Applied Biosystems,

Foster City, U.S.A.), and ran on an ABI Prism® 3100 Genetic Analyser (Applied Biosystems) using fluorescently labelled primers. Allele sizes were determined using ABI Prism® Genemappper™ Software version 3.0 (Applied Biosystems). Standardized heterozygosity (SH) (Coltman *et al.* 1999) and standardized mean d^2 (Sd^2) (Amos *et al.* 2001) was calculated. A more thorough description of the genetic analyses can be found in Fossøy *et al*. (in progress).

Sperm measurements

The sperm fixated in glutaraldehyd were prepared on a microscope slide and allowed to dry over night. A Leica DC 500 camera mounted on a Leica DM 6000B stereo microscope was used to photograph the sperm cells at a magnification of 320 X. A total of 30 sperm cells from each male were identified and photographed. All sperm cells were measured by using the Leica IM1000 software system provided by Tamro MedLab AS, Norway. Abnormal sperm cells (broken tail, damaged or missing acrosome) were not photographed. Passerine sperm are characterized by having a helical shaped head (acrosome + nuclear region) and straight flagellum (midpiece + tail), or both helical shaped head and flagellum (Koehler 1995). Bluethroat sperm is characterized as the latter, with helical shaped head and flagellum (figure 2). When examining passerine sperm it is hard to distinguish the midpiece and the tail from one another, and in this study I concentrated on the flagellum and total sperm length. Hence midpiece + tail are called flagellum in this paper. Two measures were taken; head length and flagellum length, combined giving the total length. When measuring flagellum and head length, a path tool in the Leica IM1000 software was used, as illustrated on the flagellum in figure

2a. Figure 2b & c illustrates one sperm cell from the individual exhibiting the shortest sperm cells, and one sperm cell from the individual exhibiting the longest sperm cells.

Statistical procedures

Statistical analyses were performed using STATISTICA version 6.1 (StatSoft, Inc). Constructions of graphs were done using Origin® v7.0300 (OriginLab Corporation). Nonparametric tests were used on data that were not normally distributed. All tests were two-tailed and the null hypotheses were rejected at $p<0.05$.

Results

Testis characteristics

The bluethroats in the present study had a mean mass of testes (TM) of 0.171g (n=47, SD ±0.045g). Coefficient of variance ($CV = \frac{SD}{\hat{x}} * 100$) was calculated to 26.2, illustrating a substantial variation in testis size. One potential source of variation is different stages of testicular development among the males included in the study. This would be indicated by a positive relationship between testis size and collection date. However, no such date effect was revealed (figure 3).

There was no difference in mass between right and left testis (table 1). In order to test Møller's (1994) directional asymmetry hypothesis, directional asymmetry (DA) was calculated (left testis – right testis) and correlated with secondary sexual characters (RB and UV chroma). None of the two correlations were significant (Linear regressions: RB: n=47, r=-0.002, *p*=0.99; UV chroma: n=47, r=-0.03, *p*=0.85). Thus, Møller's (1994) directional asymmetry hypothesis was not supported.

Older males had significantly larger testes than yearlings (table 2). No significant differences between older males and yearlings were revealed with respect to measures of testis asymmetry, although there was a trend with yearlings having a higher degree of directional asymmetry (DA) than older males (table 2).

One of the males caught showed an extreme testis asymmetry with the right testis weighing more than twice as much as the left testis (right testis: 0.224 g and left testis: 0.090 g). In addition I was unable to find any sperm cells in either of the seminal glomera of this male. Thus the male was classified as infertile and not included in the analyses.

I also tested whether standardized heterozygosity (SH) had any effect on TM. SH and TM were not significantly correlated (figure 4). Neither of the condition measures (body mass, haemoglobin and haematocrit) nor the body size measures (tarsus length and scull length) were significantly correlated with TM (Linear regressions: body mass: n=47, r=0.09, p=0.55; haemoglobin: n=47, r=-0.14, p=0.35; haematocrit: n=45, r=-0.18, p=0.22; tarsus: n=47, r=0.17, p=0.26; scull: n=33, r=0.25, p=0.17). The last body size measure, wing length, was positively and significantly correlated with TM (n=47, r=0.29, $p<0.05$). However this is explained by the fact that older males have significantly longer wings than yearlings (Appendix, table B), and when running linear regressions after dividing into the two age classes, none were significant (yearlings: n=22, r=0.09, p=0.69; older males: n=25, r=0.08, p=0.69).

After running separate Analyses of covariance (ANCOVA's) with TM as dependent variable, age class as categorical and one and one of several continuous variables, age class shows as the only significant variable predicting TM (Appendix, table A).

Seminal glomera characteristics

Mean seminal glomera mass (SGM) was measured to 0.080g (n=45. SD=0.019). CV was calculated to 24.3, illustrating a considerable variation in this trait as well. SGM could also have been affected by a date effect as they may require some time to be filled after the testes have reached full size. However no date effect on SGM was found (figure 5). It therefore seems evident that all the collected birds had fully developed reproductive organs. No difference in right and left seminal glomerus was found (table 1).

Older males had significantly larger seminal glomera than yearlings (table 2). In addition TM and SGM was positively correlated (figure 6), providing support for more sperm production in larger testes. TM and SGM were significantly correlated in older males, but not in yearlings (figure 6).

Sperm characteristics

Mean sperm length was measured to 216.5 µm (n=46, SD=6.0). The sperm length dataset was tested for normal distribution using a *Shapiro-Wilk's* test (W=0.98, p=0.42), giving support for normal distribution. I found a substantial variation in sperm length between the different bluethroat males (197.4 µm – 232.8 µm), but the variance within each male were relatively low (SD around 3.1 µm) (figure 7). There were significant differences in sperm length between males (one-way ANOVA $F_{1,45}$ =112, p<0.001), with a repeatability (Lessells & Boag 1987) of r=0.79. The CV for mean sperm length among males was 3.1.

