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Pre- and post-copulatory sexual selection in the collared flycatcher (*Ficedula albicollis*)

Pre- a post-kopulační pohlavní výběr u lejska bělokrkého (*Ficedula albicollis*)

Ph.D. thesis

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I declare that this thesis is my original work and has not been submitted for the purpose of obtaining the same or any other academic degree earlier or at another institution. My contribution to each of the chapters comprising this work is expressed through the authorship order of the included chapters and author's contribution statements complementing the chapters.

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Abstract

Sexual selection is one of the forces that lead evolution and may happen at different times during the reproductive period. Mate choice and extra-pair mate choice are forms of pre-copulatory selection. Secondary sexual traits may be indicators of male quality and females should express preferences for those traits. Males with larger ornaments may be able to obtain more partners and increase their number of offspring. Females may obtain direct benefits and/or indirect genetic benefits from their social mate. But when mated to non-preferred males, females may choose an extra-pair mate with superior traits than their social mate, since females usually do not receive direct benefits from extra-pair mates and are therefore expecting to receive indirect benefits. As females copulate with many males, sperm from different males compete to fertilize the set of ova. Sperm length should influence the outcome of sperm competition and the ability to produce more offspring. Under strong sexual selection (high rate of extra-pair paternity), stabilizing selection should decrease the genetic additive variance of sperm length to allow an optimum sperm morphology to outcompete sperm from other males.

This thesis focuses on the pre- and post-copulatory steps of sexual selection in the collared flycatcher (Ficedula albicollis), a migratory bird with a high rate of extra-pair paternity. Secondary sexual traits were manipulated to determine which traits females used to select mates. Genetic analyses allowed the assignment of offspring to their genetic fathers and determined the rate of extra-paternity. Traits of males that shared paternity were compared. Arrival date and proximity of males were determined as they may influence the outcome of mate choice and paternity. Sperm were sampled at different periods of the breeding season to estimate the dynamics of sperm length variation. Long-term pedigree data and a quantitative model helped to separate the genetic and environmental variances that influence sperm length.

Females did not demonstrate a consistent preference for more ornamented males as social mates. Males with large ornaments were less attractive to females late in the season and lost more within-pair paternity. Extra-pair males were not more ornamented than the males they cuckolded but both social and extra-pair males were superior to males around the females' nests. Early arrival at the breeding site allowed males to protect their within-pair paternity. Males did not win more extra-pair offspring when they were more ornamented and sperm length did not influence fertilization success. Sperm length demonstrated phenotypic plasticity throughout the breeding season and had moderate heritability.

Future research should focus on the environment in which sperm competition takes place. Indeed, studies trying to identify sperm traits that make sperm successful in fertilization focus on sperm traits before the bottleneck of cryptic female choice. More studies on the heritability of sperm traits in wild populations and especially on the effect of the environment on sperm length are needed to understand how it is influenced by the pressure of selection. The genomes of many species have been sequenced, but the genes which code for sperm morphology have largely been ignored. More knowledge about the genes coding for these traits will help in understanding the genetic process acting on sperm diversity.

Keywords: sexual selection, mate choice, extra-pair paternity, ornaments, sperm competition, heritability

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Abstrakt

Pohlavní výběr je důležitou evoluční silou, která se může projevovat v různých fázích reprodukčního období. Výběr sociálního a extra-párového partnera jsou formy pohlavního výběru, které probíhají před kopulací. Sekundární pohlavní znaky mohou být indikátory samčí kvality, a proto by pro ně samice měly mít preference. Samci s většími ornamenty mohou získat více partnerek a zvýšit tak počet svých potomků. Samice mohou volbou svých sociálních partnerů získat přímý i nepřímý, genetický užitek. Když ale mají samice nepreferovaného sociálního partnera, mohou si za toho extra-párového vybrat samce s lepšími znaky, než jaké má ten sociální, protože extra-pároví samci samicím žádný přímý užitek neposkytují a tak se dá očekávat, že jim poskytují užitek nepřímý. Když samice kopuluje s více samci, konkurují si jejich spermie o oplodnění sady vajíček. Výsledek této kompetice spermií a tedy počet potomků by měl být ovlivněn délkou spermií. Pokud je pohlavní výběr silný, jak tomu bývá v případě vysoké míry extra-párové paternity, stabilizující selekce by měla snižovat aditivní genetickou varianci pro délku spermie, v důsledku čehož by měly spermie optimální morfologii pro vítězství v konkurenci s ostatními samci.

Tato práce se zaměřuje na pre-kopulační a post-kopulační fáze pohlavního výběru u lejska bělokrkého (*Ficedula albicollis*), migrujícího pěvce s vysokou mírou extra-párové paternity. Manipulovali jsme sekundární pohlavní znaky samců, abychom zjistili, zda si na jejich základě samice vybírají partnera. Genetické analýzy nám umožnily přiřadit k mláďatům jejich genetické otce a zjistit míru extra-párové paternity. Srovnávali jsme pak znaky samců, kteří zplodili mláďata ve stejném hnízdě. Zaznamenávali jsme také přílet samců na hnízdiště a vzdálenosti mezi jednotlivými hnízdy, což jsou faktory, které mohou ovlivňovat výběr partnera a paternitu. Spermie jsme odebírali v různých fázích hnízdní sezóny, abychom zjistili, jak se v sezóně mění jejich délka a variabilita. Na základě dlouhodobých pozorování jsme sestavili rodokmen dané populace a pomocí kvantitativně genetického modelu jsme zjistili jaká část variability v délce spermií je daná geneticky a jaká prostředím.

Samice si nevybíraly za sociální partnery jen samce s většími ornamenty. Samci s většími ornamenty byli dokonce na konci sezóny pro samice méně atraktivní a měli ve svých sociálních hnízdech více extra-párových mláďat. Extra-pároví samci neměli větší ornamenty než samci sociální, ale obě tyto skupiny měly větší ornamenty než jiní samci dostupní v blízkosti sledovaných hnízd. Časný přílet umožnil samcům lepší ochranu paternity ve vlastních hnízdech. Samci s velkými ornamenty nezplodili více extra-párových mláďat v jiných hnízdech a ani délka spermií neměla vliv na úspěch oplodnění. Délka spermií vykazovala v průběhu sezóny fenotypickou plasticitu a byla středně heritabilní.

Další výzkum by se měl zaměřit na prostředí, ve kterém probíhá kompetice spermií. Dosavadní studie, které se pokoušely zjistit, jaké znaky spermií mají vliv na jejich fertilizační úspěšnost, neuvažovaly možný vliv kryptické volby samice. Pro lepší porozumění selekčních tlaků na morfologii spermií je třeba více studií, které by zjišťovaly heritabilitu tohoto znaku

v přírodních populacích a také jeho ovlivnění prostředím. Třebaže genomy mnoha druhů jsou již osekvenovány, geny zodpovědné za znaky spermií stojí dosud stranou zájmu. Jejich znalost by nám napomohla pochopit, jakou roli hrají genetické procesy v diverzitě spermií.

Klíčová slova: pohlavní výběr, volba partnera, extra-párová paternita, ornamenty, kompetice spermií, heritabilita

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Contents

General introduction

Sexual selection

In the Linnaean classification system from 1758, the description of two duck species, *Anas platyrhynchos* (Syst. Nat, 1758, ed. 10, p125) and *Anas boscha* (Syst. Nat, 1758, ed. 10, p127), stand out. Both of them have an iridescent blue patch on their wings but the first species has a green head and neck where the second species is completely brown. We now know these two species are in fact a single species, with the different descriptions referring to the male and female of *Anas platyrhynchos*, respectively. This confusion arose through the high degree of dimorphism between the sexes and particularly the presence of secondary sexual traits in males.

The question of such differences between sexes was a challenge for many years. Darwin noticed that males had some extravagant traits (like the tail feathers of the peacock) that constrain males and challenged his hypothesis of natural selection. He proposed the beginning of an answer in 1871 in *The descent of man and selection in relation to sex*, with the theory of sexual selection. This theory explains that extravagant traits increase the chance of mating. Traits play a direct role in attracting a partner from the other sex in cases of inter-sexual competition. Moreover, sexual traits allow males to be more efficient in male-male competition, also known as intra-sexual selection. In this case, extravagant traits are called “armaments” or “badges” as they provide the ability to fight and indicate their status (Andersson 1994). The strength of sexual selection can be estimated by the variation in the number of offspring produced in the next generation.

Sexual dimorphism of secondary sexual traits is common and well-explained for males in polygynous mating systems. The exaggerated traits allow males to attract more females and consequently to obtain higher numbers of offspring. However, when males have a monogamous breeding system, dimorphism is not expected (or should be less expressed). Indeed, males in monogamous systems have lower reproductive success comparatively with polygynous males. However, sexual dimorphism is also common in monogamous species. Whatever the mating system, the exaggerated traits of males are used by females to select their partner. The selection of mates allows females to obtain sperm that will fertilize their ova and produce offspring.

Sexual selection can act on two different steps. Pre-copulatory selection concerns every behavior that takes place before the copulation, such as courtship behavior, male-male competition and mate (social and/or extra-pair) choice. **Chapter** 1 and **2** of this thesis focus on this first step of selection as social and extra-pair mate choice according to ornamentation and the temporal context within which this choice is made. Sexual selection does not stop with copulation and continues after sperm is released in the female tract and reaches the ova. This post-copulatory selection is studied in **Chapter 2** and **Chapter 3**, by investigating paternity success according to sperm length and the heritability of sperm length.

