Sperm Morphology, Sperm Motility and Paternity Success in the Bluethroat (*Luscinia s. svecica*)

Camilla LoCascio Sætre

Master of Science Thesis in Ecology and Evolution

Natural History Museum
University of Oslo

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Photo: Bjørn Aksel Bjerke

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http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Acknowledgements

This thesis was written at the Natural History Museum, University of Oslo, under the supervision of Professor Arild Johnsen and Dr. Emily Rebecca A. Cramer.

To Arild and Becky: I am so grateful to have had you as my supervisors. You have been incredibly engaging, inspiring and supportive throughout this whole process, and I have learnt so much from you. Arild, I want to thank you first of all for welcoming me into your group, and most importantly, for suggesting that I choose the bluethroat as my study species. I have absolutely loved working on this project. You have been an excellent guide in introducing me to this field, and I have really appreciated your involvement in this thesis. Thank you for everything you have taught me and all your helpful comments in the writing process, for your support, and for always being in a good mood and lifting my spirits. Becky, you have been an awesome supervisor, and you have been there for me every step of the way. You have taught me so much, and even made me like Excel (which I never thought was possible). I have come to you most of all, with problems or questions, big and small, and your help has truly been invaluable. Thank you so much for everything, for being so positive and supportive, and for being a great friend.

The bird group at the museum is amazing, and I am so glad to have been part of it. Fieldwork was so much fun, both the work itself and spending the days with these wonderful people: Arild, Becky, Kristine Dobbe, Silje Rekdal, Silje Hogner, Even Stensrud, Gunnhild Marthinsen and Celeste Apolinario. I've had the best time! Special thanks to Silje Hogner for training me in the lab, Even Stensrud for measuring sperm and Silje Rekdal for proof-reading.

I want to thank everyone who has contributed to this thesis one way or another: My father, Glenn-Peter Sætre, for helpful discussions about evolutionary aspects, for excellent proof-reading and for immense support. Maria LoCascio Sætre, Helene LoCascio Sætre, Tim Dunker and Camilla Bomé: for proof-reading, for keeping me going and being family and friends of the utmost quality.

Table of contents

1 Abstract

Postcopulatory sexual selection is among the least studied forms of sexual selection. Certain male characteristics, such as sperm morphology and motility, may be selected for through sperm competition or cryptic female choice. However, how these characteristics may influence male fertilization success remains poorly understood. In this study, I investigate possible correlations between characteristics of males, their sperm, and paternity success in bluethroats (*Luscinia svecica svecica*), predicting that sperm length and sperm swimming speed would be positively correlated with paternity success. Sperm morphology and sperm motility are also expected to be correlated. From two breeding seasons, I ran paternity tests to identify which offspring were true genetic matches to their social father and which were extra-pair (i.e. sired by another male). In total, 23 % (11/48) of broods contained extra-pair offspring and 10 % (27/260) of the offspring were sired by extra-pair males. In one year, but not the other, sperm length was significantly shorter in males who had lost paternity in their own nest than in males who had not. Paired comparisons showed that males who had gained paternity in another nest tended to have longer sperm than the males they had cuckolded, though the effect was not significant. Although the evidence is only preliminary, sperm length may therefore relate to fertilization success. Sperm swimming speed was not associated with paternity success, nor correlated with sperm morphology. Thus, if sperm length does increase fertilization success, it does not appear to be due to higher swimming speed. Paternity success did not correlate significantly with any other morphological trait of the males themselves or with the age of the male. Thus, the male characteristics investigated here do not appear to be strong predictors of paternity success in bluethroats, although the sample sizes may be too small to detect any actual effects. Two of the males in the dataset lost full paternity in their own nest, suggesting that they were functionally infertile or genetically incompatible with their mate. Previous studies indicate that cryptic female choice for genetically compatible males is most likely very important in postcopulatory sexual selection in bluethroats. Apparently, however, many factors may influence patterns of paternity in these birds, including plumage traits, precopulatory behavior, age, and, according to the present study, possibly sperm length.

2 Introduction

Sexual selection occurs when individuals of the same sex differ in their ability to secure mates and/or fertilizations (Andersson 1994). Intrasexual selection takes the form of competition between members of one sex (typically males) for access to mating (Bateman 1948; Trivers 1972). Intersexual selection occurs when members of one sex (typically females) favor mates with certain characteristics over others (Fisher 1915; Andersson 1982). Sexual selection can also be divided into two main categories depending on when in the reproductive cycle it occurs: precopulatory and postcopulatory sexual selection, i.e., before and after copulation. In species where females may copulate with two or more males, postcopulatory sexual selection may take place in the form of sperm competition (Parker 1970) or cryptic female choice (Eberhard 1996). Less is known about the process of postcopulatory than precopulatory sexual selection, simply because it is more obscured from our view and hence more difficult to study. Various adaptations may have evolved in each sex to gain control over fertilization (Stockley 1997; Westneat and Stewart 2003).

Sperm competition is the competition between sperm from two or more males to fertilize a set of ova (Parker 1970). The outcome of this competition, i.e., which male gains paternity, may depend on certain qualities of the sperm cells and sperm producing tissues, such as sperm length, sperm swimming speed or sperm numbers (Snook 2005). Cryptic female choice is the ability of females to control which male fertilizes their eggs after having mated with several males (Eberhard 1996). In some species, females may eject sperm from unwanted copulations, for example from subdominant males (Pizzari and Birkhead 2000). Alternatively, females may not actively control the process. For instance, the most genetically compatible male might have the highest probability of fertilizing the egg (Pryke *et al*. 2010; Alcaide *et al*. 2012; Yeates *et al*. 2013), for example through differential chemical attraction between different sperm and the egg (Yeates *et al*. 2013). Cryptic female choice may counteract the effects of sperm competition, unless certain sperm traits are related to male qualities preferred by females (Evans *et al*. 2003).

Together, sperm competition and cryptic female choice are thought to have shaped the evolution of sperm traits. Much of the research investigating selection for various sperm traits involves comparative analyses among different taxa. In species where the risk of females mating with multiple males is higher, males have been shown to produce, for example, faster

swimming sperm and/or longer sperm cells (insects: Gage 1994, mammals: Gomendio and Roldan 1991, birds: Briskie *et al*. 1997; Kleven *et al*. 2009a; Lüpold *et al*. 2009b). However, postcopulatory sexual selection, and thus selection for various sperm traits, may operate differently in different species, in relation to factors such as differences in life histories and modes of mating.

In birds, it has been suggested that the most important selection pressure on males has been to produce larger quantities of small sperm, as sperm is diluted in the female reproductive tract (Snook 2005; Immler *et al*. 2011). However, sperm length is highly variable between different bird species (Briskie and Montgomerie 1992). This can, at least in part, be explained by variation in the female sperm storage organs (sperm storage tubules). Sperm length has been found to increase with the length of female sperm storage tubules, and to increase in species where the risk of female promiscuity is higher (Briskie and Montgomerie 1992, 1993). Similar patterns have also been found in other taxa, such as in *Drosophila* (Miller and Pitnick 2002).

Sperm morphology may affect fertilization success through effects on swimming speed. Flagellum length can be important, since a longer flagellum may propel the cell faster (Gomendio and Roldan 1991; Mossman *et al*. 2009). The length of the midpiece can be important because the midpiece contains fused mitochondria that provide energy (Lüpold *et al*. 2009a; Laskemoen *et al*. 2010). Finally, the shape and length of the head can be important, since the head produces drag which counteracts the propulsion of the flagellum (Humphries *et al*. 2008; Lüpold *et al*. 2009a; Helfenstein *et al*. 2010).

Intuitively, a faster swimming sperm cell should have higher fertilization success since it on average would reach the egg before a slower sperm cell. Sperm swimming speed, as well as the proportion of motile sperm in ejaculates, has been shown to correlate with levels of promiscuity between species (Kleven *et al*. 2009a; Rowe and Pruett-Jones 2011). In intraspecific experiments controlling for sperm quantity, faster swimming sperm has repeatedly been shown to have higher fertilization success (Birkhead *et al*. 1999; Levitan 2000; Gage *et al*. 2004; Denk *et al*. 2005; Casselman *et al*. 2006; Gasparini *et al*. 2010; Boschetto *et al*. 2011). Studies on wild birds are scarce, but sperm swimming speed may not have a similarly strong effect on fertilization success in natural settings when sperm quantity may vary between males (Laskemoen *et al*. 2010), and when sperm from different males are deposited at different times (Briskie 1996).

To what extent sperm length and shape affect fertilization success may vary between species and is a matter of current research (reviewed in Snook 2005). Mixed results have been found in intraspecific studies of wild passerine species (Laskemoen *et al*. 2010; Calhim *et al*. 2011; Cramer *et al*. 2013b). Laskemoen *et al*. (2010) found some indirect evidence that midpiece length may affect fertilization success in tree swallows (*Tachycineta bicolor*), but sperm quantity seemed to be more important. In the Australian superb fairy-wren (*Malurus cyaneus*), sperm with a longer flagellum and a relatively smaller head secured more within-pair fertilizations, whereas sperm with the opposite morphology was more successful in obtaining fertilizations in other nests (Calhim *et al*. 2011). Finally, Cramer *et al*. (2013b) did not find any association between paternity success and sperm morphology in house wrens (*Troglodytes aedon*).

There is clearly a need for continued research to understand sperm competition and cryptic female choice. Passerine birds are good model species to investigate these processes, because despite the fact that most passerines are socially monogamous, extra-pair copulations (i.e. copulations outside the social partnership) are common (Griffith *et al*. 2002). Determining the adaptive value of such copulations for males is straightforward, as males may potentially sire more offspring if they mate with multiple females (Birkhead and Møller 1992). Hence, selection should favor male traits that increase fertilization success. Whether females benefit from such behavior is more controversial. The wide occurrence of extra-pair paternity in passerines can be due to non-adaptive factors (Forstmeier *et al*. 2014), but it is also likely that there are some benefits of mating outside the social partnership (Petrie and Kempenaers 1998). Females may seek to increase the fitness of their offspring by copulating with extra-pair males that possess phenotypic traits reflecting good genes (Houtman, 1992; Michl *et al*. 2002), or by copulating with a genetically compatible male (Johnsen *et al*. 2000; Foerster *et al*. 2003; Freeman-Gallant *et al*. 2003; Fossøy *et al*. 2008, Pryke *et al*. 2010). Females may also copulate with multiple males as a means to insure fertilization of their eggs, since sterility in males may occur (Wetton and Parkin 1991; Sheldon 1994; Krokene *et al*. 1998; Lifjeld *et al*. 2007).

Testing between a number of adaptive and non-adaptive hypotheses can be challenging. Hence, a reasonable starting point is to look for correlations between male characteristics and reproductive success. In this study, I investigated postcopulatory sexual selection in a

Norwegian population of bluethroats (*Luscinia svecica svecica*; hereafter referred to as bluethroats), using such a correlative approach.

The bluethroat is a socially monogamous passerine in the Muscicapidae family with high rates of extra-pair paternity (Krokene *et al*. 1996; Johnsen and Lifjeld 2003). The social male guards his mate before egg-laying, presumably to reduce the risk of being cuckolded (Krokene *et al*. 1996; Johnsen *et al*. 2003). However, his ability to protect paternity through this strategy is far from complete (Johnsen *et al*. 1998a, b; Johnsen *et al*. 2003).

Many aspects of precopulatory and postcopulatory sexual selection have been studied in bluethroats. Male coloration has been shown to correlate with within-pair fertilization success, whereas the age of the male correlates with extra-pair fertilization success (Johnsen *et al*. 2001). Variation in fertilization success between males may be explained by precopulatory sexual selection, but postcopulatory processes may also affect the outcome of paternity. There is evidence that cryptic female choice is operating in bluethroats, as the genetic compatibility of the pair mates is important for determining paternity success (Fossøy *et al*. 2008). Since most bluethroat females engage in extra-pair copulations, but not all of them produce extra-pair offspring (Fossøy *et al*. 2006), the selection for genetically compatible genes is thought to occur at the postcopulatory stage. Genetic compatibility, specifically genetic dissimilarity between copulation partners, has been linked to increased fitness in the offspring, through increased heterozygosity and immunocompetence (Johnsen *et al*. 2000, Fossøy *et al*. 2008).