As predicted there were no differences in sperm length between older males and yearlings (table 1). In addition sperm length was not significantly correlated with testes mass (figure 8). Neither of the measures body mass, haemoglobin, haematocrit, wing length, scull length and SH were significantly correlated with sperm length (Linear regressions: body mass: n=46, r=0.09, p=0.53; haemoglobin: n=46, r=-0.09, p=0.53; haematocrit: n=44, r=-0.03, p=0.85; wing length: n=46, r=-0.24, p=0.11; scull length: n=33, r=0.07, p=0.69; SH: n=46, r=-0.06, p=0.70). Surprisingly, tarsus length was significantly correlated with sperm length (n=46, r=0.35, p=0.02). However, it is difficult to explain the biological meaning of this correlation, and after applying a Bonferroni

adjustment (0.05/7) the α-level should be set to 0.007, thus implying that the correlation was not significant.

Discussion

This study has documented a large between-male variation in several primary sexual traits in the bluethroat. Given the fact that sperm competition is intense in the species (Johnsen *et al.* 1998; Johnsen *et al.* 2001), it was interesting to test whether components of this variation were correlated with secondary sexual traits, age, condition or indicators of genetic quality. I found a strong age effect on the size of testes and seminal glomera, whereas no other correlates were significant. Neither did I find any correlates of directional asymmetry in testis size. Sperm length varied considerably between males, but was highly repeatable within males. Sperm length did not correlate with male age or any other phenotypic traits.

Average TM in the bluethroat was 0.17 g., This value is lower than that reported by Møller (1991; 0.24 g) and lies between the phylogenetically related common redstart *Phoenicurus phoenicurus* (testes mass 0.10g) and the stonechat *Saxicola torquata* (testes mass 0.30g) (Møller 1991). The expected TM for a body size of 17 g (mean body mass of the birds caught in this study) is 0.28 g, according to regression equation in Møller (1991). This suggests that the TM in the bluethroat is somewhat lower than expected for its size.

Larger testes in older birds (second breeding season or older) was expected due to the findings from several other species (e.g. greenfinch *Carduelis chloris* (Merilä & Sheldon 1999), black-throated blue warbler *Dendroica caerulescens* (Graves 2004), sedge warbler *Acrocephalus schoenobaenus* (Birkhead *et al.* 1997), dark-eyed junco *Junco hyemalis* (Deviche *et al.* 2000), wren *Troglodytes troglodytes* (Evans & Goldsmith 2000) and barn owl *Tyto alba* (Roulin *et al.* 2004)). A possibly more important finding is

that seminal glomera also were larger in older males. This is more seldom reported from other studies, although Birkhead *et al*. (1997) reported a significant positive correlation between age and seminal glomera mass in the sedge warbler. There is support for increasing number of sperm when testes increase in size (Birkhead & Møller 1992; Briskie 1993; Møller 1988; 1989; 1991; 1994). In addition earlier studies have found increasing number of sperm with increasing size of the seminal glomera (Birkhead *et al.* 1993; Sax & Hoi 1998). The seminal glomera functions as storage for mature sperm, ready to be ejaculated during copulation (Birkhead *et al.* 1994). Especially if sperm competition by numbers is the most important factor, larger seminal glomera may give older males a significant advantage over yearlings during copulations due to the relatively larger number of sperm ejaculated. They may also produce a higher number of ejaculates per day. Both of these possible advantages experienced by older males may explain why older males have a higher EPP success than younger males in the bluethroat (Johnsen *et al.* 2001), as well as in other species (e.g. purple martin *Progne subis* (Wagner *et al.* 1996); Bullock's orioles *Icterus galbula bullockii* (Richardson & Burke 1999)).

Earlier hypotheses have emphasized experience with age as an important variable explaining why older males sire significantly more EPO than yearling males (Johnsen *et al.* 2001; Richardson & Burke 1999), or even older males being the only males in a population siring EPO (Wagner *et al.* 1996). It is also a possibility that yearling males do not invest much effort in EPC behaviour during their first breeding season. This can be due to their lack of experience and their relatively less developed reproduction organs (smaller testes and smaller seminal glomera than older males). Hence a somewhat speculative theory can be that yearlings trade off effort in EPC behaviour and

development of reproduction organs during their first breeding season with gaining experience, guarding within-pair paternity (WPP) and survival. In addition to the finding of older males achieving more EPP than yearlings (Johnsen *et al.* 2001), it has been shown that yearlings mate guard at a significant higher rate than older males in the same bluethroat population (Johnsen *et al.* 2003). Further, there is no age difference in achieving WPP (Johnsen *et al.* 2003), and this may indicate that an increase in mate guarding intensity compensates for lower sperm production in the yearlings in the sperm competition.

So far I have only focused on the males' behaviour and morphology as predictors of paternity. If female choice occurs and some males are favoured over others, the sperm competition and paternity competition may be biased. Earlier studies have found significant differences between the two age classes in secondary sexual characters such as width of RB and UV chroma (Johnsen *et al.* 2001). In this study there was no difference in RB (Appendix Table B), but significantly higher value for UV chroma in the older males (Appendix table B). In addition it has been shown that males with experimentally blackened throat patch had lower pairing success than control males (Johnsen & Lifjeld 1995) and males with artificially reduced UV reflectance had lower success in attracting social mates, and furthermore had lower success in achieving extra-pair fertilizations (Johnsen *et al.* 1998). But it is important to remember that these manipulations were considerably outside of normal variation, and may even have influenced the species recognition. And given the fact that normal variation in coloration does not correlate with paternity success (Johnsen *et al.* 2001), it seems unlikely that female choice on male coloration and ornamentation plays an important role in the sperm competition.