Pre-copulatory sexual selection

Mate choice

Mate choice is defined as “the outcome of the inherent propensity of an individual to mate more readily with certain phenotypes of the opposite sex (i.e. mating preference or bias) and the extent to which an individual engages in mate sampling before deciding to mate (i.e. choosiness)” (Kokko et al. 2006). The choosiness towards a particular phenotype is due to a divergence of interests when mating. The origin of this divergence comes from anisogamy (i.e. differences in gamete investment according to the producing sex). Males make many small motile gametes, which are cheap to produce and easy to disperse. Females produce fewer and large gametes; in consequence, females are resource-constrained for producing gametes. These differences in gamete investment led to the divergence of interests of both sexes. Males should try to obtain many partners as their reproductive success increases with the number of fertilized females (Bateman 1948), while females may look to increase the quality of offspring as they are limited in the number of descendants they can produce (Trivers 1972). Thus, females are choosy towards males that will fertilize their eggs and increase the quality of offspring they will produce. As females are confronted with a crucial choice to increase their fitness (and the fitness of offspring), they may use clues displayed by the males to choose a mate. However, they may choose this partner according to their own interests. Some traits are indicators of male quality and females should use those cues to select an adequate partner. Female mate preferences may provide some benefits to them: direct or indirect.

Direct benefits occur when a gain of fitness is obtained from males which will affect the immediate viability of offspring. Males may provide resources to increase the fecundity of the female. In insects, for example, many males offer nuptial gifts (i.e. prey) to the females whose fecundity will increase as it depends on alimentary resources (Vahed 1998). Males can also secure resources for females by obtaining better territories (feeding territories, breeding territories, etc.) (Alatalo et al. 1986). This should be an important factor especially when resources fluctuate during the breeding season. An important direct benefit females obtain from males is parental care (Hoelzer 1989, Keyser and Hill 2000). This last statement is essential in monogamous bird species where both partners provide care to the offspring to ensure their survival. If one of the partners dies or deserts the nest, the survival rate of the young is reduced. But before obtaining offspring, males may provide sperm to females and this may be the most important resource that females look for (Arnqvist and Kirkpatrick 2005, Griffiths et al. 2010).

Females may ensure that the selected mate will be able to fertilize all the eggs she will produce in the reproductive event.

The preferences toward exaggerated traits also exist when males seem to provide only sperm resources (and so genetic material). Females may obtain some indirect benefits in this case. Indirect benefits involve no direct advantages for the females, but the fitness of females will rise by increasing the genetic quality of their offspring (Slatyer et al. 2012). Young will consequently have a higher survival rate and/or better reproductive success. Many theories have tried to explain the mechanisms underlying indirect benefits.

A first explanation emerges with the runaway process proposed by Fisher (Fisher 1930). Fisher's model, or the "sexy-son model", states that the preference of females for exaggerated traits may favor the reproductive success of sons. The model is based on the coevolution of the females' preference and the exaggerated traits of males. If a male trait has a genetic basis and females have a preference for males which express those traits to a greater degree, sons may obtain this gene and express it more than the mean population expression and spread the female preference. By consequence, sons may themselves be more attractive as they express the exaggerated trait. The traits and the female preference will increase by positive feedback, even if the trait becomes troublesome for survival. The benefits of reproduction associated with this trait overcome the cost of declining survival by natural selection.

Another explanation by Zahavi was proposed to explain the evolution of the exaggerated traits (Zahavi 1975). The "handicap model" or "good genes model" is based on the idea that exaggerated traits are honest signals (Zahavi 1977). Males able to express more exaggerated traits may demonstrate their ability to survive despite being handicapped by large or conspicuous traits. The traits should be condition-dependent and heritable; only good quality males may express them. In this case, the expression of the traits is costly for the male as they may invest more in the trait to obtain females. The investment necessary to develop exaggerated traits may be detrimental to other physiological aspects as the energy they can invest is limited (Jennions et al. 2001). Longer and more visible traits may attract predators and reduce the ability of the male to escape (Jennions et al. 2001, Danchin et al. 2005). By mating with males that express honest traits indicating their quality (or good genes), females may increase the quality of their offspring.

In both the good genes and sexy-son hypotheses, the male traits existed before the preference of the females, and this preference led to the extravagant development of the trait. However, another theory predicts that female preferences existed first. The sensory bias hypothesis states that males may “exploit” a sensory bias in female perception to increase their reproductive success (Basolo 1990, Ryan and Rand 1990, Fuller et al. 2005). Males developing those traits may be more attractive to females and the runaway process can begin.

All of these models are based on the idea that genes will increase fitness by increasing genetic quality and that all females express the same preference. But females may express a range of preferences that allow them to choose their ideal partner. In this case, females may select for compatible genes instead of good genes. In this model, the quality of offspring will arise from an association between different pools of genes (non-additive model) instead of the improvement of the same pool of genes (additive model) (Johnsen et al. 2000, Neff and Pitcher 2005). In this case, females can increase the heterozygosity of offspring (Foerster et al. 2003), avoid genetic incompatibility (Zeh and Zeh 1996) and also avoid inbreeding.

All females may not be able to mate with a male that will provide optimum benefits (direct or indirect). If females all express the same preference, especially in a monogamous system, they may not be able to mate with the few males that overexpress secondary sexual traits more than the other males. Being badly paired could be stressful and costly if it reduces the chance of successful reproduction (Griffith et al. 2011). After selecting a social partner that will provide parental care, females may adjust their indirect benefits through extra-pair copulations.

Extra-pair paternity

Before the rise of molecular analysis, there was no doubt that monogamous species were socially but also genetically monogamous. The idea of unfaithful partners was not considered, or only as a consequence of re-mating. Genetic assignment demonstrates that many monogamous species are not genetically monogamous (Griffith 2002) and many offspring produced by a female are half-siblings. As females may not be able to mate with their preferred male, they may adjust the initial mate choice by behaving polyandrously (i.e. females mating with more than one male). The expression of genetic polyandry is commonly found to be a female strategy (Westneat and Stewart 2003) even though the idea of coercion or alternative male strategies are not excluded (Currie et al. 1998, Stutchbury 1998a, Westneat and Stewart 2003). The Bateman gradient is the relationship between mating success, measured as the number of mates, and reproductive success. Extra-pair paternity could be a way to reduce the difference in Bateman gradient between males and females (Kvarnemo and Simmons 2013).

Once again, females and males do not gain the same benefits by engaging in extra-pair copulation. For males, strict monogamy should be a waste of benefits as they should be able to fertilize more than their own partner. Extra-pair paternity allows them to fertilize other females without providing the parental care imposed by monogamy. Males which are more attractive as social mates may also be more attractive as extra-pair mates. They should also lose less within-pair paternity as their females should be less motived to cuckold them. The existence of a trade-off between within-pair paternity and extra-pair paternity seems unlikely according to many studies where preferred males lost less within-pair and gained more extra-pair offspring (Ferree and Dickinson 2014, Araya-Ajoy et al. 2016). However, the traits influencing within-pair paternity and extra-pair paternity may be different, especially if females look for different kinds of benefits according to the context.

A cost to females seeking extra-pair copulations is being harassed by males and potentially being injured by them (Valera et al. 2003). Additional direct costs to females include the decrease of parental care if their social partners are able to estimate their paternity. The benefits of extra-pair paternity are less obvious. Direct benefits of extra-pair paternity seem scarce as females do not receive food or parental care from the extra-pair males (Arnqvist and Kirkpatrick 2005). Some studies have shown that females may benefit from accessing the territories of neighboring extra-pair males and may benefit from some protection from extra-pair mates if they are neighbors (Gray 1997, Eliassen and Jørgensen 2014). As females may receive only sperm from the extra-pair mates, it is possible that females engage in extra-pair copulation to secure their egg fertilization against their own mate’s partial or total infertility (Sheldon 1994, Birkhead and Fletcher 1995, Ihle et al. 2012). Extra-pair copulation can also act as an adjustment of mate choice. If females express strong preferences for some male traits, the availability of those males declines when they are engaged in a monogamous pair (operational sex ratio). It means that some females may pair with sub-optimal or sub-attractive males. These badly-paired females may increase the fitness of their offspring by obtaining good genes or compatible genes from their extra-pair mates. Once again, secondary sexual traits may play a role in the decision to engage in extra-pair copulation. According to the good genes hypothesis, females may have extra-pair copulations with more ornamented or older males that their social mates (Jennions and

Petrie 2000, Akçay and Roughgarden 2007). The extra-pair offspring will be more viable and/ or more attractive than their half-siblings. The compatible genes hypothesis states that females choose males with genotypes that better fit them. Another explanation for extra-pair copulation is that females make “the best of a bad job” (Forstmeier et al. 2011).

However, the advantages for offspring resulting from extra-pair copulation are not obvious. Many studies have demonstrated the superiority of extra-pair young (Sardell et al. 2011, Bowers et al. 2015) but not all of them demonstrate their superiority (Sardell et al. 2012). But this superiority can be due to the order of extra-pair offspring in the laying sequence (Krist and Munclinger 2011). It is commonly found that early chicks in the hatching sequence have an advantage on the chicks that hatch later. So the better survival of extra-pair chicks may be due to maternal effects and not to genetic quality.

Both male traits and environmental factors may lead to non-random mating in extra-pair copulations. Temporality, seasonality and social factors could influence the outcome of extra-pair paternity. While males are almost always available for copulation, the success of fertilization of the females depends on their fertile period. The fertile window of females is roughly a few days before the first egg laying and a few days after. The question of the synchrony of females’ fertile periods is still debated (Dunn et al. 1994, Spottiswoode 2004, LaBarbera et al. 2010). Synchrony may favor extra-pair paternity as it concentrates females available for copulation on a small scale of time (Stutchbury and Morton 1995, Stutchbury 1998b). However, it may also decrease the opportunity for extra-pair copulation as males may guard their mates more intensively (Emlen and Oring 1977, Thusius et al. 2001a). From another point of view, asynchrony of the fertile period for females may favor extra-pair copulation as males may be more willing to leave their partners if the fertile period of their mate is secure.