In this study, I investigate possible associations between male within- and extra-pair fertilization success and different characteristics of the males, focusing on characteristics of their sperm cells. So far, no study has investigated whether sperm traits affect paternity success in bluethroats. This study will therefore be an important step in unraveling the relative role of sperm competition in postcopulatory prezygotic selection in bluethroats. If sperm competition is important, I predict that paternity success should be positively associated with sperm length and/or sperm swimming speed (e.g. Gomendio and Roldan 1991; Birkhead *et al*. 1999). I further expect to support previous findings in bluethroats, showing that within-pair fertilization success correlates with male plumage traits, and that extra-pair fertilization success increases with male age. Finally, to improve our understanding of sperm biology in bluethroats, I also test for correlations among sperm characteristics (such as morphology and

swimming speed), and to what extent the various measures are repeatable within and between seasons.

3 Materials and methods

3.1 Study species

The bluethroat is highly sexually dichromatic. The males are strikingly colourful, with a blue throat surrounding a chestnut orange spot (Johnsen *et al*. 2006). The blue throat patch is bordered by a black and a chestnut orange band (hereafter referred to as the red border). Some females have a rudimentary version of the throat patch, but most are brown, grey and black in color (Cramp 1988). Bluethroats are migratory birds, breeding in northern parts of Europe, North America and Asia, and wintering in Africa and south Asia (Cramp 1988). In the spring, males arrive at the breeding site about a week before the females to establish territories. The males court the females with their complex song and by displaying the colors of their throats and tails (Peiponen 1960). Once a pair is formed, the female builds her nest on the ground, hidden by vegetation. During this period, extra-pair copulations may occur, despite intense male mate guarding (Johnsen *et al*. 2003). The males continue to court neighboring females and defend their territories until the eggs are hatched (Johnsen *et al*. 2001). The female lays five to seven eggs which she incubates for 13 to 15 days, and the social partners feed their young together (Cramp 1988).

3.2 Study site

Fieldwork was conducted in the valley of Øvre Heimdalen, Øystre Slidre, in Oppland, Norway (61º25'N, 8º52'E) during spring/summer in 2013 and 2014. Øvre Heimdalen lies in the eastern part of the Jotunheimen mountain range at about 1100 m altitude. The study area is situated along the lake Øvre Heimdalsvatn, and is an open sub-alpine habitat, with shrubs such as junipers (*Juniperus communis*), willows (*Salix sp.*), birch (*Betula pubescens*) and dwarf birch (*Betula nana*).

3.3 Sampling

Fieldwork was conducted from 28 May to 29 June 2013, and from 29 May to 2 July 2014. I caught adult bluethroats with mist nets. Each bird was banded with a unique set of three color rings and a metal ring for identification, photographed and measured in multiple ways. About 25 µl of blood was collected by puncturing the brachial vein under the wing and collecting

blood in a capillary tube. The samples were stored in 2 ml Sarstedt tubes with 1 ml ethanol. The age of the bird was determined as either second year (2k) or older (3k+) by inspecting the coverts of the wings (Svensson 1992). The length of the tarsus was measured (between the extreme bending points; Alatalo and Lundberg 1986) to the nearest 0.1 mm with a slide caliper, the length of the wing (flattened and straightened; Svensson 1992) to the nearest 1 mm with a wing ruler and body mass (to the nearest 0.5 g) with a Pesola 50 g spring balance. Body mass depends on both general size and temporary body fat deposits. Therefore, I use an additional variable I called body condition, which I computed from the residuals of a regression model with body mass as the response variable and tarsus length and time of day captured as predictors. For males, the width of the red border was also measured (to the nearest 1 mm) with a slide caliper.

Ejaculates were obtained by gently massaging the cloacal protuberance, as described in Wolfson (1952). The ejaculates were collected in a capillary tube and diluted in a microcentrifuge tube containing phosphate buffered saline (PBS) preheated to 40 ºC. Sperm motility was recorded immediately upon collection and the remaining sperm was fixed in 5 % formalin for later morphometry measures (see below). Some of the samples (n = 27) were used in other experiments, so there was some variation in how the sperm motility recordings were taken. For the experimental recordings, ejaculates were put into 12 µl of PBS, and 2 µl of this diluted sample was put into 5 µl of female fluid two times and 5 µl of PBS one time as a control (i.e. three times per ejaculate, n = 12), or 2 µl of the diluted sample were put into 5 µl of PBS three times (n = 15). For the non-experimental recordings, ejaculates were simply diluted into 20 – 40 µl of PBS, depending on the density of sperm cells obtained. For all samples, 3 µl of diluted sperm was placed in a preheated microscope slide (depth 20 mm; Leja Products BV, Nieuw-Vennep, the Netherlands). For the experimental recordings, the different dilutions of sperm were placed in separate chambers in a multi-chamber slide. Each slide was mounted on a MiniTherm stage warmer (Hamilton Thorne Biosciences, Beverly, MA), or a Tokai Hit TP-S heated microscope stage (Tokai Hit Co, Fujinomiya-shi, Shizuoka-ken, Japan) maintained at a constant temperature of 40 ºC. Sperm movement was recorded through a phase contrast microscope (CX41, Olympus, Japan) with a digital video camera (HDR-HC1C, Sony, Tokyo, Japan). Each sperm sample was recorded in different locations across the slide chamber to reduce the possibility of tracking the same cell twice. As the different dilution methods had no effect on sperm motility in the experimental recordings, I averaged sperm motility across the four chambers per ejaculate.

I recorded the location of male territories and searched for nests by tracking female nest-building behavior or feeding activity. Nests found (Figure 1) were visited every day until incubation began, and from two days before expected hatching, to keep track of the number of eggs and hatching time.

Chicks were weighed at least two days after hatching and bled by puncturing the femoral vein. Unhatched eggs were collected. For the nests found after the chicks had hatched, I estimated the hatching date by the weight of the heaviest chick (Rangbru 1994). I further calculated the egg-laying date as described in Rangbru (1994).

In 2013, I found 31 nests and sampled 250 birds (43 males, 31 females and 176 chicks). The chicks in one nest were not sampled due to late hatching. A total of 15 of the chick samples collected were from unhatched eggs from nests that either had been abandoned or where all the other eggs had hatched (four different nests). One sample was excluded due to contamination during extraction, and another two due to low DNA quantity. Accordingly, in my analysis I used samples from 173 chicks from 30 different nests.

In 2014, I found 29 nests and sampled 150 birds (33 males, 18 females and 99 chicks). Seven nests were depredated before I could sample the chicks, and another two were not sampled due to late hatching. From the remaining 20 nests, I collected 26 unhatched eggs from 11 different nests, and got blood samples from the other 73 chicks. Twelve of the eggs were too under-developed to yield any product in the DNA extractions, so two nests were excluded from the analysis. Thus, from 2014, I used 87 chicks from 18 nests in my analysis.

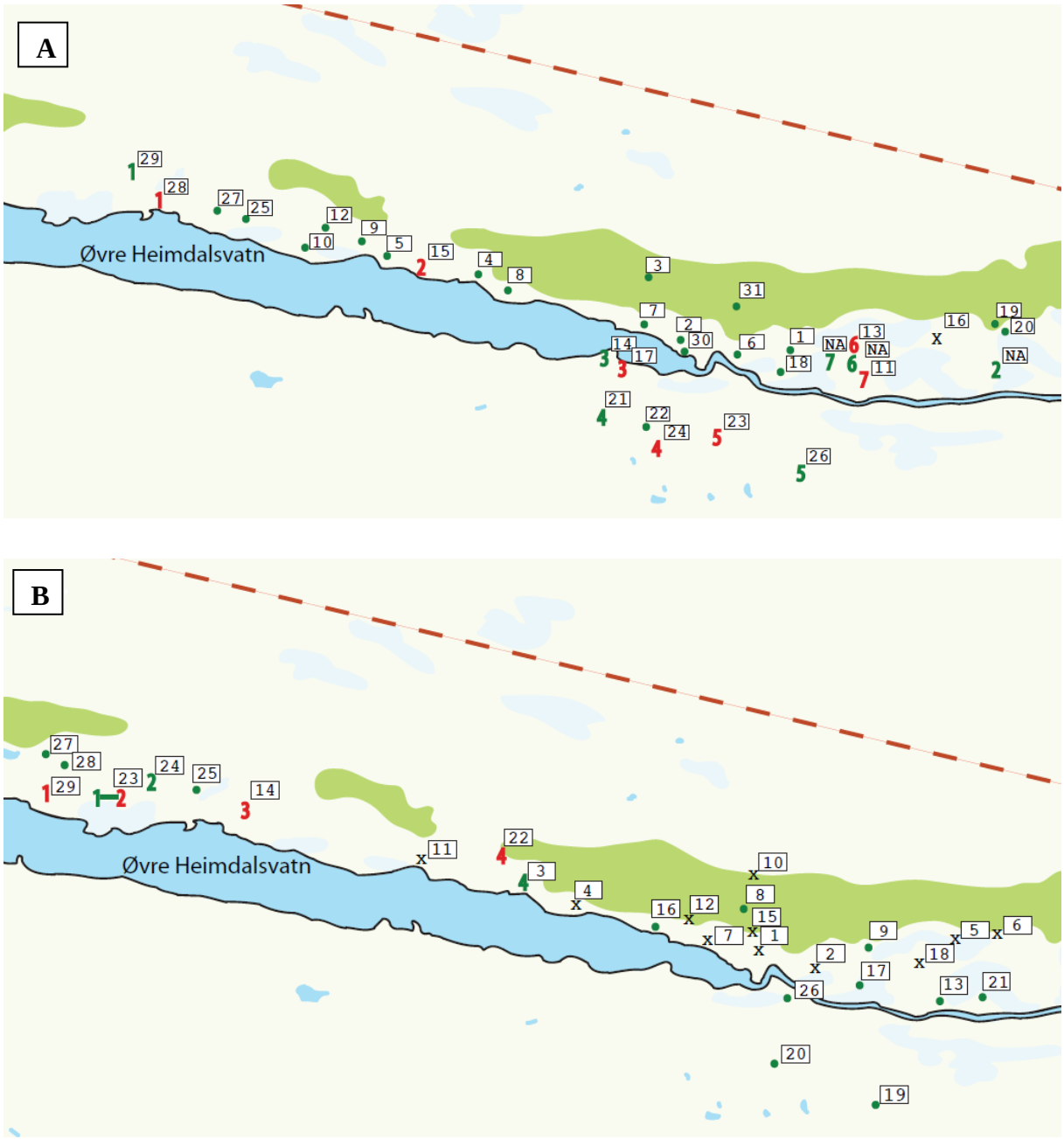

Figure 1. Maps of the study area with nests found during the field season of 2013 (A) and 2014 (B). Nest numbers are in the white text boxes in the order they were found. Boxes with NA indicate the capture location of extra-pair males without a nest. Nests with extra-pair offspring are marked with red numbers in a sequential order according to the location on the map. In cases where the extra-pair male was known he was given the same number as the male he cuckolded, but in green. Nest that had an extra-pair sire with an unknown identity have been marked with a star. One male was both cuckolded and sired an extra-pair offspring, so he has been marked with both symbols, connected by a green line. Nests without extra-pair offspring are marked with a green dot, and excluded nests (see main text) are marked with an X. The dashed line represents the border between the counties Øystre Slidre and Vågå. Open water is in blue, marshland is in light blue, green indicates dense shrubs and white represent open shrub land.

3.4 Genetic analyses

All lab work was performed at the DNA Lab of the Natural History Museum, University of Oslo.

3.4.1 DNA extraction and amplification

I extracted DNA from blood samples using an E-Z 96 Blood DNA Kit (Omega Bio-Tek (D1199-01)), following the manufacturers' protocol. From the tissue samples of unhatched eggs, DNA was extracted with an E.Z.N.A. ® Tissue DNA Kit (Omega Bio-Tek). After extraction, I diluted the DNA extracts 1:3 with Milli-Q water (30 µl DNA and 90 µl water).