The EPC-mating system in the bluethroat population of Heimdalen has shown to increase the heterozygosity of the extra-pair young (EPY) compared to the within-pair young (WPY) (Lifjeld *et al*. unpublished data). In addition an earlier study from the same population has shown that EPY have higher immunocompetence than WPY (Johnsen *et al.* 2000). When correlating SH (as a proxy for "genetic quality") with TM in the present dataset, no significant relationship was found, nor when dividing into the two age subsets.

Earlier studies have supported the prediction that increased risk and intensity of sperm competition can favour increased sperm length in e.g. insects (Gage 1994), frogs (Byrne *et al.* 2003), and some mammals (Gomendio & Roldan 1991). In birds, sperm length has shown to correlate positively with level of EPC and EPP in passerines (Briskie *et al.* 1997) and in a study of shorebirds, sperm were significantly longer in nomonogamous than in monogamous species (Johnson & Briskie 1999). A study of 20 species of North American passerines revealed no relation between sperm size and mating system (Briskie & Montgomerie 1992). However, Briskie & Montgomerie (1992) used mating system, and not level of EPP, as a proxy for sperm competition. An example of the disadvantage of that definition of sperm competition is the later revealed level of EPP in one of their monogamous species, tree swallow (*Tachycineta bicolour*), with 38% EPY and 50% broods containing EPY (Lifjeld *et al.* 1993). In addition the tree swallow exhibits relatively long sperm (approx 250 μm) (Briskie & Montgomerie 1992). The reed bunting (*Emberiza schoeniclus*) exhibits the highest reported frequency of EPP for socially monogamous species (Griffith *et al.* 2002), namely 55% EPY and 86% broods containing EPY in a population in Great Britain (Dixon *et al.* 1994). And the sperm length (292 μm) of the reed bunting is among the longest reported in birds (Briskie *et al.*

1997). In comparison, the zebra finch exhibits short sperm (approx. 60 µm) (Birkhead *et al.* 2005) and low intensity in sperm competition (Birkhead *et al.* 1990). The sperm length of the bluethroat revealed in the present study (216 µm) together with the earlier revealed frequency of EPP in this population (19-32%) (Johnsen *et al.* 2001; Krokene *et al.* 1996), coincide well with earlier findings of positive relationship between sperm competition and sperm length (Briskie *et al.* 1997; Johnson & Briskie 1999).

Having found that testis size is age-dependent in the bluethroat, it would have been interesting to study the development of the testes over subsequent breeding seasons. Measuring testis size in living birds can be a challenge, but earlier studies have shown successful laparotomy on both captive and free living birds (Bailey 1953; Partecke *et al.* 2004; Wingfield & Farner 1976). Briefly, the laparotomy method implies a small incision on left side of the bird between the last two ribs, thus permitting a satisfactory view of the left testis and allowing measurement of testis length (Bailey 1953). A recent paper describes a simple technique of collecting sperm from fresh faeces of the birds (Immler & Birkhead 2005). This method would be preferred over other methods due to the less handling and manipulation of the bird. However, this method excludes the possibility of measuring sperm motility as the sperm most likely are killed or influenced in other ways after mixing with the faeces. If measurements of sperm motility are included as an aim in the study, one should probably seek another method of obtaining living sperm. Abdominal massage, a method which has been used successfully on passerines previously (Tuttle *et al.* 1996; Vernon & Woolley 1999), could be the solution. To be able to carry out such a study over subsequent breeding seasons one would need a

philopatric study species. The return rate of bluethroats in Heimdalen is approx. 20% (J. T. Lifjeld pers. comm.), quite low but a study as mentioned above should be feasible.

In conclusion, the present study shows that both testis mass and seminal glomera mass are age-dependent in the bluethroat. The positive relationship between testis mass and seminal glomera mass revealed together with the fact that larger testes and larger seminal glomera are associated with larger number of sperm, may indicate that older males experience an advantage in sperm competition with yearling males. The bluethroat possesses relatively long spermatozoa compared to other passerines, and in combination with the high frequency of EPP in this species it provides support for earlier findings of positive relationship between frequency of EPP and length of sperm. Future studies should seek to find relationships between sperm motility and other variables in the bluethroat (already in progress (Fossøy *et al.*)). In addition, measurements of testes development over subsequent breeding seasons together with already known data on behaviour of the males would be valuable in order to understand the role of male factors in EPC behaviour and sperm competition.

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Tables and figures

Table 1. Comparisons of right and left testes and seminal glomera. Two-sample t-tests assuming equal variances

	Left			Right				
Variable	Mean	SD	n	Mean	SD	n	*t*-value	*p*
TM (g)	0.087	0.023	47	0.083	0.024	47	0.77	0.44
SGM (g)	0.040	0.010	45	0.039	0.011	45	0.33	0.74

TM=mass of testes, SGM=seminal glomera mass. Both tests two tailed.

Table 2. Means and standard deviation of testicular measurements of older male and yearling Bluethroats. Two-sample t-tests assuming equal variances

	Older males			Yearlings				
Variable	Mean	SD	n	Mean	SD	n	*t*-value	*p*
TM (g)	0.194	0.044	25	0.144	0.029	22	4.52	<0.001*
SGM (g)	0.086	0.021	23	0.073	0.010	22	2.22	<0.03*
Testes DA	0.001	0.015	25	0.007	0.012	22	1.53	0.13
Sperm flagellum length (µm)	191.3	5.7	25	192.3	6.2	21	0.57	0.57
Sperm total length (µm)	215.8	5.7	25	217.2	6.2	21	0.74	0.46

SGM=seminal glomera mass, TM=mass of testes, Testes DA1=directional asymmetry in testis mass; left testis mass minus right testis mass. All tests two tailed: $*p<0.05$.