For many species, the breeding season is limited in time, especially for migratory birds. Early in the breeding period, a lot of females should be available for copulation and the opportunity for extra-pair copulation is higher. Later in the season, the opportunity should be restrained but, as favored males should also be paired, females may favor extra-pair copulation at this time to compensate for the sub-optimal choice of mate. Social factors may also influence the extra-pair behavior: the density and proximity of potential extra-pair mates. The density may favor encounters for potential extra-pair copulations and it seems to be the case at the species level (Dunn et al. 1994, Westneat and Sherman 1997). However, the study of the influence of density is still uncertain (Westneat and Sherman 1997). Whatever the density, the proximity of potential extra-pair mates seems to be a factor that influences extra-pair paternity. Colonial species are more involved in extra-pair paternity than other species. However, even in non-colonial species, the extra-pair mates are close neighbors.

Studies about mate choice toward a preferred phenotype of males are quite common. However, if the choosiness of females is incontestable, the benefits that she will obtain in mate choice between the sexy-son and good genes hypotheses are still under debate (Prokop et al. 2012). But studies where mate preference, ecological factors and sperm competition are taken into account together are scarcer and deserve more attention as these factors may work concomitantly. According to the good genes hypothesis, females should demonstrate preferences for more ornamented males as social and extra-pair mates without consideration for the context. But the context of mate choice should lead to variation in costs and benefits and so change the mate preference. In **Chapter 1**, the consequence of the manipulation of attractiveness through male ornament modification and its influence on paternity success according to the context is examined. Females mated to ornamented males should be less motivated to cuckold their mate and consequently have half-siblings in their nests less frequently. They should also be able to select superior males that will provide indirect benefits from a pool of males available around their nests. This is the focus of **Chapter 2**. Females may select mates according to their own (direct and/or indirect) benefits they will obtain, but the benefits could vary according to the ecological context, especially according to the advance of the breeding season. Females should be less choosy late in the season as they are constrained by time, especially in migratory birds, to raise offspring in good conditions before going back to their wintering regions. The influence of the early arrival of males, called protandry, on female preference and extra-pair paternity opportunities is studied in **Chapters 1** and **2.** Females are often surrounded by males' territories. If they are looking for indirect benefits, they may be able to select the more ornamented males from a pool of males in a close area. Moreover, if ornamented males are more aggressive against intruders, the chance to find a better male close to her should decrease with male ornamentation. We can expect to find the distance between males varying in relation to their ornaments. This hypothesis is tested in **Chapter 2**.

Post-copulatory sexual selection

Sperm competition

The main consequence of the polyandrous behavior of females is that sperm from different males compete to fertilize the eggs. Extra-pair copulation does not automatically lead to extra-pair fertilization and extra-pair offspring (Schwartz et al. 1999), indicating that other processes occur after copulation. The success of fertilization can be biased in favor of some males through sperm competition (Parker 1970) and female cryptic choice (Thornhill 1983). These two selective pathways of sperm are commonly gathered together under the general term of sperm competition, and it is considered a third type of sexual selection (Danchin et al. 2005) as it causes a bias in the reproductive success of males. Many factors influence the outcome of this competition.

The success of sperm competition may be dependent on the timing and order of copulation. Males do not copulate at the same time with females, and the order in which they copulate may modify their chances of success. The last sperm precedence theory partially explains the success in sperm competition by the mechanism of passive sperm loss (Birkhead 1998). The number of sperm from the first male to copulate will decrease as time to oviposition decreases. If a second male copulates with the female, this male will have an advantage as more of his sperm will be available for fertilization. The fertile period of females is short and a “good shot” may be enough to be successful. However, this is risky and males may prefer to copulate frequently with their partners during the fertile period to avoid losing paternity. To secure paternity, males may adapt their behavior by guarding their partner and/or copulating frequently with their social partners to favor their sperm to outcompete other sperm. Mate guarding is a common behavior especially during the female fertile period, indicating that males protect their paternity by this behavioral approach. However, this behavior does not always secure the partners completely (Kempenaers et al. 1992) and also depends on the willingness of the females to escape this surveillance to obtain extra-copulation (Kokko and Morrell 2005). Another way to ensure securing the paternity of the mate is that males may invest more sperm by having a high rate of copulation with the partner (Birkhead et al. 2009). Species with a higher risk of extra-pair paternity are known to have larger testes size, indicating that those species produce more sperm as the risk of losing paternity is important. Intensive copulation (social and extra-pair) requires high sperm production.

Males may physiologically invest in traits directly linked to spermatogenesis, such as large testes and/or in the morphology of spermatozoa (Dunn et al. 2001, Pitcher et al. 2005, Lüpold et al. 2011) when under strong selection. Larger testes will allow the production of more spermatozoa, especially under strong sexual selection (Tuttle et al. 1996, Tuttle and Pruett-Jones 2004). However, the production of numerous spermatozoa may be costly (Nakatsuru and Kramer 1982) and they may adjust their investment in reproduction by modulating the quality and number of sperm they invest in a reproductive event (Reinhold et al. 2002, Wedell et al. 2002, Cornwallis and Birkhead 2007). The length of sperm is the other factor that may lead to more success in fertilization (Snook 2005, Immler et al. 2011, Bennison et al. 2015). Size may lead to an exclusion of other sperm from the female storage organ (LaMunyon and Ward 1998). Length is not always directly linked to the direct exclusion of competitors' sperm, but is also linked to sperm velocity. Mobility and velocity may allow the sperm to reach the ova first before the other sperm from different males. Spermatozoa are commonly described as having three main parts. The head is the part where the genetic material is stored. The midpiece is commonly the part of sperm which houses mitochondria and provides the energy necessary for the propulsion of sperm through the female tract. The tail allows the sperm to swim. The midpiece and tail are often regrouped as the flagellum as it is the mechanistic part of the spermatozoa. In many species, longer sperm and longer flagella lead to an increase in sperm velocity (Helfenstein et al. 2010 but see Kleven et al. 2009). However, the length of the flagellum alone cannot explain the higher success of paternity; the relative length of the midpiece to the flagellum length makes the sperm more efficient (Knief et al. 2017). This last result is the consequence of an inversion of the Z-chromosome in the zebra finch (*Taeniopygia guttata*). This inversion provides an advantage for heterozygous males and consequently maintains genetic diversity (Fisher 2017, Kim et al. 2017, Knief et al. 2017). But not all species have their genome sequenced and the region coding for sperm traits is often still unidentified.

Quantitative genetics and sperm traits

Under strong sexual selection, longer and less variable sperm length are expected as they will favor fertilization. This lack of variation, reflecting stabilizing selection, unifies sperm length around an optimal mean length by favoring one type of genotype and homogenizing the phenotype of spermatozoa (Lifjeld et al. 2010 but see for heterozygote advantage Kim et al. 2017; Knief et al. 2017). It is possible to estimate the additive genetic variance of spermatozoa length by measuring the length (total length and/or length of each sperm section) and using quantitative genetic tools. The phenotypic variance and the additive genetic variance will allow the determination of the heritability. Under strong stabilizing selection, the additive genetic variance should be low. Heritability is the proportion of the phenotypic variance attributable to additive genetic effects (Falconer et al. 1996): $h^2 = Va/Vp$. Va is the genetic additive variance and Vp the phenotypic variance. Heritability is considered a base for evolvability, the capability of species to respond to selection. Traits closely tied to fitness generally have lower heritability than morphological traits (Houle 1992, Kruuk et al. 2000, Teplitsky et al. 2009) because of depleted Va (Charmantier and Garant 2005, Kruuk et al. 2008). Sperm length heritability is mainly studied in farmed species and in captive species (Birkhead et al. 2005, Mossman et al. 2009). In those unnatural conditions, the effect of environment may be less important and may bias the estimation of heritability (Charmantier and Garant 2005). Studies of sperm length from wild populations are lacking. In **Chapter 3**, the long-term monitoring of a population allows the development of a multigenerational pedigree able to determine the heritability of the total length of spermatozoa in a wild population. To our knowledge, this has never been done before and will elucidate the process of selection on this particular trait.