Using polymerase chain reaction (PCR), 37 microsatellite markers were amplified (GeneAmp® PCR System 9700 (Applied Biosystems)). These markers were originally designed for use in pied flycatchers (*Ficedula hypoleuca*), but have also been found to amplify well for bluethroats (Leder *et al*. 2008). The markers were sorted into five panels (1-5), depending on which primers could be run together in multiplex analysis.

The PCRs were run in 10 µl volume (per sample) containing 5 µl Qiagen Buffer, 1 µl primer-mix, 3 µl Milli-Q water and 1 µl diluted DNA extract. The primer-mixes contained forward and reverse primers for each marker. The PCR programs used are shown in Appendix Table 1.

To confirm amplification success, I performed gel electrophoresis with 3 µl of PCR product on a 1 % agarose gel. I used 2 µl of GelRed™ per gel as a fluorescent nucleic acid gel stain. The PCR product was mixed with 4 µl 6X MassRuler™ DNA Loading Dye in each well. The gels were run for about 40 minutes on 90 V with FastRuler™ LR DNA Ladder as a ladder, and then visualized by taking a picture in Kodak GelLogic with UV transillumination.

In 2013, I extracted five females from 2012, and in 2014, I re-extracted three females, five males and six chicks (14 in total) from 2013, due to various ambiguities (see 3.4.3).

3.4.2 Genotyping

PCR products were diluted 1:99 with Milli-Q water and length separated on an ABI Prism® 3130 XL Genetic analyzer (Applied Biosystems) using fluorescently labeled primers. In each

well of the ABI plates, 9.5 µl Hi-Di™ and 0.5 µl GeneScan™ 600 Liz® size standard were added, along with diluted PCR product (2 µl for panel 1-4 and 1 µl for panel 5).

Allele sizes were determined using ABI Prism® GeneMapper™ Software version 4.0 (Applied Biosystems).

3.4.3 Paternity analyses

I ran paternity analyses with Cervus version 3.0.7, using 22 of the 37 microsatellite markers (Appendix Table 2). Two markers did not show any product. For the remaining 35 markers, I ran an allele frequency analysis in Cervus, using genotypes from the adult birds, which gave an estimate of null allele frequencies for each marker. Null alleles are alleles that fail to amplify in PCR, which may lead to misinterpretations of paternity (Callen *et al*. 1993), so I excluded all markers with null allele frequencies above a limit set to 0.10 (N = 9). I also computed the allelic richness of each locus and ran a genotypic disequilibrium test between all pairs of loci (595 combinations, 11900 permutations) in FSTAT version 2.9.3.2 (Goudet 1995). The test indicated strong linkage between loci ZF-C59 and EST16 (p value under the adjusted alpha level of 0.000084). Accordingly, I excluded one locus to avoid pseudoreplication. ZF-C59 and EST16 had identical and low null allele frequencies, but since ZF-C59 had a marginally higher allelic richness than EST16, I chose to exclude the latter. Finally, I excluded three more markers due to stutter bands that made it difficult to distinguish true peaks from noise. The combined exclusion probability of the 22 markers I chose was greater than 0.9999 for the first parent, meaning that the probability of excluding individuals that are unrelated to the offspring based on these markers is very high, even without knowing the identity of one of the parents.

In 2013, I knew the identity of the social parents in 29 out of 30 nests. In the remaining nest, I knew the identity of the social male. I failed to capture the female, but since she had a metal ring from the year before, I searched for her identity among five females from 2012 that I had extracted. In 2014, I knew the identity of the social parents in 15 of 18 nests. In two nests, I failed to sample the social male, while in one nest I failed to sample the female. One of the males was banded, but the exact combination of the bands was not seen, so I searched for his identity among the males from 2013.

For each year, I ran maternity tests with all candidate mothers (2013: $N = 36$, 2014: $N = 20$), one paternity test with the known social parent pair, and one paternity test with the social mother and all candidate fathers (2013: $N = 43$, 2014: $N = 33$). In 2014, I also re-ran a family from 2013 where an extra-pair male had obtained full paternity, to verify my results (one female, two males and six chicks). For the offspring of 2014 I ran an additional test with all the males from 2013, in search of missing extra-pair males.

As brood parasitism is extremely rare in bluethroats (no cases found in Krokene *et al*. 1996 or Questiau *et al*. 1999; one case found in Johnsen *et al*. 2000, total number of nests in the three studies = 153), the likelihood that a social mother is the true genetic mother of her offspring is high. Still, there may be some mismatches between mother and offspring due to genotyping errors or mutations. In my dataset, all offspring either had zero, one or two mismatches with their mothers, except for one offspring that had three mismatches. Since the proportion of alleles shared was within the same range as the other offspring (0.875 - 1, see Figure 2), I concluded that it was most likely a true genetic match. I set the limit to two mismatches for a male to be considered the true sire of his offspring (there was no father-offspring pair with three mismatches), and a proportion of alleles shared of at least 0.875 (Figure 3).

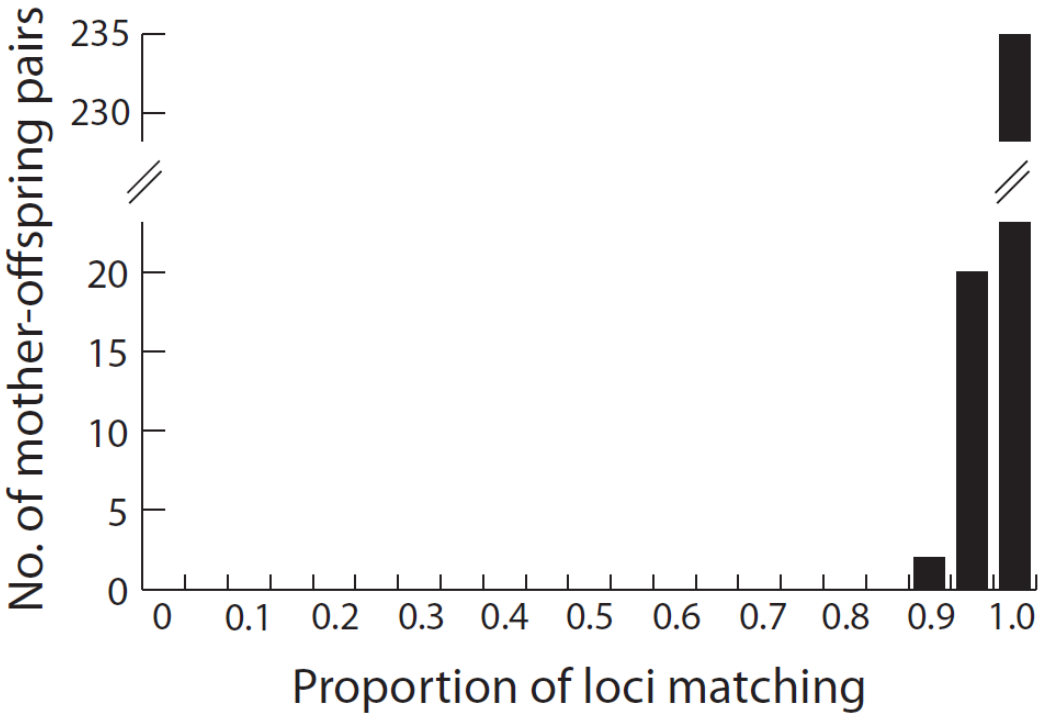

Figure 2. Frequency histogram of female-offspring pairs with different degrees of allele-sharing, in bins of 0.05, with putative mother.

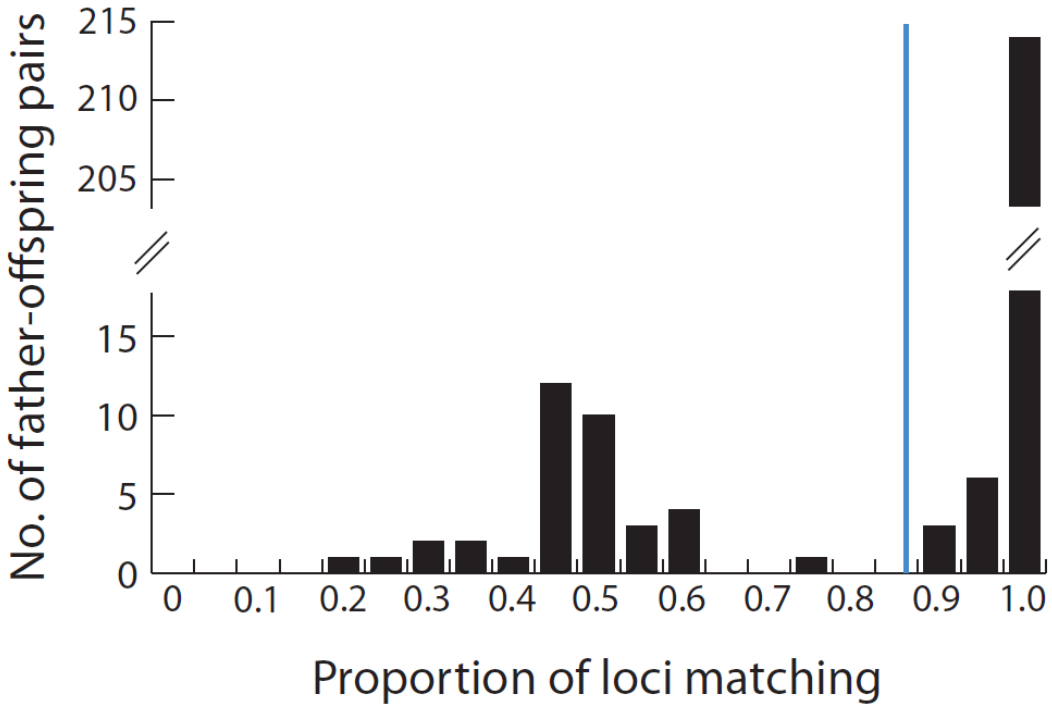

Figure 3. Frequency histogram of male-offspring pairs with different degrees of allele-sharing, in bins of 0.05, with putative (i.e. social) father. Frequencies to the right of the blue line are considered to be true genetic matches, while the ones to the left are considered offspring sired by an extra-pair male.

3.5 Sperm analyses

3.5.1 Morphometry

All sperm morphometric analyses were performed by Even Stensrud, without knowledge about extra-pair and within-pair success status of the males. A total of 10-15 µl of diluted sperm was spread out on a microscope slide with a pipette and left overnight to air-dry. The following day, the slides were washed with distilled water to remove salt crystals, and left to dry for at least one hour. Digital pictures were taken with a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope at 160 x magnification, and the images were processed in Leica Application suite version 4.1. Sperm cells consist of three components: head, midpiece and tail (Figure 4). The lengths of these components were measured separately, and a number of variables were calculated based on these measurements, including total sperm length (head + midpiece + tail), flagellum length (midpiece + tail), F:H ratio (flagellum/head), and M:TSL (midpiece/total sperm length). The within-male coefficient of variation in total sperm length (CV_{wm}, where CV = (standard deviation/mean)*100) was also calculated.

A total of 58 males were measured for sperm morphology (36 in 2013, 16 in 2014 and an additional six males that were measured both in 2013 and 2014). For each male, 30 sperm cells were measured. The lengths of the sperm components were averaged, and the F:H ratio and M:TSL were calculated independently for the 30 cells and then averaged.

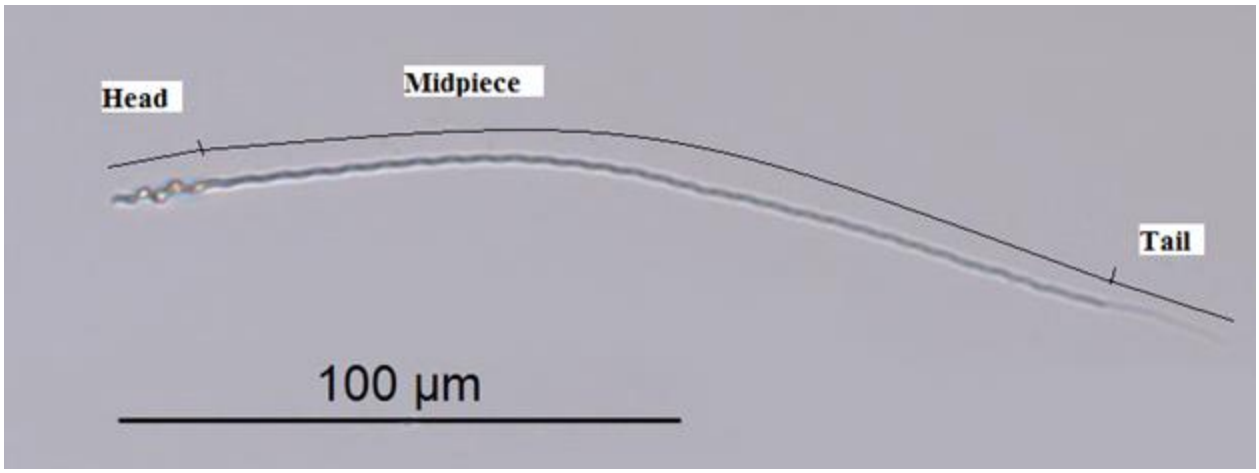

Figure 4. Microscope image of a bluethroat sperm cell. The three components of the sperm are indicated: head, midpiece and tail. Photo: Even Stensrud.