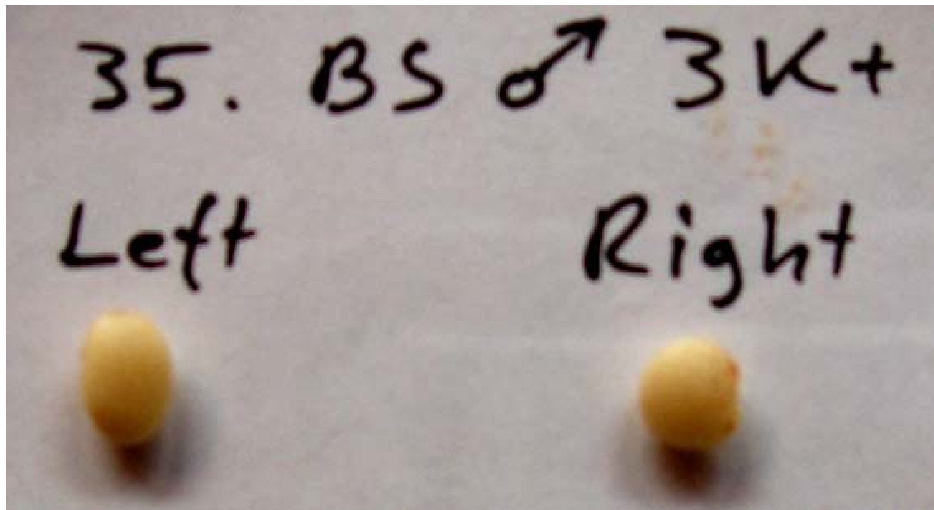

Figure 1. Photography showing the morphological difference between the left and right testis. Left testis bean-shaped and right testis pea-shaped.

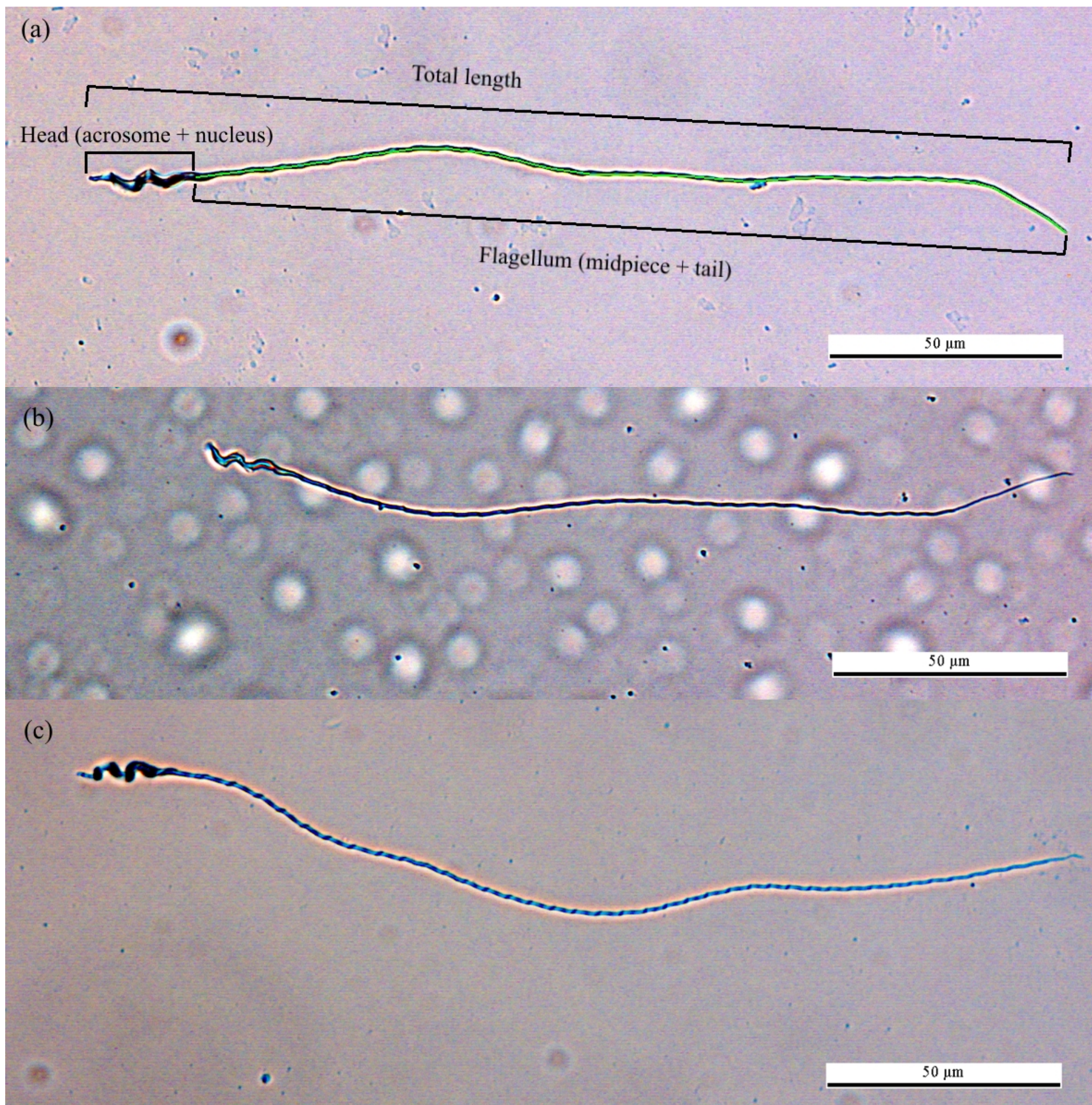

Figure 2. (a-c) Digital photos of bluethroat sperm. (a) Illustrating the helical shaped head and flagellum. Head (acrosome + nucleus), flagellum (midpiece + tail) and total length indicated by straight bars. Green path illustrates the measuring method of flagellum length. (b) Sperm cell from the individual possessing the shortest spermatozoa measured in this study (197.4 µm); (c) Sperm cell from the individual possessing the longest spermatozoa measured in this study (232.8 µm). (Photos taken with a Leica DC 500 camera mounted on a Leica 600B stereo microscope.)