The collared flycatcher, a case study for sexual selection

The collared flycatcher (*Ficedula albicollis*) is a small migratory passerine bird. This species is widespread in Eastern Europe and some populations breed on the Swedish island of Gotland (Cramp and Perrins 1993). As a migratory bird, it spends winter in Africa and returns to Europe for the breeding season (Cramp and Perrins 1993). The breeding season ranges from the beginning of April to late July for the northern population (Cramp and Perrins 1993). Males generally arrive first to the breeding area. Males are highly territorial and defend a suitable nest site before the females' arrival. To do so, males sing in proximity of the nest area and can react aggressively to intrusive males (Garamszegi et al. 2006). The collared flycatcher is a cavity-nesting bird and easily accepts breeding in artificial nest-boxes. Females generally lay about 5-7 eggs, one per day, and the female incubates alone (Cramps and Perrins 1993). Chicks hatch about twelve days after egg laying and fledge about fourteen days after hatching. Bi-parental care increases the chicks' chance of survival. Females are dull brown with white patches on the wings. Males are black and white with two secondary sexual traits: a white forehead patch and white wing patches on the primaries. These two ornaments on the forehead and wing are cues for female mate choice. These patches are involved in male-male competition but also in inter-sexual selection (Qvarnström et al. 2000). Interestingly in collared flycatchers, the secondary sexual traits are not expressed similarly between two populations. In the isolated population of Gotland, the forehead patch is a condition-dependent trait (Gustafsson et al. 1995) used in both intra- and inter-sexual selection (Pärt and Qvarnström 1997, Sheldon et al. 1997, Sheldon and

Ellegren 1999, Qvarnström et al. 2000). In the Eastern European population (so a priori Czech population), the wing patch is condition-dependent and varies according to the age of the male (Hegyi et al. 2002, de Heij et al. 2011). Female collared flycatchers may use different cues to choose a mate during the breeding season. In addition to secondary sexual traits, females may also use ecological factors like territory quality to select their social partner. The collared flycatcher is a migratory bird and the males arrive earlier than females (Cramps and Perrins 1993). The timing of arrival at the breeding site may influence territory settlement as males that arrive first should be able to monopolize the best territories. In a closely-related species, the pied flycatcher (*Ficedula hypoleuca*), females choose mates according to the breeding territory (Alatalo et al. 1986). Ornamentation plays a role in male-male competition (Pärt and Qvarnström 1997) and males with large ornaments settle more easily on good territories (Pärt and Qvarnström 1997). Like many monogamous passerine birds, the collared flycatcher is not genetically monogamous. The rate of extra-pair paternity varies (Gotland population : 15.5% (Sheldon and Ellegren 1999), Hungarian population : 20.61% (Rosivall et al. 2009), Czech population : 24.2 % (Krist et al. 2005), 26.8 % (**Chapter 2**) (Edme et al. 2016), 23.4% (Edme et al. 2017) (**Chapter 1**). As with mate choice, females may prefer more ornamented males (wing patch or forehead patch according to the population) (Michl et al. 2002). In **Chapter 2**, the traits (age, ornament) that influence the loss of paternity are studied. We also look closer at the choice of extra-pair partner as we compare the social male against the males that cuckold it and also the set of males around the female’s nest. The polyandry of the female collared flycatcher leads to sperm competition to fertilize the eggs. Little information is available about sperm competition in this species and competition seems to take place early in the female fertile period (Sheldon and Ellegren 1999, Michl et al. 2002, Krist et al. 2005). According to the sexy sperm theory, sons may inherit their fathers' sperm's competitive ability and so increase their competitiveness in sperm competition. **Chapter 3** focuses on the heritability of spermatozoa length and the phenotypic change in the breeding season that can influence the success of paternity.

Thesis outline

Mate choice, extra-pair mate choice and the role of ornamentation have been plentifully studied. However, these studies have mainly considered mate choice as being static during the breeding season. The study of variation in mate choice according to seasonal context but also between populations of the same species in two ecological contexts is lacking. Moreover, sperm traits are also factors that influence the outcome of paternity and are rarely considered in combination with mate choice and ecological factors. The heritability of sperm length in wild populations has never previously been studied. The key aims of my thesis are to determine (1) which traits influence mate choice and extra-pair mate preference, (2) the influence of early arrival on breeding site on paternity success and (3) the heritability of sperm length in a wild population of birds with a high risk of sperm competition.

Chapter 1 of this study explores all of the stages of sexual selection. A secondary sexual trait known to be preferred by females in other population was modified and all components of sexual selection were investigated: mate choice, female investment, paternity and extra-pair paternity success, the influence of sperm length on paternity and finally fledgling and recruit success. Large ornaments were not preferred by females early in the season and were less attractive late in the season as males with enlarged ornaments needed more time to pair. Females modulate their investment in reproduction according to the arrival date of their mate but not according to ornaments. More ornamented males did not obtain more offspring, chicks did not fledge or recruit more than less ornamented males, and sperm length did not influence paternity success. We discuss the variation in preference for different secondary sexual traits in two populations of collared flycatchers and the implication of the absence of an effect of sperm length on paternity.

In **Chapter 2**, the factors that influence paternity within the social nest were investigated and the traits of males who share paternity were compared. As females are expected to choose extra-pair mates according to their preference, they should prospect in a close area to find males with superior traits to their social mate. The proximity of other males may favor the encounter of unfaithful birds. If females are paired to highly ornamented males, they should prospect farther to find a suitable extra-pair partner. Here, females' social, extra-pair mates and available males in a close area around the female's nest were compared. We found that more ornamented males more often share paternity in their nest. The cuckolded males were similar to the males sharing paternity in their nest, but both of them were superior to neighboring males. We discuss the strategies of both males and females in obtaining extra-pair copulations and their potential costs and benefits.

Chapter 3 is, to our knowledge, the first to estimate the heritability of sperm length in a wild bird population under a high level of sexual selection. In this study, sperm were sampled for five years and during two periods of the breeding season. The collared flycatcher is under strong sexual selection that should lead to stabilizing selection toward sperm morphology and consequently decrease the genetic additive variance of sperm traits. Many studies of sperm length have been conducted in farmed or laboratory species with environmental control that could influence the strength of environmental effects. High environmental variance and stabilizing selection could decrease the heritability of traits. Animal models are strong tools to tease apart the environmental and genetic parts of phenotypic variance. We discuss the role of migration and the rate of copulation on the phenotypic plasticity of sperm length. We found moderate additive genetic variance; we discuss the influence of a controlled environment and high sexual selection pressure via a high rate of extra-pair paternity. We also discussed our results according to the lower sperm traits heritability in the zebra finch (*Taeniopygia guttata*), a bird with a high heritability of sperm length and an inversion on the Z-chromosome which maintains genetic diversity for sperm traits through heterozygote advantage.

General discussion

This thesis focuses on the role of ornamentation in female social and extra-pair mate choice, the role of ecological factors on paternity and the influence of sperm length on sperm competition and its heritability. The results indicate that females' social and extra-pair mate choice is not influenced by ornamentation and paternity success is not influenced by large ornaments but by age (**Chapter 1** and **Chapter 2**). Arrival at the breeding site and the progress of the breeding season influence mate choice and the within-pair paternity of males (**Chapter 1** and **Chapter 2**). Sperm length did not influence fertilization success and sperm length expressed phenotypic plasticity during the breeding season and has a moderate heritability (**Chapter 2** and **Chapter 3**).

Mate choice and extra-pair paternity

In birds, males often display large ornaments that are preferred by females and those ornaments should reflect male quality and benefits (direct or indirect) they could provide to females (Andersson 1994). In **Chapter 1**, the appearance of males was manipulated by increasing or decreasing the forehead patch size, a sexually selected trait (Pärt and Qvarnström 1997; Sheldon et al. 1997; Sheldon and Ellegren 1999; Qvarnström et al. 2000). Mating success (paired/ non paired) and the time necessary for pairing (time between arrival at the breeding site and the start of nest building) were estimated. Females do not express a constant preference for the size of this ornament. Males with larger patches are less preferred late in the breeding season. In **Chapter 2**, the secondary sexual traits of males sharing paternity in the same nest were compared. Females expressed no preference for more ornamented males as both extra-pair partners and social partners had the same size range of secondary sexual traits. However, females discriminated across available males as males unable to share paternity are less ornamented. Females demonstrated a preference for older males and males with longer wings. Many studies have demonstrated the preference of females for more ornamented (or dominant) males (Andersson 1994, Danchin et al. 2005).

Male ornaments may not reflect the direct quality of males for females, but they can indicate other behavioral aspects useful for female choice. Secondary sexual traits could provide clues to females about their fertility or parental care they will provide. The phenotype-linked fertility hypothesis states that secondary sexual characters indicate the quality of ejaculate (sperm/ejaculate traits that increase fertilization) (Mautz et al. 2013). This hypothesis is not well supported even though many studies have found a positive trend (Mautz et al. 2013). In our population, forehead and wing patches do not indicate the fertility of males (unpublished data.) Females are probably unable to estimate the fertility of their mates according to their ornaments, but they may be able to obtain enough sperm to fertilize their eggs through multiple matings with social and extra-pair mates. Males should face a trade-off by investing more in reproductive effort or in parental effort (Qvarnström 1999, Magrath and Komdeur 2003).

Secondary sexual traits, if condition-dependent, could indicate the capacity of males to invest in offspring (Gustafsson et al. 1995, Sanz 2001). Attractive males (with large ornaments) should spend more energy to obtain mating opportunities and dispose of fewer resources for parental care. In this case, females may modulate their choice toward males according to their own benefits: select a less attractive social mate and obtain more paternal care. Indeed, more ornamented males are known to invest less in paternal care (Mitchell et al. 2007). So females may trade off genetic quality against higher parental care. This should be important for females to have a social partner who will provide enough parental care to successfully fledge offspring. In our study, males with large ornaments did not fledge more chicks or favor their survival than less ornamented males (**Chapter 1**). However, paternal care, body condition of chicks at fledging and their reproductive success were not tested and certainly deserve more attention.

If females do not choose a social mate for the genetic qualities they will provide to offspring, they can adjust their choice by being involved in extra-pair copulation. Females' indirect benefits could be through "good genes" increasing the viability of all offspring (Zahavi 1975, 1977), "sexy sons" where sons will have an advantage in mating success (Fisher 1930), or compatible genes (Johnsen et al. 2000, Neff and Pitcher 2005, Griffith and Immler 2009).