3.5.2 Motility

Motility measurements were performed by Becky Cramer. Sperm motility was measured with computer-assisted sperm analysis (HTM-CEROS II Sperm Analyzer; Hamilton Thorne Research, Beverly, MA), as described in Kleven *et al*. (2009a). The sperm analyzer was set at a frame rate of 50 Hz for 25 frames (i.e. sperm cells were tracked for 0.5 seconds). Three estimates of sperm velocity were calculated:

1. Average path velocity (VAP), which is the average velocity over a smoothed sperm track.
2. Straight line velocity (VSL), where the velocity is measured on a straight line between the start and end points of the sperm tracks.
3. Curvilinear velocity (VCL), which is the velocity of the point-to-point sperm track.

The latter measure is thought to be the most accurate (Kleven *et al*. 2009a; Rowe *et al*. 2013; Laskemoen *et al*. 2013), since the sperm track is not expected to be linear, as there is no egg to attract or guide the sperm cells in any specific direction (Eisenbach and Giojalas 2006). Since the three tracking methods have been shown to intercorrelate strongly (Kleven *et al*. 2009a; Rowe *et al*. 2013; Laskemoen *et al*. 2013), I chose to only use VCL in the further analyses, and I refer to this measure as sperm velocity.

The number of static and motile cells, and the proportion motile cells were also calculated. Filters were applied to exclude inaccurate tracks and incorrect detections for all the measurements in both years, except for proportion motile in 2013 (see below). In order to qualify as good motile tracks, and contribute to the mean sperm velocity, sperm tracks had to have at least 10 detection points, zero gaps in the detection series, linearity (=(VSL/VCL)*100) of 60 or greater, straightness (=(VSL/VAP)*100) of 80 or greater, and elongation (ratio of sperm head width to head length) of 50 or less. Also, no single movement could be more than five interquartile ranges greater than the median length of movements for that sperm track. Moving cells with VAP under 50 or VSL under 25 were considered static (they were likely moving because of drift or software analysis issues). I set a cutoff of 10 good motile tracks per male, and excluded all males with sperm velocity measurements under this value (N = 10). One exception was made when testing the repeatability of sperm velocity between the years. To avoid losing multiple data points, I lowered the cutoff to 5 good motile tracks in this analysis.

In the estimates of proportion motile cells, different settings were used in 2013 and 2014, because of different video quality. For 2013 the number of motile tracks (including motile tracks that fail the above filters) was divided by total number of sperm cells. For 2014, an elongation filter was applied, so anything with elongation over 50 and VAP under 50 was eliminated from the dataset. I set a limit of 30 cells in total for calculating proportion motile cells, and excluded males with measurements under this value (N = 2).

I was able to use sperm velocity measurements from 60 males (28 in 2013, 29 in 2014, and three males that were measured in both years. For proportion motile cells, I used a total of 66 males (34 in 2013, 26 in 2014, and six males were measured in both years).

3.6 Statistical analyses

I used R version 3.1.1 for all statistical analyses (R core team, 2014). To control for multiple testing, I used false discovery rate correction (Benjamini and Hochberg 1995). This minimizes the chance of making a type 1 error, and is more powerful than for example the Bonferroni procedure (Verhoeven *et al*. 2005).

3.6.1 Data handling

In total, I had sperm and body morphology measurements from 70 males (76 recordings, as six males were measured in both years), and paternity data on a subset of these (N = 46 males, 49 recordings). Some of these males were measured multiple times within the same season (details in 3.6.4), but I only used one measurement per male per year, except in repeatability analyses. I chose the measurement that had been taken closest to the egg-laying date, since this is the most crucial period for male fertilization success. For males without a known egg-laying date, I chose the measurement with the highest number of good motile tracks, as more tracks give a better representation of actual sperm velocity. I account for the presence of the same males in both years in the following ways: 1) In correlation analyses I only included the first recording of each male (i.e. from 2013), and 2) In analyses of associations between different variables and paternity success, I kept both recordings and included male identity (ring number) as a random variable in my models. I checked my results by running each test without the second recordings, but this did not change any of my conclusions qualitatively.

To compare mean differences in the variables between the years, I ran t-tests for normally distributed variables, and Mann-Whitney U tests for non-normal variables. I ran them separately both for the paternity dataset and the sperm dataset, because different variables might have caused different biases in the two datasets. Within each dataset, I chose to center all of the variables to the mean of each year separately, as many of them were significantly different between years (Appendix Tables 3 and 4).

3.6.2 Paternity success

I used three different measures of paternity success in my analyses: 1) within-pair fertilization success, 2) extra-pair fertilization success, and 3) total number of offspring sired (the number of sired offspring in the social nest plus the number of offspring sired in other nests). For the first two measures I used generalized linear mixed models with binomial distributions, and for the third I used linear mixed models with normal distributions, to test for possible associations between different variables and paternity success.

I also directly compared pairs consisting of within-pair males and the extra-pair males who had cuckolded them in paired t-tests (N = 9 pairs). One male was a cuckolder in both years, but since he cuckolded different males in the two years, I kept both as independent data points.

To rule out the possibility that males had been cuckolded because they had been captured in the most fertile period of their partners, I calculated the difference between capture date and egg-laying date for each male and used this variable in a generalized linear mixed model predicting either a linear or a quadratic relationship between within-pair paternity and distance to the most fertile period.

3.6.3 Correlations among variables

I tested for shape variation between sperm cells by correlating the various size measures with each other in linear regression models. I also investigated correlations between sperm measures and body morphology, and correlations between different measures of body morphology.

3.6.4 Repeatability

I tested the repeatability of the variables by comparing both the males who had been sampled in both years (N = 6), and the males who had been measured multiple times in the same year (N = 20 for sperm motility, N = 14 for sperm morphometry, N = 5 for body morphology). One male was sampled in both years and measured twice in each year. Many of the repeated measurements within the same season were taken on the same day (sperm velocity: 7/8, proportion motile: 12/16, sperm morphometry: 9/14, body morphology: 0/5). I ran linear regression models between first and second measurement of all variables to find the correlation values (Nakagawa and Schielzeth 2010).

4 Results

4.1 Patterns of paternity

In 2013, 23 % (7/30) of broods contained extra-pair offspring and 13 % (22/173) of the offspring were sired by extra-pair males. In 2014, 24 % (4/18) of broods contained extra-pair young and 6 % (5/87) of the offspring were extra-pair. The total proportion of offspring that were extra-pair was lower in 2014 than in 2013, which was close to significant (Fisher's exact test: $p = 0.09$). However, the difference between the years was not significant with respect to proportion of broods containing extra-pair young (Mann-Whitney U: $W = 251$, $p = 0.73$) or the number of extra-pair offspring within the nests that contained extra-pair offspring ($W = 255.5$, $p = 0.63$). In 2014, date of egg-laying was later, clutch sizes tended to be smaller and the number of chicks that hatched was lower than in 2013 (Appendix Table 3). There was no bias in within-pair paternity with respect to when males were sampled relative to their mates fertile period (linear relationship: $Z = 0.6$, $p = 0.55$; quadratic relationship: $Z = -0.14$, $p = 0.89$).

In total, eight of the 11 broods where cuckoldry occurred had more than one extra-pair offspring, and two males experienced total loss of within-pair paternity. Both of these two putative social fathers were captured near the nest location, but only one of them was later observed feeding the chicks, thus confirming that he was in fact the social male. Out of the 11 broods with extra-pair paternity, nine (82 %) broods had a single extra-pair sire, while the remaining two most likely had two extra-pair sires. I identified 10 males who had sired a total of 70 % (19/27) of the extra-pair young. Seven of these had nests in my study area, and one of them had been cuckolded in his own nest. I tested whether cuckolders were less likely to be cuckolded than non-cuckolders for both years combined, but this was not significant (Fisher's exact test: $p = 0.67$). Among the males siring extra-pair young, the number sired ranged from one to six. One of the extra-pair males obtained full paternity of the brood, which I confirmed by re-extracting and re-genotyping all chicks and putative parents (see section 3.4.1). The total number of offspring sired per male ranged from 0 to 8 (mean ± SE: 5.06 ± 0.27).

There was no significant difference in proportion of males who had sired extra-pair young between the years ($W = 270.5$, $p = 0.85$), or number of extra-pair offspring sired ($W = 272.5$,

p = 0.81). The total number of offspring sired per male in 2014 was not significantly different from 2013 (W = 300, p = 0.16).

In all, I assigned the sires of 95 % (247/260) of the offspring (2013: 96% (166/173), 2014: 93 % (81/87)). Hence, I lack information on 5 % (13/260) of the offspring, and males may also have sired additional offspring outside of my study area. In two of the nests from 2014, I failed to sample the within-pair male, but one of them was color banded, and turned out to be a male caught in 2013. To avoid pseudoreplication, and because I had not obtained a sperm sample from him in 2014, he was left out of further analyses.

Figure 1 A and B show overviews over the nests I found and the occurrence of extra-pair paternity in 2013 and 2014, respectively. In all cases where both the within-pair male and the extra-pair male had known nest locations, they were neighbors. The extra-pair males without a known nest location are indicated on the map where they were captured (Figure 1), which may not necessarily be close to their own territory.

Four males had nests in both years. Three of them were not cuckolded in either year, whereas the last was cuckolded in 2013, but not in 2014. The cuckolded male did not sire extra-pair offspring in either year. One of the males sired extra-pair offspring in both years and another sired extra-pair offspring in 2013, but not in 2014. I could not see any specific trend regarding paternity success between the years.

4.2 Male characteristics and paternity success

4.2.1 Sperm characteristics

For the 2013 data set, I found that males who had been cuckolded had shorter sperm than males who had not been cuckolded (Figure 5). This was supported by the generalized linear mixed model for both total sperm length (Z = -2.09, p = 0.04), and length of flagellum (Z = -0.21, p = 0.04). However, in 2014, no such effect was found (total sperm length: Z = 0.21, p = 0.83, flagellum: Z = 0.25, p = 0.80). In 2013, the interquartile ranges were non-overlapping, whereas in 2014, the variation among males was lower and the difference between the two groups was smaller and in a weakly opposite direction compared to 2013 (Figure 5). Thus, in the generalized linear mixed model combining both years, where sperm length had been centered within each year to control for year effects, there was an almost

significant overall effect for total sperm length (mean ± SE: cuckolded: 206.27 ± 1.93 µm, not cuckolded: 209.98 ± 0.79 µm, Z = -1.87, p = 0.06) and flagellum (cuckolded: 190.76 ± 1.85 µm, not cuckolded: 194.3 ± 0.78 µm, Z = -1.83, p = 0.07).