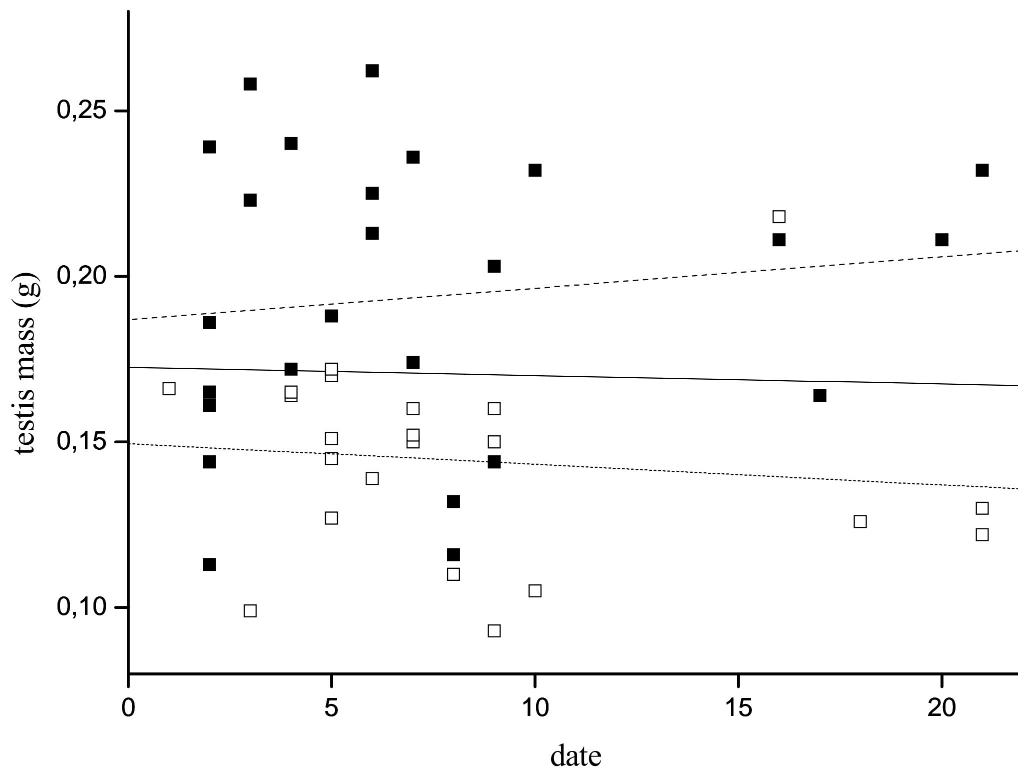

Figure 3. No date effect on testis mass revealed with respect to capture date. (Spearman: whole sample: n=47, r_s=-0.15, p=0.32; yearlings: n=22, r_s=-0.35, p=0.11; older males: n=25, r_s=0.08, p=0.70) Lines shown to illustrate the lack of date effect (linear regressions: solid line: whole sample: n=47, r=-0.03, p=0.83; dotted line: yearlings: n=22, r= -0.12, p=0.59; dashed line: older males: n=25, r=0.12, p=0.56). Symbols: □ = yearling (2k) and ■ = older male (3k+).