Taken together, the results of **Chapter 1** and **Chapter 2** do not indicate that females choose partners according to the sexy sons hypothesis. In the sexy sons hypothesis, females are expected to choose a partner with larger secondary sexual traits because they will provide indirect benefits to sons. Sons will inherit large ornaments from their father and this will provide them with an advantage in obtaining partners. But in our studies, females did not prefer males with larger ornaments, despite finding in **Chapter 2** that extra-pair males had longer wing patches than social males; this is a sub-product of age. Older males often express secondary sexual traits more; this is the case for collared flycatchers where older males have longer wing patches than sub-adult males. When only old males (*versus* sub-adult males) were considered, wing patch size was not preferred. So females did not demonstrate preferences in their mates (social or extra-pair mates) according to their secondary sexual ornaments. Moreover, in this Czech population of collared flycatchers, the extra-pair young did not have larger ornaments that within-pair young (Krist and Munclinger 2011), indicating that females do not increase their indirect benefits through the attractiveness of offspring. Moreover, males did not fledge more chicks according to their ornament (**Chapter 2,** Hegyi et al. 2011). If females do not increase their fitness by the sexy sons hypothesis, they may increase their fitness by increasing the viability of all chicks.

In **Chapter 2**, females preferred older males and males with longer wings as extra-pair mates. The age of males is a common trait preferred by females in extra-pair paternity (Sundberg and Dixon 1996, Bouwman et al. 2007, Cleasby and Nakagawa 2012) and may indicate the ability to survive (Evans et al. 2011). Another interesting point is the preference of males with long wings as extra-pair mates. This morphological trait could improve flying ability, which can be essential for long-distance migratory species. Wing length also changes with age as young have different wing shape and shorter wings (Alatalo et al. 1984, Garcia Peiro 2003). If shorter wings provide an advantage for young birds against predation, longer wings are useful for endurance during migration (Alatalo et al. 1984). Long wings can be advantageous for offspring, especially sons, as they could help during their migration and may allow males to arrive first at the breeding site (Alatalo et al. 1984, Perez-Tris and Tellaria 2001). Arriving first at the breeding site can provide advantages to males, first in terms of territories as early arrival could allow males to obtain better territories. In a closely-related species, the pied flycatcher, female choice is expressed not on ornaments (as in this study) but on the quality of the territory (Alatalo et al. 1986). This hypothesis was not tested in the collared flycatcher and deserves to be examined. Moreover, females invest differently according to the arrival date of males. Females mated with males that arrived early lay more, but smaller, eggs compared to females that breed later. Those females have smaller clutches with large eggs (**Chapter 1**). This indicates a different strategy by the females according to the arrival date of males.

In monogamous species, males are constrained by the number of offspring their females can produce. In consequence, males may look for extra-pair copulations to obtain more extra-pair paternity, which for the males is considered an opportunity to increase their reproductive success (Thusius et al. 2001b). The reproductive success of males is then estimated according to the number of offspring they father inside their own nest and in nests of extra-pair females. In **Chapter 1**, the number of within-pair and extra-pair offspring per male was determined. Males did not have more paternity success according to their secondary sexual traits. In **Chapter 2**, the sharing of paternity within nests was estimated and ornaments influenced the loss of within-pair paternity as males with larger ornaments had a higher risk of losing paternity in their nests. This was a surprising result as females should be less motivated to cuckold their mate if they have a large ornament. A first explanation to this result is that females may be harassed by males to obtain extra-pair copulations and may copulate with those males to avoid harassment and possible injuries. In **Chapter 2**, the comparison between the social males, extra-pair males and potential males around the nests seemed to indicate that females were able to choose their extra-pair mates as the potential males were less attractive. This may indicate that females do not suffer from forced copulation by less ornamented males, or at least those males were not able to fertilize the eggs. A second explanation is that males with large ornaments may protect their partner less via mate guarding. Attractive males may spend more time looking for extra-pair copulation opportunities than closely guarding their mates. Moreover, if females are willing to seek extra-pair copulations, mate guarding may become inefficient (Kokko and Morrell 2005).

For males, a trade-off exists between not losing within-pair paternity and winning extra-pair young (Webster et al. 1995, Grunst and Grunst 2014). If males lose more within-pair paternity, they may also be able to obtain more extra-pair young in other nests and compensate the loss in their own nest. In the study in **Chapter 2**, males with large ornaments shared more paternity in their nests than less ornamented males. However, we did not count the number of within-pair young lost. Males with large ornaments may lose within-pair paternity more often in their nest but lost less within-pair offspring (Balenger et al. 2008). But in **Chapter 1**, males with large ornaments did not obtain more paternity as they did not obtain more extra-pair young. This may indicate that males with large ornaments invest more time in finding extra-pair mates, but are able to compensate the loss of within-pair paternity through extra-pair paternity. However, ecological factors may modulate this loss of paternity.

Environmental and population context-dependence of choice

Chapter 1 is the replicate of a study conducted in the Swedish population by Qvarnström et al (2000), where the size of the ornament was manipulated. In her study, she found that females express context-dependent mate choice. In accordance with her study, **Chapter 1** found no preference early in the season but contrary to Qvarnström et al (200), the more ornamented males are not preferred late in the season. On the contrary, they needed more time to pair. Our results are in opposite directions but this supports the hypothesis of context-dependent mate choice. The difference of direction of mate preference late in the season can be explained by a population difference in mate preference in our species.

In the collared flycatcher, sexual selection on forehead and wing patches vary according to the geographical population. In the most studied isolated population of Gotland (Sweden), the forehead patch is condition-dependent (Pärt and Qvarnström 1997, Sheldon et al. 1997, Sheldon and Ellegren 1999, Qvarnström et al. 2000). The forehead is preferred by females and also provides an indication about paternal care (Gustafsson et al. 1995, Qvarnström 1997). In central Europe, the wing patches are condition-dependent and used in both intra- and inter-sexual selection (Hegyi et al. 2002, Török et al. 2003, de Heij et al. 2011). This difference in populations' sexual selection may partially explain our findings of female mate preferences in **Chapter 1**. A change in forehead patch should not reflect the quality of males and females may focus on other clues. Wing patches are involved in extra-pair paternity preferences in the Central European population (de Heij et al. 2011) and **Chapter 2** provides an indication that females base their extra-pair mating behavior on this ornament. The variation of mate choice according to different sexual ornaments in two different populations of the same species may indicate different directions of selection (Robinson et al. 2012) reflecting different environmental conditions and the variation of associated costs and benefits. Female preferences of mate choice are often considered static but this view seems simplistic; they may adaptively adjust their mate choice according to context (Griffith et al. 1999, Qvarnström 2001, Robinson et al. 2012). Preferences could be costly and weakly or strongly expressed according to the costs and benefits (Cotton et al. 2006). If females pay a high cost in choosing a mate, they may express their preference less, but if the benefits of expressing this preference override the costs, females may be choosier. Females may trade off their mate choice between direct and indirect benefits provided by males. In monogamous bird species, paternal care is essential for the survival of chicks. Females may base their choice of social partner on the care they will provide especially if a trade-off exists between pre-copulatory investment and parental care (Qvarnström 1997). Males with large ornaments may invest more in pre-copulatory behavior and dispose of fewer resources to invest in the care of chicks later in the season. Gustafsson (1995) found that paternal investment modified the size of sexual ornaments. Females may prefer slightly less attractive males (with smaller patches) but who provide an indication of their capacity to raise offspring.

The opportunity for extra-pair copulation may be influenced by the opportunity for encounters with suitable males. These encounters may be shaped by the spatial and temporal distribution of males and females. Extra-pair mates are often neighboring males (Smith 1988, Neudorf et al. 1997, Mays and Ritchison 2004, Pedersen et al. 2006) but information about the distribution of suitable males near females is lacking (but see Schlicht et al. 2015). The traits that influence the distance between males that obtain offspring in the same nest are not clear. In **Chapter 2**, the traits of each partner involved in extra-pair paternity were tested to examine whether they influenced the distance between them. Collared flycatchers are territorial birds and secondary sexual traits influence their aggressiveness against intruders (Garamszegi et al. 2006, Hegyi et al. 2008). But we found no influence of the ornamentation of social and extra-pair males on the distance that separated them. The only trait that influenced distance was the wing length of social males. Once again, wing length had a role in extra-pair paternity and the role of this morphological trait in female preference needs to be investigated more precisely. Importantly, this trait may influence migration and protandry.

In migratory birds, males often arrive before the females; this phenomenon is call protandry (Morbey and Ydenberg 2001). Many explanations for this phenomenon have arisen and two principal hypotheses may explain why early arrival and breeding have fitness advantages. The rank advantage hypothesis states that protandry is favored because competition for territories selects males that arrive earlier. Males that arrive first will obtain better territories. Territory quality may be a factor that influences female mate choice (Alatalo et al. 1986). As females do not seem to select males based on ornaments, they may use indications of territory quality to select mates. Male collared flycatchers defend cavities (and nest-boxes) and females may attach importance to the site of nesting and breeding. The choice of a good quality nest cavity could decrease predation and protect the eggs and chicks more efficiently from climatic variation. So females may indirectly select males that arrive earlier. Another advantage of protandry is related to mating opportunity. This hypothesis states that early-arriving males will obtain more opportunities for mate competition and increase their chances for extra-pair paternity. In **Chapter 1**, the arrival of mates did not increase the chance to mate, the speed of mating, or the within- and extra-pair paternity. However, early arrival in **Chapter 2** reduced the chance to share paternity, supporting the hypothesis that early arrival provided some benefit in terms of within-pair paternity (Møller et al. 2003). In consequence, early arrival may not favor extra-pair paternity but could be a mechanism to protect within-pair paternity, especially if the probability to lose paternity increases with the advance of the season. The risk and/or intensity of the sperm competition can be different according to the advancement of the breeding season. Sperm competition is known to influence sperm traits and the outcome of fertilization.