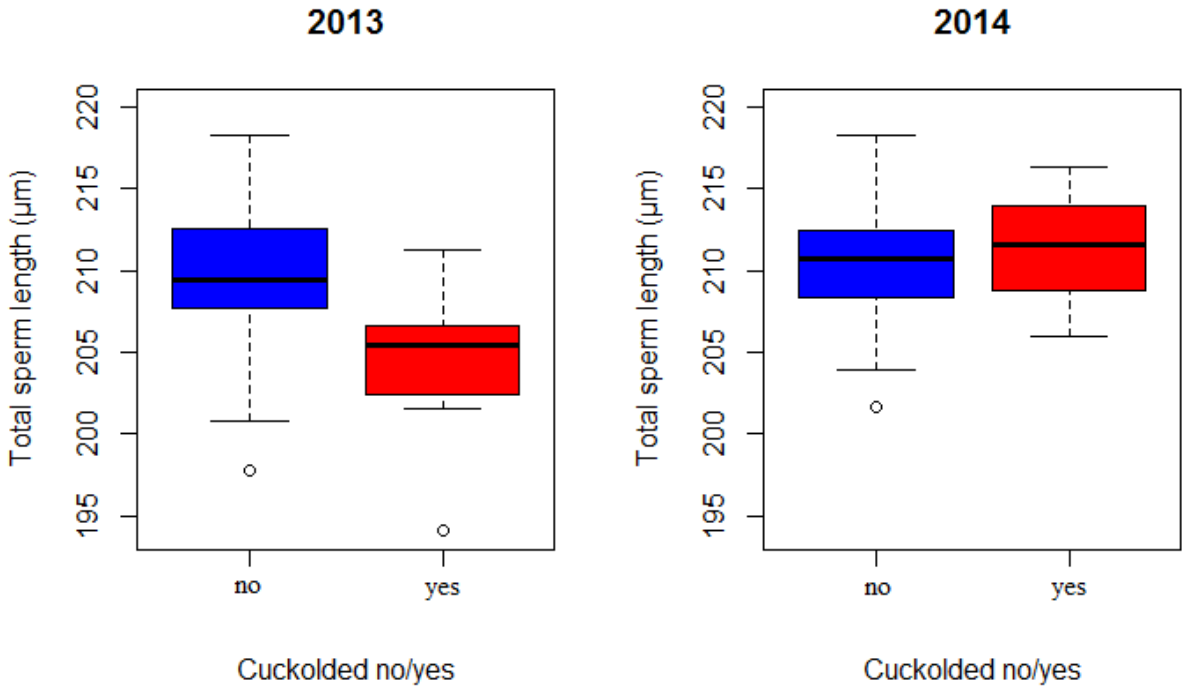

Figure 5. Boxplot showing how total sperm length differed between males that had been cuckolded and males that had not been cuckolded in 2013 (left) and 2014 (right). The bottom and the top of the boxes are the first and third quartiles, and the band inside the box is the second quartile (the median). The whiskers represent the lowest and highest points still within the 1.5 interquartile range, and dots outside of the whiskers are outliers.

I found a similar, almost significant, trend that the extra-pair males had longer sperm than the males they had cuckolded, in the paired t-test (Figure 6; total sperm length: mean ± SE: within-pair male: 205.68 ± 2.34 µm, extra-pair male: 211.73 ± 2.21 µm, t = -1.97, p = 0.09).

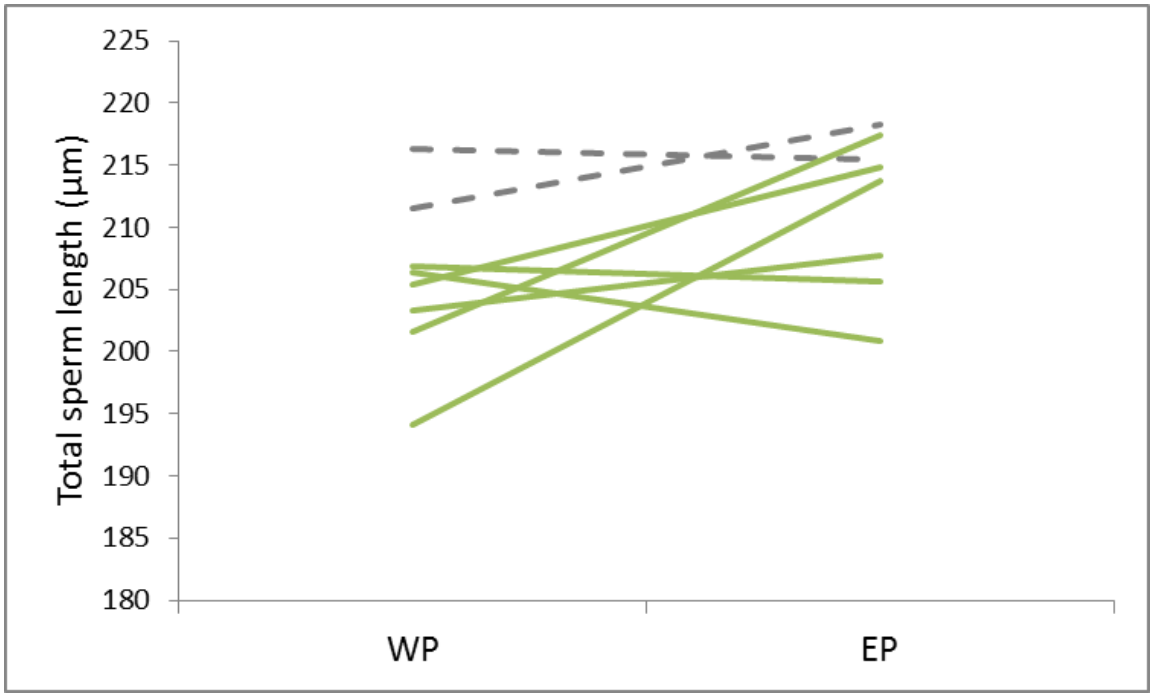

Figure 6. Paired comparisons of total sperm length of within-pair males and the extra-pair males that cuckolded them. Years are indicated as follows: green, solid lines = 2013; grey, dashed lines = 2014.

There was no statistically significant difference in total sperm length between males who had sired extra-pair offspring and males who had not (sired one or more extra-pair young: 211.71 ± 1.95 µm, sired no extra-pair young: 208.61 ± 0.79 µm, Z = 0.27, p = 0.79). Similarly, there was no detectable association between sperm length and total number of chicks sired ($F_{1,42}$ = 0.11, p = 0.74). No other sperm morphometric variable was associated with extra-pair fertilization success or total number of offspring sired (Appendix Table 5).

Neither sperm velocity or proportion motile cells had any effect on paternity success (Appendix Table 5).

4.2.2 Body morphology and age

I investigated whether different male characteristics (wing length, tarsus length, mass, red border width, body condition and age) were associated with paternity success. I found no significant associations in generalized linear mixed models (Appendix Table 5). Older males sired on average more offspring in total than young males, but not significantly so (mean ± SE: 2k: 4.65 ± 0.48, 3k+: 5.34 ± 0.33, $W = 178$, $p = 0.17$). There was no significant difference in number of males that sired extra-pair offspring between the age groups (one-tailed Fisher's exact test: $p = 0.26$). In a paired test, although the cuckolder was an old male in 7 of the 9 pairs, the difference in age distribution was not significant (sign test: $p = 0.18$). Pairwise t-tests did not show any significant difference in other male characteristics between the within-pair males and the extra-pair males that cuckolded them (Appendix Table 6).

4.3 Relationships among male characteristics, and repeatability of measures

4.3.1 Sperm morphology

Across all males (N = 58 samples), mean (± SE) total sperm length was 209.90 ± 0.71 µm, with most of the length consisting of the flagellum (midpiece: 178.84 ± 0.85 µm and tail: 15.41 ± 0.55 µm). Mean head length was 15.66 ± 0.07 µm. Thus, almost all of the variation in total sperm length can be explained by the flagellum ($R^2 = 0.99$, estimate ± SE = 1.02 ± 0.01, $F_{1,58} = 10090$, $p < 2 \times 10^{-16}$). Total sperm length was correlated with all sperm morphometric variables, except for sperm tail length and M:TSL (Appendix Table 7). Correlations among other sperm morphometric variables are shown in Appendix Table 7.

Total sperm length and F:H ratio were significantly correlated with body mass in simple models (Table 1). I found that sperm tail length decreased throughout the season, and thus the M:TSL ratio increased (Table 1). However, the significance of these correlations was not robust to correction for multiple testing.

Table 1. Correlations between sperm variables and body mass and capture date, respectively. The direction of the relationships are shown with the estimate (± SE), and the correlation strength is shown by *F* value and *p* value. *N* is the number of males. Uncorrected *p* values are shown. No correlations were significant after correcting for multiple testing with false discovery rate (Benjamini and Hochberg 1995).

		Estimate ± SE	*F*	*N*	*p*
Body mass (g)	Total sperm length (µm)	0.04 ± 0.02	4.79	56	0.03
	Head length (µm)	-0.11 ± 0.23	0.21	56	0.65
	Midpiece length (µm)	0.03 ± 0.01	2.94	56	0.09
	Tail length (µm)	0.004 ± 0.02	0.04	56	0.85
	Flagellum length (µm)	0.04 ± 0.02	5.28	56	0.03
	F:H[a]	0.55 ± 0.23	5.65	56	0.02
	M:TSL[b]	1.13 ± 4.76	0.06	56	0.81
Capture date	Total sperm length (µm)	-0.02 ± 0.28	0.005	58	0.94
	Head length (µm)	-3.92 ± 3.61	1.18	58	0.28
	Midpiece length (µm)	0.32 ± 0.23	1.92	58	0.17
	Tail length (µm)	-0.74 ± 0.34	4.63	58	0.04
	Flagellum length (µm)	0.003 ± 0.29	0.0001	58	0.99
	F:H[a]	2.75 ± 3.69	0.56	58	0.46
	M:TSL[b]	155.74 ± 71.14	4.79	58	0.03

[a] Flagellum to head ratio
[b] Midpiece to total sperm length

4.3.2 Sperm motility

Sperm velocity and proportion motile cells were significantly correlated (N = 60, estimate ± SE = 67.21 ± 15.45, $F_{1,58}$ = 18.92, p = 0.00006). I tested correlations between sperm motility and sperm morphology. In a simple model, there was a weak tendency for sperm velocity to decrease with sperm midpiece length (N = 50, estimate ± SE = -1.12 ± 0.58, $F_{1,48}$ = 3.73, p = 0.06). There were no significant correlations with other morphology measures (Table 2, all $p > 0.05$). The proportion of motile cells was not correlated with any sperm morphology measure (Table 2).

Sperm velocity increased significantly with male age (N = 59, W = 260, p = 0.03) and decreased significantly throughout the season (N = 60, estimate ± SE = -0.80 ± 0.31, $F_{1,58}$ = 6.77, p = 0.01). In a multivariate model, 44 % of the variation in sperm velocity could be explained by proportion motile cells, male age and capture date. Proportion motile cells

was not correlated with male age (N = 65, W = 497, p = 0.97) or capture date (N = 66, estimate ± SE = 0.001 ± 0.002, $F_{1,64}$ = 0.29, p = 0.6).

Table 2. Correlations between sperm motility (velocity and proportion motile) and different sperm morphometric variables. The direction of the relationships are shown with the estimate (± SE), and the correlation strength is shown by F value and p value. N is the number of males.

		Estimate ± SE	***F***	***N***	***p***
Velocity (µm/s)	Head length (µm)	-4.08 ± 10.10	0.16	50	0.69
	Midpiece length (µm)	-1.12 ± 0.58	3.73	50	0.06
	Tail length (µm)	1.25 ± 0.87	2.06	50	0.16
	Flagellum length (µm)	-0.81 ± 0.73	1.25	50	0.27
	Total sperm length (µm)	-0.78 ± 0.70	1.24	50	0.27
	F:H[a]	-7.95 ± 10.99	0.52	50	0.47
	M:TSL[b]	-288.15 ± 180.87	2.54	50	0.12
Proportion motile	Head length (µm)	0.004 ± 0.07	0.003	56	0.96
	Midpiece length (µm)	-0.0004 ± 0.004	0.01	56	0.92
	Tail length (µm)	0.001 ± 0.01	0.04	56	0.84
	Flagellum length (µm)	0.0002 ± 0.01	0.002	56	0.97
	Total sperm length (µm)	0.0002 ± 0.005	0.002	56	0.96
	F:H[a]	0.002 ± 0.07	0.0005	56	0.98
	M:TSL[b]	-0.24 ± 1.26	0.04	56	0.85

[a] F:H = Flagellum to head ratio
[b] M:TSL = Midpiece to total sperm length

4.3.3 Body morphology

Body mass was positively correlated with tarsus length (N = 68, estimate ± SE = 0.32 ± 0.12, $F_{1,66}$ = 6.93, p = 0.01) and time of day captured (N = 61, estimate ± SE = 0.002 ± 0.0005, $F_{1,59}$ = 9.79, p = 0.003). The birds in 2014 were significantly heavier than the birds in 2013 in the paternity data set (2013: N = 31, 16.83 ± 0.12 g; 2014: N = 16, 17.4 ± 0.18 g, p = 0.01). Wing length increased significantly with age, i.e., from the first breeding season to the second (N = 69, mean ± SE: 2k: 75.22 ± 0.35 mm, 3k+: 76.33 ± 0.25 mm, W = 354.5, p = 0.009), which is a general pattern among passerines, and related to a lack of spring molt of the wing feathers in their first breeding season in many species (Alatalo *et al*. 1984).