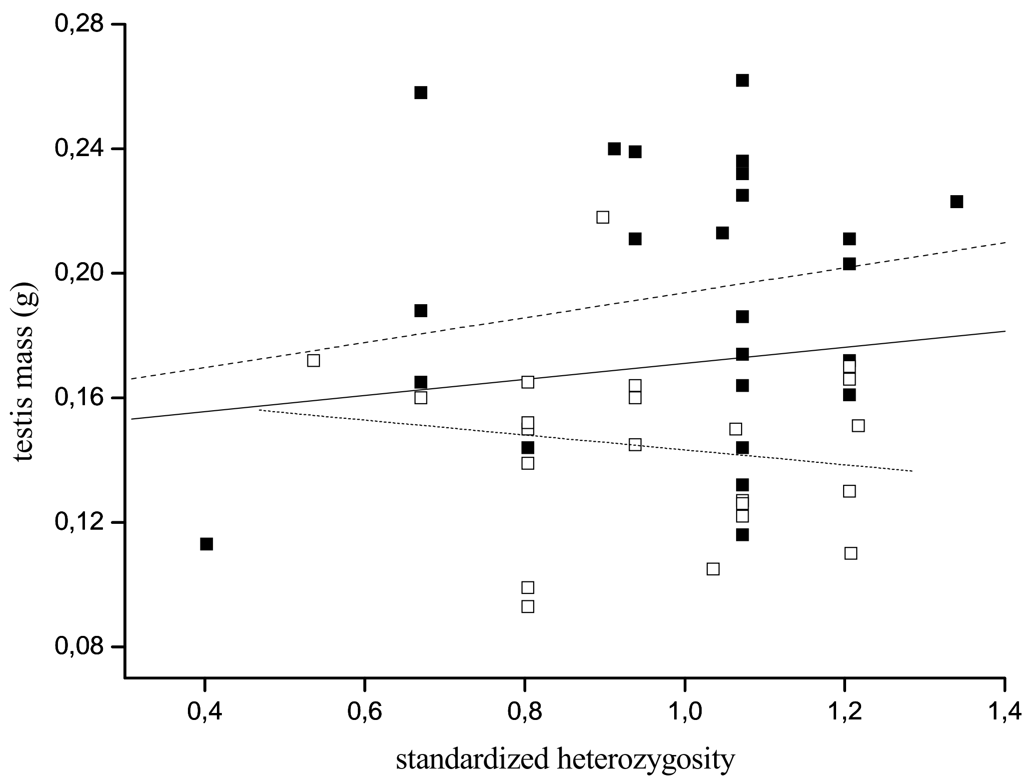

Figure 4. Showing correlation between standardized heterozygosity (Coltman *et al.* 1999) and testis mass. (Linear regressions: whole sample: n=47, r=0.12, *p*=0.43; yearlings: n=22, r=-0.16, *p*=0.48; older males: n=25, r=0.19, *p*=0.35) Solid line: whole sample; dotted line: yearlings; dashed line: older males. Symbols: □ = yearling (2k) and ■ = older male (3k+).

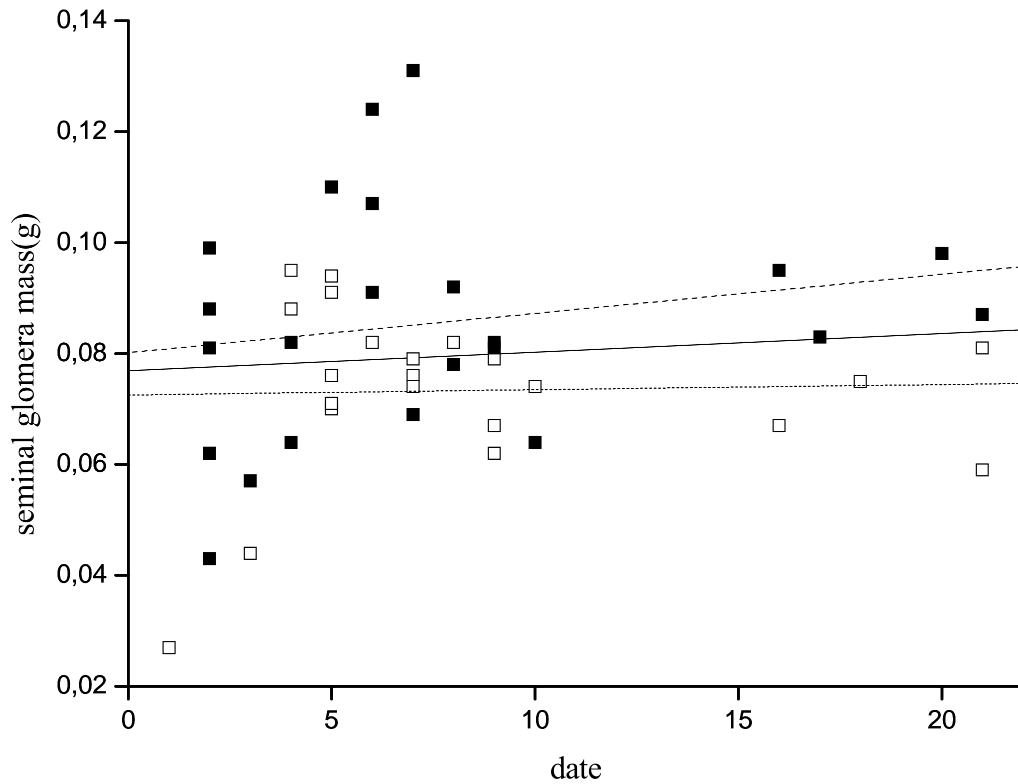

Figure 5. No date effect on seminal glomera mass revealed with respect to capture date. (Spearman: whole sample: n=45, r_s=0.07, p=0.66; yearlings: n=22, r_s=-0.15, p=0.50; older males: n=23, r_s=0.22, p=0.31). Lines shown to illustrate the lack of date effect (Linear regressions: solid line: whole sample: n=45, r=0.10, p=0.52; dotted line: yearlings: n=22, r=0.03, p=0.88; dashed line: older males: n=23, r=0.19, p=0.38). Symbols: □ = yearling (2k) and ■ = older male (3k+).