Sperm competition and heritability of sperm length

Before the rise of sperm competition studies, in monogamous species, males were expected to provide enough sperm to fertilize eggs. However, with sperm competition occurring in the female genital tract, males should provide not only enough sperm but also “victorious” sperm that will be able to outcompete other sperm to fertilize the eggs. Among many sperm traits that influence the outcome of sperm competition, sperm length seems to increase fertilization success (Bennison et al. 2015). In **Chapter 1**, the effect of sperm length on paternity success was estimated for a single breeding season. No effect of sperm length on within-pair or extra-pair paternity was found. Males with longer sperm did not improve their total fitness. As no impact of length was found in **Chapter 1**, it may indicate that length only does not determine the outcome of sperm competition. In a recent study, males that were more successful in fertilizing females were not the males with longer spermatozoa but those with spermatozoa with the longest midpiece relative to the flagellum (Knief et al. 2017). The relative size of each sperm section may be a better proxy for estimating successful sperm rather than total length. Other factors like viability (Smith 2012) or number (Laskemoen et al. 2010) should improve the success of fertilization when sperm compete. Moreover, in **Chapter 3**, spermatozoa were longer during the feeding of the chicks, when the chance to fertilize females was lower, indicating that spermatozoa successful in fertilization are shorter. In **Chapter 3**, spermatozoa were sampled at two stages of the breeding season and demonstrated phenotypic plasticity during the breeding season. Spermatozoa were shorter early in the breeding season. Phenotypic plasticity according to season seems to be common even though the direction is not the same as what we found (Calhim et al. 2009; Lüpold et al. 2012; Cramer et al. 2013, Schmoll, Kleven and Rusche, submitted). The dynamic of spermatozoa change across the breeding season may correspond to a maturation delay. Males do not produce sperm while wintering in Africa and start producing sperm during migration (Bauchinger et al. 2007). In many migratory species, such as the collared flycatcher, males arrive first at the breeding area, and females follow a few days later (Rubolini et al. 2004 but see Briedis et al. 2016). Males should be ready to fertilize females when arriving at the breeding site. Males should face a trade-off in sperm production: size *versus* number of spermatozoa. Males may produce a lot of sperm that will drown out the sperm of other competitors or produce longer sperm that can reach the egg first (Immler et al. 2011). Spermatogenesis takes time (about two weeks, Aire 2007) and is not as cheap as commonly expected (Pitnick 1996). Males are not capable of overproducing spermatozoa, especially if longer spermatozoa are more expensive to produce. So males may produce short and numerous sperm or longer but less numerous spermatozoa. Early in the breeding season, males need to copulate a lot with their social (and extra-pair) partners to be able to fertilize their mates' eggs. In a short time, they may produce a lot of sperm able to fertilize eggs successfully. In **Chapter 3**, we found that males may produce a lot of immature, therefore smaller, spermatozoa. Spermatozoa may not reach their maximum length, and therefore efficiency, before being expulsed in an ejaculate. Later in the breeding season, during the feeding period of the chicks, males should have less opportunity to copulate, allowing sperm to mature and consequently to be longer. The trade-off between the number of sperm and the length of sperm is known and birds seem to allocate in favor of sperm number rather than length (Immler et al. 2011). If longer sperm could be advantageous for males in some cases, waiting for spermatozoa to reach their optimal length may lead to a risk of decreased opportunities for fertilization when sperm competition is high. It may be dangerous for males to wait for sperm to increase in efficiency when other males copulate with females and it also increases the risk of missing the females' fertile period. If fertilization efficiency is due to the number and not size of sperm, this could explain why longer sperm did not influence paternity success in **Chapter 1**. Moreover, it was more difficult to find spermatozoa (at least 20) in the experimental ejaculates late in the breeding period (personal observation), indicating that the production of sperm starts declining in June. The genetic and environmental components influencing this variance are unclear and were the subject of the last chapter of this thesis.

Quantitative genetics allow the disentangling of the roles of genetics and the environment in the variation of a trait. The measurements of sperm length were matched to a pedigree, which allowed us to obtain values for additive genetic variance and environmental variance in **Chapter 3**. The additive genetic variance was moderate relative to permanent environmental and residual variance, and the heritability was also moderate (44%). In other species, the heritability of sperm length is higher (Ward 2000, Morrow and Gage 2001, Birkhead et al. 2005, Baer et al. 2006, Mossman et al. 2009). Many factors could explain the moderate heritability: environmental conditions and the selection acting on this trait through sperm competition.

Almost all studies of the heritability of sperm length were conducted in captive species. If the environment (especially a stressful environment) has strong effects on variance, as our results suggest, the unnatural conditions (very consistent through time and not resource-limited) may have biased the results upward (Charmantier and Garant 2005). Charmantier and Garant (2005) suggested the variation of heritability could be due to a decrease of Va or an increase in environmental effect.

Va could decrease throughout unfavorable conditions due to the inability to express genetic potential under constrained growth conditions. Moreover, environmental variance (or residual variance) could increase as the environment will be more determinant under stressful conditions than genetic effects. In consequence, for species in captivity living in a comfortable environment, the local conditions could decrease environmental variance and let the genetic component be more expressed. **Chapter 3** is the first study that may reflect the real strength of the environment on sperm traits and consequently lower heritability compared to other studies.

Spermatozoa are expected to be under stabilizing selection (Lifjeld et al. 2010) and stabilizing selection decreases the genetic additive variance (and heritability). Indeed, fitness and traits closely related to it have lower heritability (Gustafsson 1986, Houle 1992, Kruuk et al. 2000, Teplitsky et al. 2009). This can be explained by the strong selection acting on them. Variation in fitness should be low because genes beneficial to fitness should have reached fixation and so express no variation (or little), and deleterious genes for fitness should have been lost. Our results suggest this is true, as the Va is moderate compared to other morphological traits and the same trait in other species. In the collared flycatcher, the high rate of extra-pair paternity (about 25%) leads to intensive sperm competition. If longer sperm length was under strong pressure, the longest spermatozoa should be more successful at fertilization and reduce the variation around the mean size of sperm (stabilizing selection). This should lead to a decrease in genetic additive variance as alleles increasing size should be increasing in the population. More alleles decreasing size should be lost as it would reduce the chance of fertilization success. However, sperm length did not influence paternity in our population (**Chapter 1**) indicating that stabilizing selection is not currently acting strongly. However, it should have shaped sperm length earlier in the evolutionary process.

In birds, only two studies have focused on sperm length heritability in the zebra finch (*Taeniopygia guttata*) (Birkhead et al. 2005, Mossman et al. 2009). This species is known to have low extra-pair paternity in the wild. The pressure of the selection on sperm traits should be less intense and erode the additive genetic variance less. Moreover, the phenotypic diversity of sperm length and high genetic variation in the zebra finch could be explained by a heterozygote advantage of the pool of genes coding for sperm length (Fisher 2017, Kim et al. 2017, Knief et al. 2017). A large inversion on the Z-chromosome shapes sperm with medium-sized flagella but long midpieces. Males with the heterocaryotype have the advantage of long flagella and long midpieces compared to the homozygous males, and are consequently more successful in fertilization. This heterozygote advantage maintains a high phenotypic variance of sperm length in the zebra finch. In the collared flycatcher, even though the genome is sequenced, the genes coding for sperm morphology are unknown (Ellegren et al. 2012, Kawakami et al. 2014).

General conclusion and future research

General conclusion

The aim of this thesis was to understand how females and males increase their fitness through two steps of sexual selection. Male collared flycatchers with large ornaments are not preferred by females as mates or extra-pair mates, rather females prefer old males and males with longer wings. Extra-pair paternity success is not influenced by the size of secondary sexual traits, but ornaments influence within pair-paternity. Age is the main factor influencing the outcome of extra-pair paternity; this indicates that females may obtain good genes from their extra-pair mates. Females demonstrate seasonal variation in mate preference, supporting the theory of context-dependence of mate choice. This could reflect a population difference in collared flycatchers but also a change in costs and benefits during the breeding season. Moreover, spermatozoa length does not influence paternity success, and expresses phenotypic plasticity and moderate heritability.

Future research and perspective

As female mate choice is plastic, the role of the environment (seasonal and/or social) needs to be further investigated to better understand the process of sexual selection in changing environments. If sperm traits are an important factor for fertilization, the lack of knowledge on the environment in the female genital tract where this competition takes place could lead to biased conclusions. Indeed, the studies trying to identify sperm traits that make them successful in fertilization focus on sperm traits before the bottleneck of cryptic female choice. Further study on the effect of the environment on sperm length is needed to understand the variation in heritability and whether the variation in heritability is due to an increase in environmental variance or a decrease in genetic additive variance. The genomes of many species have been sequenced but the genes coding for sperm morphology are largely unknown. More knowledge about the genes coding for these traits will help in our understanding of the genetic basis of sperm phenotypic diversity and may be the mechanism that could maintain additive variance at the genetic level like the heterozygous advantage in the zebra finch.

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Chapter 1: Do ornaments, arrival date, and sperm size influence mating and paternity success in the collared flycatcher?

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ORIGINAL ARTICLE

Do ornaments, arrival date, and sperm size influence mating and paternity success in the collared flycatcher?

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Abstract

Males advertise their intrinsic parental and/or genetic qualities by the size of secondary sexual ornaments. Moreover, they compete with one another for the best territory and males who arrive first at the breeding ground usually have an advantage in this competition. Females may consider multiple male qualities simultaneously and prefer the one most important for their fitness in the current context. They can further improve their fitness by selecting the best care-giver as their social mate and engaging in an extra-pair copulation with a genetically superior male. In such cases, sperm competition arises in the female reproductive tract and its outcome may be affected by the sperm morphology of both the social and extra-pair male. Here, we tested these ideas in the collared flycatcher (*Ficedula albicollis*), a species with context-dependent choice of social partners and frequent extra-pair paternity. We recorded male arrival to breeding sites, manipulated their forehead patches, and measured sperm size. In contrast to a previous study in a Swedish population, males with enlarged patches were non-significantly less successful late in the season while no such difference was found early in the season. Besides this tendential seasonal interaction, arrival date did not affect mating and paternity success or male fitness, and the same was true for sperm size. These results suggest different benefits of male ornamentation and female mate choice between populations and call for more replicated research within and between species.