4.3.4 Repeatability

Within-season, body morphology measures were highly repeatable, except for body mass, which is more variable (Table 3). All sperm component lengths and variables were highly repeatable, whereas sperm motility measures had low repeatability (Table 3).

Table 3: Within-season repeatability, comparing measurements of males that have been sampled twice in the same year. R^2 is the repeatability, mean ± SE is shown for first and second measure, along with *F* value, number of males (*N*), and *p* value. All significant correlations ($p < 0.05$) were robust to correction for multiple testing, and are marked in bold.

Variables	R^2	**Mean ± SE**		*F*	*N*	*p*
		1st measure	**2nd measure**			
Body morphology						
Wing length (mm)	0.92	76.35 ± 0.31	76.60 ± 1.17	36.87	5	**0.009**
Tarsus length (mm)	0.89	30.57 ± 0.15	30.30 ± 0.34	24.13	5	**0.02**
Body mass (g)	0.70	17.05 ± 0.13	17.08 ± 0.30	2.37	3	0.37
Red border width (mm)	0.86	8.10 ± 0.56	9.08 ± 1.80	18.63	5	**0.02**
Sperm morphology						
Head length (µm)	0.65	15.51 ± 0.14	15.55 ± 0.13	22.23	14	**0.0005**
Midpiece length (µm)	0.93	175.81 ± 2.02	175.67 ± 1.95	148.48	14	**4.08E-08**
Tail length (µm)	0.93	16.87 ± 1.47	16.8 ± 1.35	160.94	14	**2.60E-08**
Flagellum length (µm)	0.93	192.68 ± 1.45	192.46 ± 1.47	162.19	14	**2.49E-08**
Total sperm length (µm)	0.93	208.18 ± 1.55	208.01 ± 1.54	170.76	14	**1.86E-08**
F:H[a]	0.51	12.46 ± 0.08	12.41 ± 0.10	12.32	14	**0.004**
M:TSL[b]	0.93	0.84 ± 0.01	0.84 ± 0.01	149.16	14	**3.97E-08**
CV_{wm}[c]	0.30	1.77 ± 0.19	1.65 ± 0.12	5.11	14	**0.04**
Sperm motility						
Velocity (µm/s)	0.02	145.85 ± 7.58	115.94 ± 5.27	0.12	8	0.74
Proportion motile	0.06	0.32 ± 0.07	0.16 ± 0.03	0.89	16	0.36

[a] Flagellum to head ratio

[b] Midpiece to total sperm length

[c] Within-male variation in total sperm length, where CV = (standard deviation/mean)*100

Between years, body morphology measures had moderate repeatability, except for wing length which decreased from one year to the next (Table 4, four out of the six males were 3k+ in both years). Sperm component lengths were quite highly repeatable, whereas sperm motility measures again had very low repeatability (Table 4).

Table 4: Between-year repeatability, comparing measurements of males sampled in both years. R^2 is the repeatability, mean ± SE is shown for 2013 and 2014, along with *F* value, number of males (*N*) and *p* value. Correlations which are still significant after correcting for multiple testing ($p < 0.05$) are marked in bold.

Variables	R^2	**Mean ± SE**		*F*	*N*	*p*
		2013	**2014**			
Body morphology						
Wing length (mm)	0	76 ± 0.37	75.17 ± 0.31	0	6	1
Tarsus length (mm)	0.69	30.58 ± 0.29	30.52 ± 0.13	8.90	6	0.04
Body mass (g)	0.57	17.33 ± 0.21	17.33 ± 0.28	5.33	6	0.08
Red border width (mm)	0.62	7 ± 0.68	7.33 ± 0.80	6.65	6	0.06
Sperm morphology						
Head length (µm)	0.87	15.63 ± 0.24	15.98 ± 0.22	26.93	6	**0.007**
Midpiece length (µm)	0.89	179.53 ± 2.94	181.01 ± 2.91	33.37	6	**0.004**
Tail length (µm)	0.45	13.64 ± 0.84	12.29 ± 1.01	3.32	6	0.14
Flagellum length (µm)	0.97	193.16 ± 2.90	193.3 ± 2.94	140.90	6	**0.0003**
Total sperm length (µm)	0.98	208.8 ± 3.03	209.27 ± 3.04	160.96	6	**0.0002**
F:H[a]	0.80	12.39 ± 0.18	12.13 ± 0.19	16.06	6	**0.02**
M:TSL[b]	0.52	0.86 ± 0.00	0.86 ± 0.01	4.25	6	0.11
CV_{wm}[c]	0.17	1.59 ± 0.12	1.6 ± 0.15	0.80	6	0.42
Sperm motility						
Velocity (µm/s)	0.09	132.95 ± 20.52	166.23 ± 2.97	0.30	5	0.62
Proportion motile	0.10	0.17 ± 0.09	0.49 ± 0.12	0.43	6	0.55

[a] Flagellum to head ratio

[b] Midpiece to total sperm length

[c] Within-male variation in total sperm length, where CV = (standard deviation/mean)*100

5 Discussion

In this thesis, I have investigated postcopulatory sexual selection in bluethroats by looking at correlations between characteristics of the males, their sperm, and paternity success. I found a significant relationship between sperm length and within-pair paternity in one year, and a similar tendency in the combined dataset. There was no relationship between sperm motility and paternity success. Below I discuss this and other results in relation to different hypotheses for postcopulatory sexual selection.

5.1.1 Sperm characteristics and fertilization success

Longer sperm may have a competitive advantage in sperm competition, for example through higher swimming speed (e.g. Gomendio and Roldan 1991). In this study, I did not find that total sperm length was related to sperm velocity (see section 5.1.4). Moreover, sperm velocity was not correlated with paternity success, corroborating the findings of Laskemoen *et al*. (2010) in tree swallows. This may suggest that if longer sperm are better competitors, it is not because they swim faster. However, sperm velocity had low repeatability (see section 5.1.4) and correlated with the proportion of motile sperm in the sample. It may be premature to rule out any effect of sperm swimming speed, since one cannot know how conditions are at the time of fertilization. In controlled laboratory experiments, swimming speed has indeed been found to affect fertilization success in birds (Birkhead *et al*. 1999; Denk *et al*. 2005). In natural settings, however, other factors, such as the order in which males copulate with the female or the relative number of sperm cells each of them transfer, may be more important. Laskemoen *et al*. (2010) found sperm quantity to be the most important predictor of fertilization success in a study on tree swallows. Other studies have found that copulation order is an important predictor of fertilization success (Birkhead and Møller 1992; Briskie 1996). However, extra-pair males do not appear to time inseminations better than within-pair males in bluethroats (Johnsen *et al*. 2012). Unfortunately, I do not have measures on sperm quantity or copulation order in my study.

Sperm length may also covary with other characteristics that influence sperm competition ability or female choice. For instance, it has been suggested that longer sperm might live longer (Helfenstein *et al*. 2008), but the evidence for this is not strong (Kleven *et al*. 2009b). In fact, shorter sperm have increased longevity in some passerines (Helfenstein *et al*. 2010;

Lifjeld *et al*. 2012). Furthermore, sperm length may in itself increase fertilization success, for example through securing storage or displacing rival sperm in the female sperm storage tubules (Briskie *et al*. 1997; Calhim *et al*. 2011).

It is possible that the lack of strong correlations between sperm characteristics and fertilization success is because cryptic female choice for genetically compatible sperm counteracts possible effects. Females may produce more extra-pair offspring when paired with a genetically similar, thus incompatible mate, as in savannah sparrows (*Passerculus sandwichensis*, Freeman-Gallant *et al*. 2003). In bluethroats, extra-pair offspring have been found to be more heterozygous than their within-pair half siblings, likely because extra-pair mates are less genetically similar to the female than within-pair mates (Fossøy *et al*. 2008). This has also been found in other passerines, such as in blue tits (*Cyanistes caeruleus*, Foerster *et al*. 2003). Genetic similarity often reflects closeness in kin, and in the more extreme cases, inbreeding depression can result from the expression of deleterious recessive alleles in homozygotes. Thus, overall heterozygosity may be positively related to survival, for example through positive correlations with variability at major histocompatibility complex (MHC) genes, which play a critical role in fighting and eradicating pathogens. The degree of MHC polymorphism is associated with the range of pathogens an individual can respond to (Alcaide *et al*. 2012). This means that the more genetically similar a pair is, the fewer pathogens their offspring can fight off. In bluethroats, extra-pair offspring have been shown to have higher immunocompetence than their half siblings both on the maternal and paternal side (Johnsen *et al*. 2000, Fossøy *et al*. 2008). This suggests that the combination of parental genotypes may be important for offspring fitness. There might be a relationship between effective fertility and genetic compatibility. In an *in vitro* experiment on house mouse (*Mus musculus*) gametes it was demonstrated that MHC compatibility affects fertilization success and/or the likelihood that the fertilized egg develops beyond the second meiotic division (Wedekind *et al*. 1996). Postcopulatory associations between fertilization success and genetic compatibility have also been demonstrated in birds (Pryke *et al*. 2010). I was not able to investigate genetic compatibility in this thesis, but the relationship between effective fertility and genetic compatibility should be investigated further in bluethroats.

It is difficult to imagine how cryptic female choice for compatible sperm could select for certain sperm traits, since it should lead to variable mate choice. However, the characteristics of a male, and his sperm, may still be important in male fertilization success, both in gaining

access to females, achieving copulations and possibly in outcompeting sperm from rival males. Indeed, the occurrence of mixed broods (i.e. broods sired by two or more males) indicates that genetic compatibility is not the only postcopulatory process determining the outcome of paternity. A high proportion of offspring are sired by the social father, even when the offspring sired by an extra-pair male are more heterozygous and immunocompetent (Johnsen *et al*. 2000; Fossøy *et al*. 2008). This suggests that even though the extra-pair male is more compatible with the female, the pair male may still gain fertilizations. This may for example be due to higher sperm numbers for the pair male compared to extra-pair males as a result of higher frequency of copulations, or different qualities of the sperm.

5.1.2 Other factors that may determine fertilization success

Male coloration has been suggested to be important for within-pair paternity success in bluethroats (Johnsen *et al*. 1998a, b; Johnsen *et al*. 2001). The width of the red border was not related to fertilization success in my data set. In a previous study, red border width was found to correlate positively with within-pair paternity in one year, but not in another (Johnsen *et al*. 2001). Possibly, there may be temporal variation in female choice (Chaine and Lyon 2008) or any positive effect may be too weak to consistently show up in data sets of moderate sizes. The blue components of the throat patch may be more important for fertilization success than the red border. Indeed, the quality of the blue color has been found to correlate with within-pair paternity (Johnsen *et al*. 2001). In this study, I did not measure the blue coloration of the males.

Male age has been found to correlate with extra-pair fertilization success in previous studies (e.g. Wetton *et al*. 1995; Bitton *et al*. 2007; Bouwman *et al*. 2007), including in bluethroats (Johnsen *et al*. 2001). I did not find a significant effect of male age on paternity success in my study, although most of the extra-pair males were old. As my results are in the same direction as previous studies, I find it likely that male age is associated with extra-pair fertilization success, but that my sample size was insufficient to detect a significant relationship. Johnsen *et al*. (2001) had data from three breeding seasons and larger sample sizes.