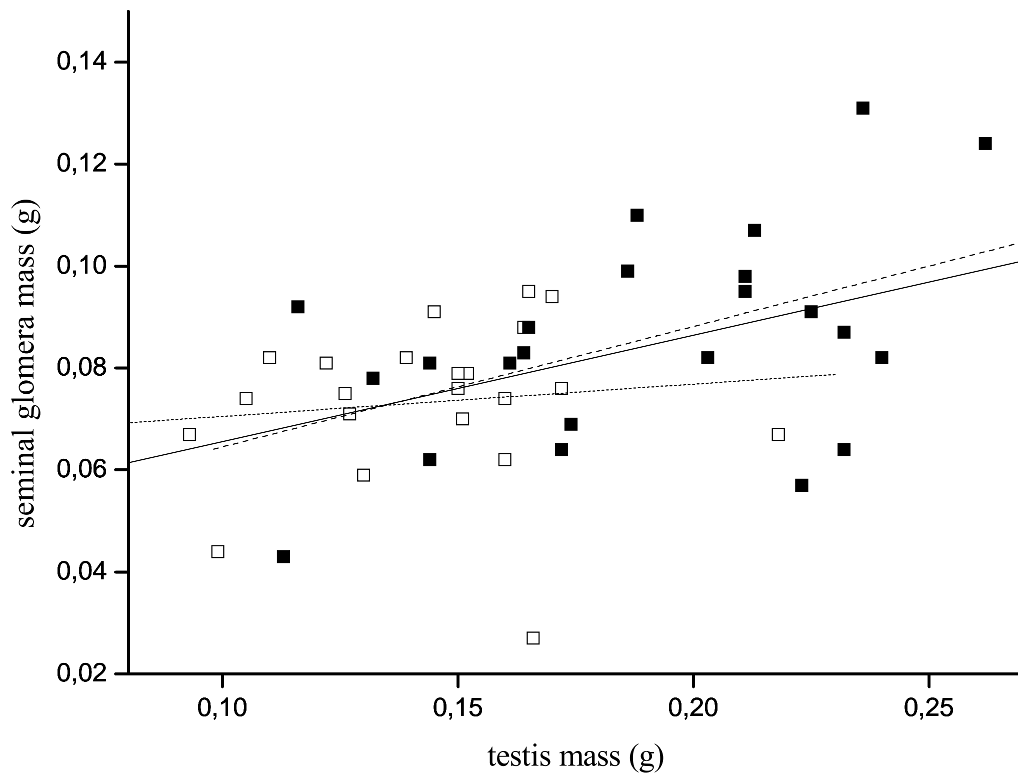

Figure 6. Correlations between testis mass and seminal glomera mass. (Linear regressions: whole sample: n=45, r=0.46, *p*=0.002; yearlings: n=22, r=0.12, *p*=0.60; older males: n=23, r=0.48, *p*=0.02). Solid line: whole sample; Dotted line: yearlings; Dashed line: older males. Symbols: □ = yearling (2k) and ■ = older male (3k+)

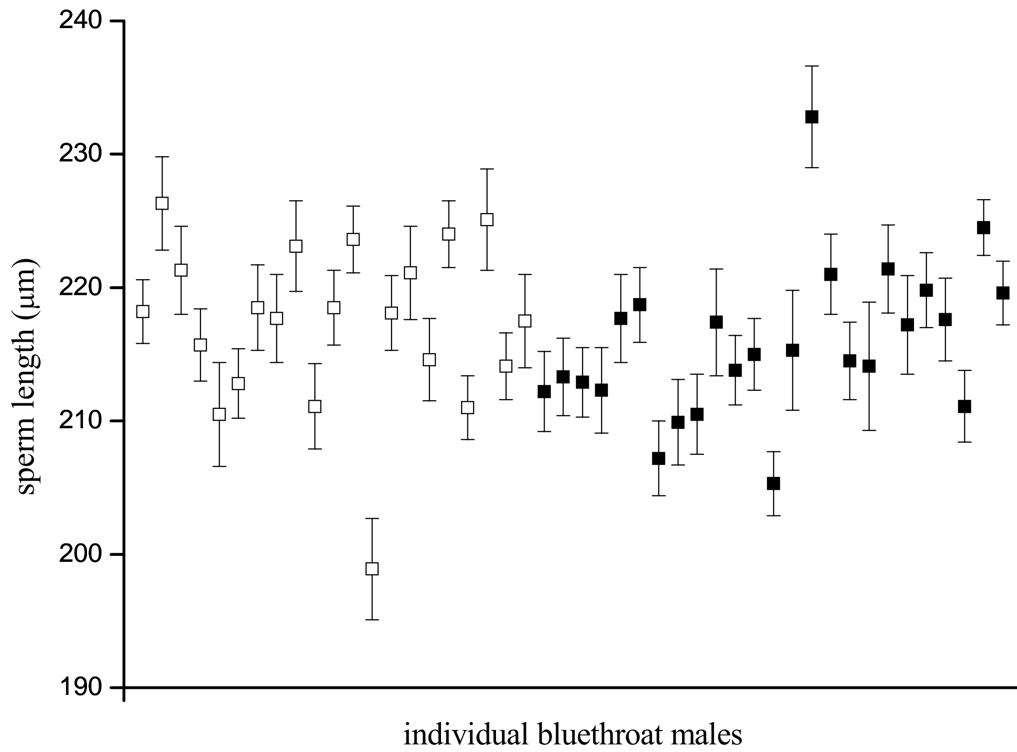

Figure 7. All sperm lengths measured (n=46, mean ± SD µm). Symbols: □ = yearling (2k) and ■ = older male (3k+). Sorted by capture date (from left to right), yearlings and older males respectively.