Significance statement

The fitness of a male of a migratory species might be affected by several pathways. First, early arrival should confer benefits in the form of best territory choice. Second, in a dichromatic and sexually promiscuous species, secondary sexual ornaments are considered by females both in the choice of social and extra-pair mates. Third, by modifying sperm traits, males may outmatch their rivals in sperm competition. These ideas have usually been tested in isolation. In this experimental study, we tested the joint effect of all of these factors on the genetic fitness of males. We found little evidence for the dependence of male reproductive success on either sperm morphology or plumage ornamentation which is in contrast to other populations of the species. Our study calls for replicated research both in well-established fields like mate choice and emerging ones like sperm competition.

Keywords Mating success · Extra-pair paternity · Differential allocation · Sexual ornament · Sperm size

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Introduction

Many studies have shown that females have preferences for males with more elaborate secondary sexual traits such as more diverse songs (Gentner and Hulse 2000; Drăgănoiu et al. 2002) and larger sexual ornaments (Sheldon et al. 1997). Females may have preferences for those males with superior traits because males will provide either direct benefits such as parental care or indirect genetic benefits to the offspring (Andersson 1994).

However, female mating preferences may vary according to the context (Qvarnström 2001). Indeed, females have displayed differences in preference according to social context (such as presence or absence of competitors) (Callander et al. 2012), environmental conditions (Hale 2008), and timing of breeding (Qvarnström et al. 2000). This last factor can be particularly important for migratory birds that are constrained by their migration schedule. It is common that males arrive earlier than females (Møller 2004; Tottrup and Thorup 2008). Early arrival allows males to settle on the best territories (Aebischer et al. 1996) and also to obtain females more easily as females may also use territories as cues for mate selection (Alatalo et al. 1986). Among males arriving at the same time, those with brighter and/or larger plumage ornaments usually win competitions for territories (Pärt and Qvarnström 1997; Beck 2013). Therefore, females can choose these highly ornamented males to have access to necessary resources like nest sites or food. However, choosing dominant mates at the start of the season may also be costly as such males often try to attract a secondary or extra-pair female instead of caring for the primary nest (Qvarnström 1997, 1999). Consequently, it may be advantageous to only choose males with larger ornaments later in the season as their chances to find another mate are low at that time, and thus, they are expected to invest more in the feeding of nestlings (Qvarnström et al. 2000).

In addition to the choice of social mate, females might use several other mechanisms to increase their fitness. First, they may be unfaithful to their social mate. Extra-pair young obtained with another male of superior quality may be of higher quality compared to within-pair young (Akçay and Roughgarden 2007, but see Krist and Munclinger 2011). Many studies show that females cuckold their mates with older males (Cleasby and Nakagawa 2012) and more ornamented males (Kempenaers et al. 1992; Richardson and Burke 1999; Akçay and Roughgarden 2007), though the role of ornaments remains controversial (review in Akçay and Roughgarden 2007). In contrast to female preferences for social mates, which may be context-dependent due to the trade-off between the direct and indirect benefits of mate choice (Qvarnström 2001), preferences for extra-pair males with large ornaments may be consistent during the course of the season as these males can provide only genetic benefits, and thus, there is no trade-off with their paternal care even at the start of the season.

Extra-pair copulations are a prerequisite for another process that has come to the center of attention of ecologists in recent years. Whenever females copulate with more than one male, different ejaculates compete to fertilize the eggs, which is known as sperm competition. Many factors may influence the success of sperm in fertilizing eggs: the timing of copulation (Birkhead et al. 1989), the frequency of copulation (Møller and Birkhead 1993; Mougeot 2004), and sperm traits (Snook 2005). Among these sperm traits, viability (Smith 2012), speed of swimming, (Birkhead et al. 1999), number (Laskemoen et al. 2010), and size of the sperm (Lifjeld et al. 2010; Bennison et al. 2015) may modulate the success of egg fertilization.

Although it has previously been shown that male arrival date (Aebischer et al. 1996), secondary sexual ornaments (Sheldon and Ellegren 1999), and sperm size (Bennison et al. 2015) can have fitness effects, these factors were usually tested in isolation which complicates the evaluation of their relative importance. One remarkable exception is the study of Qvarnström et al. (2000) that tested how benefits of female choice of male ornaments depend on the time of male arrival to the breeding ground. However, this study did not take sperm competition pathways of sexual selection into account. Here, we tested the effects of male ornamentation, arrival time, and sperm morphology on their ability to sire offspring and gain fitness.

We studied these questions in the collared flycatcher (*Ficedula albicollis*), a migratory bird in which males arrive on the breeding grounds before females. Males of this species display two white patches, one on the forehead and the other on the wing, that have been found to be sexually selected in Swedish population (e.g., Sheldon and Ellegren 1999; de Heij et al. 2011). However, there may be differences in the strength of sexual selection between populations. For example, large forehead patch has been found to be preferred in extra-pair mates in the Swedish (Sheldon et al. 1997; Sheldon and Ellegren 1999) but not Hungarian (Rosivall et al. 2009) or Czech (Edme et al. 2016) populations. This calls for replicative research both within and between populations to test if the differences between studies really represent differences in the strength of selection between populations, which would have important consequences for the evolution of the species (see Scordato and Safran 2014) or if they are merely caused by sampling variance.

Methods

Study site and species

This study was carried out in an oak forest with approximately 350 nest boxes that are distributed among five study plots in Velky Kosir (49°32′N, 17°04′E) in Moravia, Czech Republic, from 2013 to 2015. Collared flycatchers (*F. albicollis*) are migratory passerine birds, and males arrive first (around mid-April) at the breeding site to obtain territories. Males are black with one white patch on the forehead and another on their wing feathers. Females selected their social mates based on both white ornaments in a Swedish population (Sheldon and Ellegren 1999; de Heij et al. 2011) and usually lay between four and eight eggs after pair bond formation. Chicks can hatch asynchronously as females start to incubate before the completion of the clutch. Both parents feed the chicks.

Adult measurements and forehead patch manipulation

In 2013, the first male arrived on April 15, and we started to trap males the following day. In total, we trapped males on 12 different days between April 16 and May 15. Each trapping day, we captured males in all empty or abandoned nest boxes with string nest box traps. We did not activate traps in nest boxes where nest material appeared unless these were apparently abandoned for several days (i.e., no progress in nest building). For individual males, we considered the first day of capture as their date of arrival. Our trapping scheme was highly efficient as the first day of capture was highly correlated ($r = 0.96$) with true arrival date as inferred from 16 males bearing geolocators in 2015 (M. Briedis et al., unpublished data). Immediately after each new male was captured, it was brought to the central site located among study plots. This transfer lasted up to half an hour.

At the central site, body mass, wing length, and tarsal length were measured. The wing patches were determined by summing the visible length of white patches on primaries 3 to 8 from the tip of the coverts to the distal part of the wing (in mm). All of these measurements were done by one person (MK). A blood sample was taken from the tarsal vein and stored in alcohol. A cloacal massage allowed us to obtain a sperm sample (see Quay 1986), which was stored in 4% formaldehyde. The age of males was determined by wing plumage as subadult males have brownish primaries. The forehead patch area was photographed two times before and another two times after the manipulation. The original patch size was computed as the mean of the two measurements before the manipulation delimited to the nearest 0.1 mm^2 in ImageJ software.

We regularly rotated among three treatments: (1) we increased the height of white forehead patches by painting black feathers with a white marker (Alteco Paint Marker no.15). Using this technique, the size of the white patch was enlarged by ca. 50% (Table 1, Supplementary Online Material). We decided to use Alteco markers instead of Tippex used in former studies (e.g., Qvarnström et al. 2000) since they proved to be more durable during our pre-experimental manipulation done on caged zebra finches. Tippex usually started to erode within a week of manipulation, while Alteco still looked good after 7 days. Both Alteco and Tippex have similarly shaped reflectance curves that differ from natural white feathers. At low and high wavelengths, natural white reflects more than Tippex and especially Alteco (see Fig. 1). (2) Control birds were only measured and then released without any manipulation of the forehead patch. (3) We decreased the height of the white forehead patch to about half (Table 1, Supplementary Online Material) by painting it with a Copic 110 special black marker that has previously been used in flycatchers (de Heij et al. 2011). This manipulation resulted in naturally low reflectance (Fig. 1) but started to fade within a few days of manipulation. Our rotation scheme led to a random distribution of treatments among plots as indicated by a non-significant relationship between plot and frequency of treatment ($\chi^2 = 9.23$, $p = 0.324$, df = 8, $n = 73$).

Because our manipulations were relatively short-term, they could mainly affect processes at the start of the breeding season like female choice of social partners, which usually takes place during the days after arrival to breeding sites (8 days for control males on average; see Table 1, Fig. 2). However, they might be less effective for female choice of extra-pair partners which might continue for a long time after males are socially mated, although most extra-pair copulations likely take place early in the female fertile period (Krist et al. 2005; Krist and Munclinger 2011) which peaks 2 days before laying of the first egg (Lifjeld et al. 1997). In the nests attended by our control males, laying started 6 days after social mating, i.e., 14 days after male arrival.

After manipulation, males were released on the same plot as they were caught. We caught the males a second time during the feeding period, and the same measurements were taken as well as blood and sperm samples. Females were also caught during the feeding period and were measured in the same way as males except for the forehead patch.