The association between male age and fertilization success may be male driven. Older males may simply be better at finding and courting neighboring females than younger ones through their increased experience (Johnsen *et al*. 2001). It is also possible that females prefer older males as they are more colorful (Johnsen *et al*. 2001; Bitton *et al*. 2007), or that older males

do better in sperm competition as they may be able to produce more sperm (Laskemoen *et al*. 2008). Interestingly, I found a positive association between male age and sperm swimming speed in this study. Although swimming speed was not found to be related to fertilization success, the positive association between age and swimming speed suggests that it may be well worth investigating these patterns further.

The higher fertilization success of older and more colorful males (Johnsen *et al*. 2001) may be condition dependent and positively associated with sperm characteristics. For instance, males in better condition may be able to invest more resources in the production of secondary sexual traits and courtship, as well as in sperm quantity and quality, which would increase the likelihood of successful fertilization.

5.1.3 Do females seek extra-pair copulations to secure fertilization of their eggs?

Two males lost full paternity in their own nest. Possibly the two males were functionally infertile. Insuring fertilization of their eggs has been proposed as an important reason for why females may engage in extra-pair copulations (Wetton and Parkin 1991; Sheldon 1994; Krokene *et al*. 1998; Lifjeld *et al*. 2007). An experimental study suggested that almost all female bluethroats engage in extra-pair copulations, although a high proportion of the copulations do not lead to extra-pair offspring (Fossøy *et al*. 2006). Only a small proportion of males are sterile (2 % (1/48) in Lifjeld *et al*. 2007). However, should a female pair up with a sterile male one year and not produce any offspring, this would lead to a serious drop in her lifetime reproductive success (Lifjeld *et al*. 2007). Thus, engaging in one or a few extra-pair copulations effectively secures the probability of producing fertile eggs. However, the rates of extra-pair paternity in bluethroats appear to be higher than expected from the proportion of infertile males only (Lifjeld *et al*. 2007). Thus, it is probably not the only factor driving extra-pair copulations in this species. Males may differ in fertilization success without being functionally sterile. This gradient of fertility may depend on the level of genetic compatibility of the copulation mates, as discussed above. It may also be correlated with certain qualities of the male (Sheldon, 1994).

5.1.4 Sperm form-function relationships and repeatability

The lack of correlation between sperm length and velocity found in my data set corroborates previous findings in a study on bluethroats (Dobbe 2014), as well as in other intraspecific studies on passerines (Helfenstein *et al*. 2010; Laskemoen *et al*. 2010; Lifjeld *et al*. 2012, but see Mossman *et al*. 2009). In my dataset, sperm velocity decreased throughout the season. The collection of sperm samples took place in two separate periods: the first was before/during egg-laying, and second was when the chicks started hatching. This suggests that sperm velocity is fastest during the most critical time for male fertilization, i.e., around the time of egg-laying. After the eggs are laid, there should be less pressure on producing sperm of the highest quality, but males still produce sperm as females may re-nest. Thus, the timing in which sperm velocity is recorded may influence results. Furthermore, since sperm is stored in the female sperm storage tubules some time before fertilization (Briskie and Montgomerie 1992), and fluid in the female reproductive tract may influence sperm velocity (Møller *et al*. 2008), one cannot know exactly how sperm velocity functions after insemination.

I found that total sperm length and the lengths of sperm components were highly repeatable within the same season, consistent with previous findings in other passerine species (house wren: Cramer *et al*. 2013a; tree swallow: Laskemoen *et al*. 2013). However, some plasticity was observed, in that sperm tail length tended to decrease throughout the season. Sperm lengths were also highly repeatable between the years, except for sperm tail length, which decreased from the first year to the second. Whether the decrease in sperm tail length has any effect on sperm function is unclear, but does not seem likely in my dataset. It appears that patterns of sperm component co-variation and how repeatable the measurements are within the same individual may differ greatly between species. For instance, Cramer *et al*. (2013a) found that the flagellum: head ratio increased throughout the season in house wrens.

Sperm velocity was not repeatable within or between seasons, in contrast to what was found in Laskemoen *et al*. (2013) in tree swallows. As most of the recordings were taken on the same day, this might suggest that the second ejaculate is of lower quality than the first, but I have not been able to test this.

5.1.5 Methodological concerns

One problem in studies on wild populations such as this is that one may fail to obtain complete data on paternity. Males could have had extra-pair offspring in nests that were not found or not sampled. Investigating differences between males that did not lose paternity in their own nest and males that did lose paternity should be less problematic, however (Cramer *et al*. 2013b). Furthermore, I only have data on extra-pair offspring; I did not observe actual copulations. Males with the least successful sperm, i.e., those that copulated with females but did not fertilize her eggs, would have been an informative group to look at (Cramer *et al*. 2013b). Males that did not lose paternity in their own nest may have had sperm that outcompeted all other extra-pair males, or his mate might not have engaged in extra-pair copulations. Similarly, males that did not sire extra-pair offspring within the study area might have failed to copulate with additional females, or their sperm may have been outcompeted. Since my data are correlative, I cannot tell whether the loss of paternity is due to precopulatory or postcopulatory processes. Certain precopulatory behaviors might affect which sperm are in competition and therefore affect what patterns one might be able to find.

The levels of extra-pair paternity in the two years of my study was lower than the average levels found in 12 years of research on this population. On average, 44 % of broods contained extra-pair offspring, and 23 % of offspring were extra-pair (Johnsen and Lifjeld 2003; Fossøy *et al*. 2008). In contrast, 23 % of broods and 10 % of offspring were extra-pair in my data set. However, the rate of extra-pair paternity has been found to vary among years, and two of the years in the previously mentioned studies had similar or even lower levels than what I found. The rate of extra-pair paternity may vary between the years due to ecological factors such as weather conditions (Johnsen and Lifjeld 2003). However, as weather conditions were favorable in the two years of my study, I do not know what caused the relatively low levels of extra-pair paternity.

6 Conclusion and future prospects

This is the first study investigating possible associations between sperm characteristics and paternity success in bluethroats. My results suggest that sperm length might relate to within-pair fertilization success, but there are most likely other factors which affect male fertilization success more strongly. Previous studies indicate that certain male characteristics, such as throat coloration and age, are of significance, but these characteristics may be most important in precopulatory sexual selection. An important reason that female bluethroats engage in extra-pair copulations may be to secure fertilizations of their eggs, and further to give their offspring a genetic benefit through finding a male with compatible genes. Thus, postcopulatory sexual selection might be mostly female driven. The level of compatibility of pair mates and copulation partners may constrain effects of sperm traits on fertilization success, although the competitive ability of sperm may increase the chance of gaining access to the egg. It is possible that several traits involved in male fertilization success are interrelated. Further research is needed to investigate functional relationships between sperm traits and fertilization success, and to disentangle the relative roles of sperm competition and cryptic female choice on the outcome in terms of paternity. In addition to correlative studies with good sample sizes that include all potentially relevant traits, it would be informative to run controlled experiments on isolated factors. For instance, it would be useful to investigate whether sperm swimming speed predicts fertilization success in a numerically balanced sperm competition and to conduct fertilization experiments where sperm from males with known MHC-genotypes compete.

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8 Appendix

Appendix Table 1. PCR programs used to amplify five multiplex panels of 37 markers. Differences between the programs are marked in bold.

Step	Program panel 1	Program panel 2-5	Process
1	95 ºC for 15 min	95 ºC for 15 min	Denaturing
2	94 ºC for 30 s	94 ºC for 30 s	Denaturing
3	**59** ºC for 90 s	**56** ºC for 90 s	Annealing
4	72 ºC for 60 s	72 ºC for 60 s	Elongation
5	Repeat step 2-4 another 34 times	Repeat step 2-4 another 34 times	
6	60 ºC for 15 min	60 ºC for 15 min	Elongation
end	12 ºC until stopped	12 ºC until stopped	

Appendix Table 2: Characteristics of the 35 markers that successfully amplified in PCR, where *k* is the number of alleles, *N* is the number of adult individuals, HObs is the observed heterozygosity, PIC is the mean polymorphic information content and F(Null) is the estimated frequency of null alleles. Finally I have listed the allelic richness of each marker. Markers I excluded from further analyses are in bold.

Panel	Locus	*k*	*N*	HObs	PIC	F(Null)	Allelic r.
1	**EST10[c]**	**10**	**79**	**0.696**	**0.787**	**0.0795**	**9.84**
1	ZF-C59	7	79	0.506	0.479	0.0130	6.92
1	EST9	35	78	0.949	0.942	-0.0026	29.48
1	GG-C25	7	79	0.709	0.725	0.0353	6.92
1	**EST31[b]**	**9**	**79**	**0.316**	**0.449**	**0.2152**	**8.84**
1	ZF-S8	6	79	0.443	0.441	0.0407	5.92
1	ZF-S9	13	79	0.823	0.831	0.0145	12.77
1	**EST16[a]**	**7**	**79**	**0.506**	**0.479**	**0.0130**	**6.91**
2	EST46	11	80	0.800	0.748	-0.0158	10.82
2	**EST62[b]**	**9**	**78**	**0.564**	**0.628**	**0.1001**	**8.82**
2	FH310	26	80	0.838	0.842	0.0065	24.09
2	**FH326[b]**	**11**	**80**	**0.475**	**0.747**	**0.2365**	**10.73**
2	**FH336[c]**	**15**	**78**	**0.731**	**0.858**	**0.0858**	**14.86**
2	FH350	5	79	0.304	0.287	-0.0102	5
2	FH361	6	80	0.438	0.401	-0.0131	5.91
2	FH407	23	80	0.925	0.899	-0.0109	22.53
3	FH413	3	80	0.425	0.421	0.0777	3
3	**FH304[b]**	**23**	**79**	**0.291**	**0.921**	**0.5220**	**21.96**
3	FH408	33	80	0.913	0.940	0.0155	32.01
3	FH403	16	80	0.700	0.771	0.0693	15.55
3	**FH405[b]**	**25**	**77**	**0.442**	**0.926**	**0.3567**	**24.73**
3	FH356	3	80	0.263	0.279	0.0923	3
3	**FH344[b]**	**9**	**80**	**0.600**	**0.771**	**0.1437**	**7.91**
4	FH431	12	80	0.713	0.717	0.0332	11.72
4	FH448	20	80	0.825	0.897	0.0465	19.71
4	FH452	8	80	0.538	0.487	0.0184	7.82
4	FH465	27	80	0.900	0.906	0.0047	26.44
4	FH466	11	80	0.763	0.742	0.0074	10.72
4	**EST17[b]**	**19**	**80**	**0.550**	**0.866**	**0.2348**	**18.54**
5	**FH221[b]**	**19**	**75**	**0.600**	**0.812**	**0.1603**	**18.76**
5	FH227	9	75	0.573	0.556	0.0484	8.95
5	**FH224[b]**	**36**	**73**	**0.425**	**0.948**	**0.3841**	**35**
5	**FH225[c]**	**27**	**75**	**0.800**	**0.899**	**0.0632**	**24.84**
5	FH230	13	75	0.840	0.807	-0.0056	12.87
5	FH359	16	75	0.880	0.908	0.0185	15.97

[a] This marker showed a linkage to ZF-C59

[b] These markers had a high estimate of null alleles

[c] These markers had ambiguous peaks which were difficult to call

Appendix Table 3: Comparison of mean values (± SE) for each year of the variables in the paternity dataset. Significant differences that were robust to correction for multiple testing ($p < 0.05$) are marked in bold.