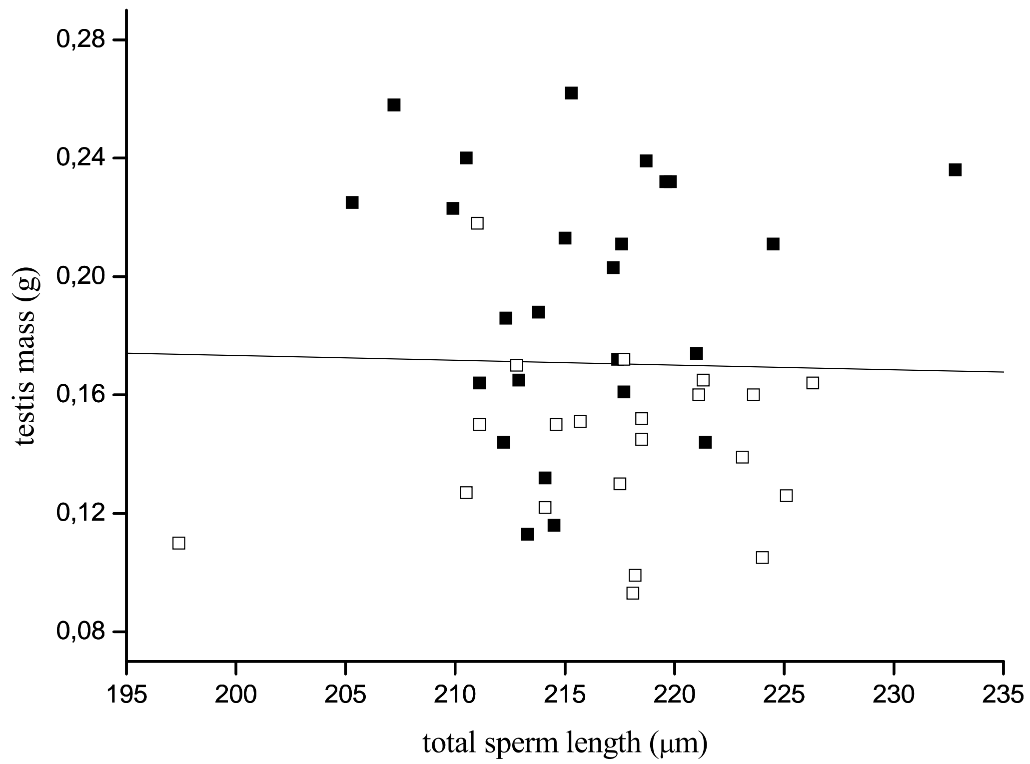

Figure 8. No significant correlation between testes mass and total sperm length (Linear regression: n=46, r=-0.02, *p*=0.89). Symbols: □ = yearling (2k) and ■ = older male (3k+).

Appendix

Table A. Independent analyses (ANCOVA) for TM.

Dependent variable	Independent variables	df	MS	*F*-ratio	*p*
TM (g)	Date	1	0.00007	0.05	0.83
	Age class	1	0.03	19.96	<0.001*
	Error	44	0.001		
TM (g)	Body mass (g)	1	0.001	0.85	0.36
	Age class	1	0.03	20.67	<0.001*
	Error	44	0.001		
TM (g)	Wing length (mm)	1	0.0004	0.31	0.58
	Age class	1	0.02	14.98	<0.001*
	Error	44	0.001		
TM (g)	Tarsus (mm)	1	0.003	2.28	0.14
	Age class	1	0.03	21.34	<0.001*
	Error	44	0.001		
TM (g)	RB	1	0.0003	0.21	0.65
	Age class	1	0.03	20.05	<0.001*
	Error	44	0.001		
TM (g)	Haemoglobin	1	0.0008	0.57	0.45
	Age class	1	0.03	19.50	<0.001*
	Error	44	0.001		
TM (g)	SH	1	0.0003	0.23	0.63
	Age class	1	0.03	19.40	<0.001*
	Error	44	0.001		
TM (g)	UV chroma	1	0.00004	0.03	0.88
	Age class	1	0.02	16.90	<0.001*
	Error	44	0.001		
TM (g)	Scull (mm)	1	0.005	3.46	0.07
	Age class	1	0.03	17.70	<0.001*
	Error	30	0.002		
TM (g)	Liver	1	0.0006	0.40	0.53
	Age class	1	0.02	10.27	0.003*
	Error	34	0.002		

TM=mass of testes; RB=width of chestnut band; SH=standardized heterozogosity. *$p<0.05$

Table B. Means and standard deviation of all measures of older male and yearling bluethroats. Two-sample t-tests assuming equal variances.

	Older males			Yearlings				
Variable	Mean	SD	n	Mean	SD	n	*t*-value	*p*
Wing length (mm)	74.3	1.4	25	73	1,5	22	2.98	0.005*
Body mass (g)	16.75	1.34	25	16.85	0.85	22	0.26	0.80
Tarsus length (mm)	30.54	0.68	25	30.58	0.90	22	0.18	0.86
Scull length (mm)	34.69	0.52	17	34.68	0.84	17	0.05	0.96
RB (mm)	7.9	2.1	25	7.9	2.6	22	0.02	0.99
Amount red in tail	0.65	0.03	25	0.66	0.03	22	0.12	0.90
Haemoglobin	17.7	2.7	25	18.1	1.7	22	0.56	0.58
Haematocrit	0.47	0.07	25	0.49	0.04	20	1.06	0.29
Liver (g)	0.588	0.078	21	0.659	0.078	16	2.77	0.009*
SH	1.00	0.19	25	0.96	0.21	22	0.69	0.49
Sd^2	0.11	0.05	25	0.13	0.08	22	1.08	0.29
Chroma	1.150	0.102	25	1.061	0.129	22	2.66	0.01*
UV chroma	0.320	0.014	25	0.306	0.016	22	3.15	0.003*
l(max)	368.8	5.5	25	373.5	10.1	22	2.04	0.05*
Left seminal glomerus (g)	0.042	0.012	23	0.038	0.008	22	1.57	0.12
Right seminal glomerus (g)	0.043	0.011	23	0.036	0.009	22	2.51	0.02*
SGM (g)	0.086	0.021	23	0.073	0.016	22	2.22	0.03*
Left testis mass (g)	0.097	0.023	25	0.076	0.016	22	3.66	<0.001*
Right testis mass (g)	0.096	0.023	25	0.069	0.015	22	4.86	<0.001*
TM (g)	0.194	0.044	25	0.144	0.029	22	4.52	<0.001*
Testes DA	0.001	0.015	25	0.007	0.012	22	1.53	0.13
Left testis volume (mm^3)	86.8	21.7	25	67.9	15.9	22	3.38	0.002*
Right testis volume (mm^3)	85.2	22.1	25	60.5	15.0	22	4.41	<0.001*
Total testis volume (mm^3)	172.0	41.4	25	128.4	28.4	22	4.17	<0.001*
Sperm length (µm)	215.8	5.7	25	217.2	6.5	21	0.74	0.46

RB=width of the chestnut band; SH=standardized heterozygosity; Sd^2=standardized mean d^2; SGM=seminal glomera mass; TM=mass of testes; Testes DA=directional asymmetry (left testis-right testis). All tests two-tailed, *$p<0.05$