Variables	2013	2014	Test statistic	*p*
Paternity				
Clutch size	6 ± 0.11	5.5 ± 0.19	*W* = 317.5	0.04
Egg-laying date[a]	5.27 ± 0.50	8.38 ± 1.06	*W* = 131	**0.01**
N chicks	5.77 ± 0.15	4.83 ± 0.27	*W* = 353	**0.006**
Proportion EP broods	0.23 ± 0.08	0.22 ± 0.10	*W* = 251	0.73
N EP chicks/N broods	0.73 ± 0.31	0.28 ± 0.14	*W* = 255.5	0.63
N WP chicks	5.03 ± 0.34	4.56 ± 0.33	*W* = 305.5	0.12
N EP chicks	0.45 ± 0.21	0.22 ± 0.13	*W* = 272.5	0.81
Total N chicks	5.27 ± 0.37	4.78 ± 0.34	*W* = 300	0.16
N siring EP chicks	0.21 ± 0.07	0.17 ± 0.09	*W* = 270.5	0.85
Sperm morphometry				
Head length (µm)	15.44 ± 0.07	16.01 ± 0.11	*W* = 61	**4.64E-06**
Midpiece length (µm)	177.24 ± 1.19	181.07 ± 1.36	*t* = -2.12	0.04
Tail length (µm)	15.71 ± 0.73	13.71 ± 1.18	*W* = 351	0.04
Flagellum length (µm)	192.95 ± 0.93	194.78 ± 1.16	*t* = -1.24	0.23
Total sperm length (µm)	208.39 ± 0.94	210.79 ± 1.16	*t* = -1.61	0.12
F:H[b]	12.53 ± 0.07	12.2 ± 0.11	*W* = 365	0.02
M:TSL[c]	0.85 ± 0.00	0.86 ± 0.01	*W* = 179	0.09
CV_{wm}[d]	1.74 ± 0.09	1.62 ± 0.07	*W* = 278	0.64
Sperm motility				
Velocity (µm/s)	139.9 ± 5.94	139.83 ± 6.79	*t* = 0.007	0.99
Proportion motile	0.24 ± 0.04	0.37 ± 0.05	*W* = 154	0.05
Body morphology				
Wing length (mm)	76.24 ± 0.34	75.75 ± 0.43	*W* = 329	0.16
Tarsus length (mm)	30.27 ± 0.10	30.16 ± 0.16	*t* = 0.56	0.58
Body mass (g)	16.83 ± 0.12	17.40 ± 0.18	*t* = -2.61	**0.01**
Red border width (mm)	8.39 ± 0.43	7.63 ± 0.47	*W* = 323.5	0.20
Body condition[e]	-0.12 ± 0.11	0.22 ± 0.18	*t* = -1.64	0.11

[a] Days after first egg-laying date
[b] Flagellum to head ratio
[c] Midpiece to total sperm length
[d] Within-male variation in total sperm length, where CV = (standard deviation/mean)*100
[e] Residuals from a regression model of body mass versus tarsus length and capture date

Appendix Table 4: Comparison of mean values (± SE) for each year of the variables in the complete sperm dataset. Differences that are significant after correction for multiple testing ($p < 0.05$) are marked in bold.

Variables	2013	2014	Test statistic	*p*
Sperm morphometry				
Head length (µm)	15.48 ± 0.06	16.08 ± 0.10	t = -5.22	**6.08E-06**
Midpiece length (µm)	178.32 ± 1.07	180.41 ± 1.15	t = -1.33	0.19
Tail length (µm)	15.51 ± 0.59	14.35 ± 1.00	W = 577	0.11
Flagellum length (µm)	193.83 ± 0.84	194.77 ± 1.12	t = -0.66	0.51
Total sperm length (µm)	209.32 ± 0.86	210.85 ± 1.15	t = -1.07	0.29
F:H[a]	12.56 ± 0.06	12.14 ± 0.09	t = 3.82	**0.0004**
M:TSL[b]	0.85 ± 0.00	0.86 ± 0.00	W = 391	0.32
CV_{wm}[c]	1.74 ± 0.08	1.60 ± 0.07	W = 529	0.35
Sperm motility				
Velocity (µm/s)	139.92 ± 5.13	144.90 ± 4.76	W = 429	0.36
Proportion motile	0.24 ± 0.03	0.41 ± 0.04	W = 329	**0.0003**
Body morphology				
Wing length (mm)	76.12 ± 0.28	75.39 ± 0.27	W = 920.5	0.02
Tarsus length (mm)	30.34 ± 0.10	30.10 ± 0.15	t = 1.35	0.18
Body mass (g)	16.99 ± 0.10	17.09 ± 0.15	W = 618.5	0.53
Red border width (mm)	8.07 ± 0.33	7.42 ± 0.33	W = 840	0.17
Body condition[d]	-0.05 ± 0.10	0.06 ± 0.13	t = -0.64	0.53

[a] Flagellum to head ratio
[b] Midpiece to total sperm length
[c] Within-male variation in total sperm length, where CV = (standard deviation/mean)*100
[d] Residuals from a regression model of body mass versus tarsus length and capture date

Appendix Table 5: Correlations between male characteristics and fertilization success in generalized linear mixed models. Fertilization success was measured as within-pair (WP) fertilization success (n = 46, males that had not been cuckolded = 0; males that had been cuckolded = 1), extra-pair (EP) fertilization success (n = 49, males that had not sired extra-pair offspring = 0; males that had sired extra-pair offspring = 1), and total fertilization success (n = 46, total number of offspring sired). The two first are from binomial error structures, whereas the third is from a normal error structure. The direction of the relationships are shown by the estimate (± SE), and the strength of the correlations are shown with the *Z* value and the *p* value (in brackets). Uncorrected *p* values are shown.

	WP fertilization success		**EP fertilization success**		**Total fertilization success**	
	Estimate ± SE	***Z (p)***	**Estimate ± SE**	***Z (p)***	**Estimate ± SE**	***t (p)***
Sperm morphology[a]						
Head length (µm)	-0.92 ± 0.98	-0.94 (0.35)	2.10 ± 6.33	0.33 (0.74)	0.11 ± 0.67	0.17 (0.88)
Midpiece length (µm)	-0.06 ± 0.06	-1.07 (0.29)	0.06 ± 0.36	0.16 (0.87)	-0.04 ± 0.05	-0.84 (0.43)
Tail length (µm)	-0.06 ± 0.10	-0.66 (0.51)	0.01 ± 0.42	0.03 (0.97)	0.10 ± 0.07	1.48 (0.16)
Flagellum length (µm)	-0.14 ± 0.08	-1.83 (0.07)	0.12 ± 0.47	0.25 (0.81)	0.01 ± 0.06	0.21 (0.84)
Total sperm length (µm)	-0.14 ± 0.08	-1.87 (0.06)	0.13 ± 0.48	0.27 (0.79)	0.01 ± 0.06	0.22 (0.83)
F:H[b]	-0.87 ± 0.97	-0.90 (0.37)	-0.27 ± 5.16	-0.05 (0.96)	0.11 ± 0.68	0.16 (0.88)
M:TSL[c]	7.81 ± 18.90	0.41 (0.68)	-1.65 ± 88.13	-0.02 (0.99)	-19.57 ± 13.49	-1.45 (0.17)
Sperm motility						
Velocity[d] (µm/s)	0.01 ± 0.02	0.75 (0.46)	-0.0009 ± 0.07	-0.01 (0.99)	0.005 ± 0.00002	261.25 (1)
Proportion motile[e]	1.83 ± 1.69	1.08 (0.28)	0.33 ± 7.94	0.04 (0.97)	-0.77 ± 1.08	-0.71 (0.57)
Body morphology						
Wing length (mm)	0.37 ± 0.22	1.63 (0.10)	0.32 ± 1.03	0.31 (0.76)	-0.11 ± 0.14	-0.74 (0.48)
Tarsus length (mm)	-0.93 ± 0.68	-1.37 (0.17)	-0.67 ± 3.20	-0.21 (0.83)	-0.05 ± 0.48	-0.10 (0.92)
Body mass[f] (g)	-0.28 ± 0.55	-0.50 (0.62)	-0.52 ± 3.11	-0.17 (0.87)	0.04 ± 0.43	0.09 (0.93)
Red border width (mm)	0.14 ± 0.16	0.89 (0.37)	-0.12 ± 0.81	-0.15 (0.88)	0.001 ± 0.12	0.01 (0.99)
Body condition[g]	-0.77 ± 0.69	-1.12 (0.26)	-0.47 ± 3.68	-0.13 (0.90)	0.27 ± 0.48	0.57 (0.61)

[a] Sperm morphology: *N* = 46/48/46

[b] Flagellum to head ratio

[c] Midpiece to total sperm length

[d] Velocity: *N* = 37/38/37

[e] Proportion motile: *N* = 44/47/44

[f] Body mass: *N* = 45/47/45

[g] Body condition (residuals from a regression model of body mass versus tarsus length and capture date): *N* = 40/42/40

Appendix Table 6: Paired comparisons of within-pair (WP) males and the extra-pair (EP) male that cuckolded them, with mean values (± SE) of sperm traits and body morphology traits. Uncorrected *p* values are shown.

Variables	WP	EP	*t*	*N* pairs	*p*
Sperm morphology					
Head length (µm)	15.46 ± 0.13	15.78 ± 0.23	-2.12	8	0.07
Midpiece length (µm)	174.9 ± 2.47	180.53 ± 2.54	-1.54	8	0.17
Tail length (µm)	15.32 ± 1.47	15.42 ± 1.04	-0.07	8	0.95
Flagellum length (µm)	190.22 ± 2.24	195.95 ± 2.13	-1.84	8	0.11
Total sperm length (µm)	205.68 ± 2.34	211.73 ± 2.21	-1.97	8	0.09
F:H[a]	12.33 ± 0.09	12.45 ± 0.18	-0.49	8	0.64
M:TSL[b]	0.85 ± 0.01	0.85 ± 0.01	-0.28	8	0.79
CV_{wm}[c]	1.54 ± 0.13	1.56 ± 0.09	-0.19	8	0.86
Sperm motility					
Velocity (µm/s)	141.51 ± 10.26	149.65 ± 5.35	-0.65	6	0.54
Proportion motile	0.35 ± 0.09	0.3 ± 0.09	0.50	8	0.63
Body morphology					
Wing length (mm)	77.11 ± 0.48	76.78 ± 0.36	0.44	9	0.67
Tarsus length (mm)	30.13 ± 0.18	30.08 ± 0.13	0.31	9	0.77
Red border width (mm)	8.83 ± 0.79	7.6 ± 0.69	1.46	9	0.18
Body mass (g)	17.06 ± 0.29	16.75 ± 0.28	1.00	8	0.35
Body condition[d]	-0.15 ± 0.23	0 ± 0.22	-0.56	6	0.60

[a] Flagellum to head ratio
[b] Midpiece to total sperm length
[c] Within-male variation in total sperm length, where CV = (standard deviation/mean)*100
[d] Residuals from a regression model of body mass versus tarsus length and capture date

Appendix Table 7: Correlations between sperm morphology variables. The direction of the relationships are shown by the estimate (± SE), and the strength of the correlations are shown with the *F* value and *p* value. Significant correlations ($p > 0.05$) which were also significant after correcting for multiple testing, are marked in bold. All sperm lengths are measured in μm.

		Estimate ± SE	*F*	*p*
Total sperm length	Head length	4.51 ± 1.62	7.74	**0.007**
	Midpiece length	0.62 ± 0.07	72.39	**1.13E-11**
	Tail length	0.11 ± 0.17	0.41	0.53
	Flagellum length	1.02 ± 0.01	10090	**<2e-16**
	F:H[a]	7.3 ± 1.46	25.03	**5.93E-06**
	M:TSL[b]	17.61 ± 34.98	0.25	0.62
Head length	Midpiece length	0.01 ± 0.01	1.35	0.25
	Tail length	0.01 ± 0.01	0.67	0.42
	Flagellum length	0.02 ± 0.01	4.68	0.03
	F:H[a]	-0.59 ± 0.11	28.75	**1.62E-06**
	M:TSL[b]	-2.77 ± 2.69	1.07	0.31
Midpiece length	Tail length	-0.9 ± 0.16	30.13	**1.01E-06**
	Flagellum length	0.94 ± 0.11	75.34	**5.96E-12**
	F:H[a]	8.11 ± 1.82	19.85	**4.06E-05**
	M:TSL[b]	224.5 ± 29.82	56.67	**4.64E-10**
Tail length	Flagellum length	0.06 ± 0.11	0.35	0.56
	F:H[a]	-0.21 ± 1.39	0.02	0.88
	M:TSL[b]	-204.1 ± 4.87	1759	**<2e-16**
Flagellum length	F:H[a]	7.9 ± 1.35	34.20	**2.68E-07**
	M:TSL[b]	20.38 ± 34.10	0.36	0.55
F:H[a]	M:TSL[b]	3.53 ± 2.63	1.80	0.19

[a] Flagellum to head ratio

[b] Midpiece to total sperm length