Monitoring of reproductive success

Nests were checked daily when the first egg was expected after nest building. Each egg was marked to obtain the laying order. The width and length were measured with digital calipers (±0.01 mm). The volume of the egg was calculated as volume = 0.51 × length × width^2 (Hoyt 1979). When females ended the laying sequence and began incubation, we stopped the daily checks and started once again when the hatchlings were expected (around 10 days after the last egg was laid). A blood sample was obtained from chicks 6 days after hatching, and their fate was monitored until fledging. Unhatched eggs were collected 4 days after the last chicks hatched, and embryos were stored in ethanol, as were all of the other chicks found dead before day 6. Blood and tissue samples were used for paternity analyses.

In 2014 and 2015, we captured all of the males at arrival and both sexes during the breeding season, so we were able to

Table 1 Summary of means ± SD for different traits according to patch treatment

	Patch treatment		
	Decreased	Control	Enlarged
Number of males manipulated	51	50	52
Number of males breeding	27	25	21
Polygynous males	1	3	1
Number of males used in most analyses	24	23	20
Arrival date (Julian day, January 1 = 1)	112.3 ± 5.7	112.7 ± 7.0	113.8 ± 5.4
Mating speed (days)	10.1 ± 7.29	8.0 ± 4.01	11.4 ± 8.0
Forehead patch before treatment (mm^2)	58.7 ± 7.4	56.1 ± 11.7	57.1 ± 8.6
Forehead patch after treatment (mm^2)	29.4 ± 5.8	56.1 ± 11.7	85.9 ± 12.8
Wing patch (mm)	50.8 ± 15.5	58.9 ± 12.6	49.2 ± 11.1
Clutch size	6.16 ± 0.81	6.21 ± 0.85	5.75 ± 1.16
Egg volume (mm^3)	1658 ± 131	1603 ± 135	1604 ± 121
Number of WPY	4.65 ± 1.52	4.81 ± 2.10	4.29 ± 2.22
Number of EPY	1.43 ± 1.61	1.59 ± 2.21	1.47 ± 1.69
Total paternity	6.08 ± 2.48	6.40 ± 3.48	5.76 ± 2.30
Number of fledglings	5.08 ± 2.24	5.47 ± 2.76	4.05 ± 2.72
Number of recruits	1.00 ± 1.17	1.65 ± 1.15	1.05 ± 1.39

Sample size was 67 males except for number of WPY, number of EPY, and total paternity, which were based on $n = 62$. For polygynous males, only their primary nests were considered for the calculation of means. The exceptions were the variables "number of extra-pair young" and "total paternity" that also included young sired by the polygynous males in their secondary nests

count the number of recruits as all the chicks were ringed during the field season in 2013. We did not record whether those recruits bred during those 2 years, but only their survival since fledging. So our recruitment data concerned the number of chicks who survived and were able to come back to our field area. Despite natal fidelity being relatively high in our study area (Krist 2009), some individuals surely dispersed and thus our estimate of recruitment represents only the lower limit of the real value.

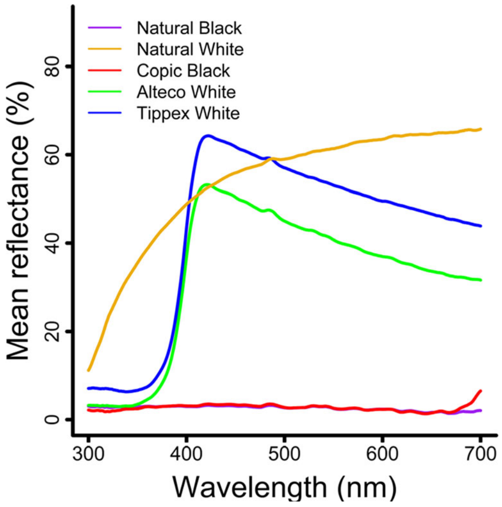

Fig. 1 Reflectance of primaries of adult males before and after coloration with black or white markers. Five measurements were taken from the feathers of two males, and the lines are averages of these five measurements. The reflectance of primaries of adult males likely closely reflects that of their foreheads but the former was easier to measure on dead birds that were available before breeding season. These dead birds were killed by great tits that destroyed their foreheads

Genotyping and parentage assignment

DNA extraction was performed with DNeasy® Blood & Tissue Kit (Qiagen) for blood samples and tissue from dead

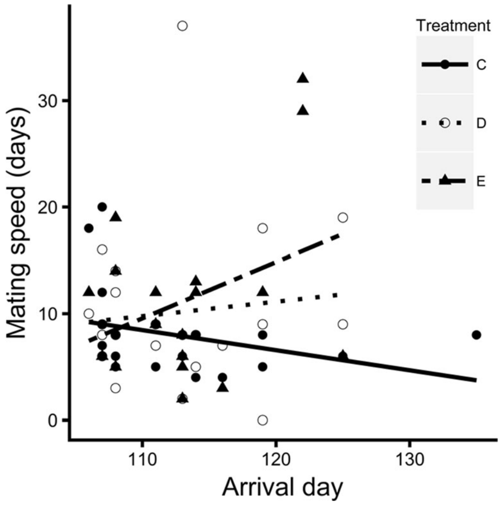

Fig. 2 Relationship between arrival date and mating speed for the three treatments of forehead patch size. Control treatment, *solid circles* and *solid line*; decreased treatment, *open circles* and *dotted line*; enlarged treatment, *triangles* and *dashed line*

embryos and chicks. All of the samples were genotyped at eight polymorphic microsatellite autosomal loci: Fhu2 (or PTC3) (Ellegren 1992), Cuμ04 (Gibbs et al. 1999), Fhy310, Fhy405, Fhy407, Fhy428, Fhy431, and Fhy452 (Leder et al. 2008). A single multiplex PCR using fluorescently labeled primers and a Type-it® Microsatellite PCR Kit (Qiagen) were used to amplify the microsatellites. The samples were treated with the following reaction conditions: 5 min at 95 °C, then in 30 cycles of 30 s at 95 °C, 90 s at 65 °C, 30 s at 72 °C, and finally 30 min at 60 °C. PCR products were mixed with GeneScan™-500 LIZ® Size Standard (Applied Biosystems) and analyzed with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). GeneMarker® version 1.9 was used to score the genotypes, and locus characteristics based on allele frequencies were obtained with Cervus 3.0.3 (Kalinowski et al. 2007).

We obtained the genotypes of 262 adults (104 females and 158 males). For the first parent, the combined non-exclusion probability for that group of loci was found to be 7.03×10^{-4}. We only considered the individuals that were genotyped at five loci or more for parental analysis. When female genotype was known, we compared it with its chick genotypes to check for egg dumping. One chick did not correspond to its social mother and was excluded. Secondly, when the social male was known, we compared the genotype of the male with the chicks he fed. If trio confidence (female-social male-chicks) based on Delta (difference in overall likelihood ratio scores between the most likely candidate parent and the second most likely candidate parent) and simulations of parentage was superior to 95%, we considered the chicks to be within-pair young. In cases where the mother was unknown, we took into account the duo confidence (male-chicks) with the same criterion. All chicks that were not assigned as within-pair young were classified as extra-pair young. Finally, we tried to determine the males who sired the extra-pair young. We selected all the males from the breeding season and compared their genotypes with the extra-pair chicks using the same criterion of 95% trio or duo confidence.

Sperm analyses

Two hundred and forty-two sperm samples were stored in 4% formaldehyde (152 from males at arrival and 90 during the feeding period) either at room temperature or at 8 °C in a refrigerator. We created slides for microscopy by spreading 7 μl of a sperm sample and letting it dry. The slide was then carefully rinsed with distilled water in order to remove dirt and salt crusts and air-dried again. For each sample, 20 pictures of morphologically normal-looking sperm were taken at ×400 magnification under light-field conditions using an Olympus CX41 microscope equipped with an Infinity 2 camera. If 20 sperms were not found on the first slide, a second slide was prepared. If after those two slides, no sperm at all was found, we did not prepare a third slide. For samples where the number of sperm was between 1 and 19 sperms after two slides, an ultimate slide was analyzed to complete the number of sperm pictures. We obtained 130 samples with the required number of sperm at arrival and 39 at feeding. Heads, mid-pieces and tails were measured (μm) in ImageJ software 1.49v (see Laskemoen et al. 2010). All of these measurements were done blindly by one person (PZ). Total sperm length was calculated by adding the three parts. Mean sperm length was calculated for each male.

Statistical analyses

All statistical analyses were conducted in RStudio, version 0.99.878 (R Core Team 2014), and we used the “lm” or “glm” function from the package “Stats” (R Core Team 2014). Since males were trapped at arrival ($n = 153$) and recaptured during the feeding period ($n = 73$), it was possible to identify those who were successful at pairing and establishing a nest in that particular season. To test this, we fitted a generalized linear model with a binomial link function (glm function from Stats package in R). The response variable was mating success (obtaining a nest: yes/no), and the predictors were the arrival date, original forehead patch size, wing patch size, the relative age (adult/subadult), and treatment (enlarged, decreased, and control). We also tested the interaction between arrival date and treatment, as the effect of ornament manipulation was found to be dependent on the time of the season in a previous study (Qvarnström et al. 2000). When this interaction was non-significant, it was removed from the final model. Continuous predictors in our models (i.e., male arrival and size of original forehead patch, wing patch, and sperm length) were not strongly intercorrelated (all r between −0.4 and +0.4, $n = 63$), indicating that multicollinearity was not a serious problem.

Another factor that we were interested in was mating speed. We calculated this as the time between male arrival date and the start of nest building by its social female. Six out of 73 males presented a negative value for the time lapse between those two dates, indicating that we trapped them well after their arrival. These males were trapped during the searching of secondary nest sites after they had started to breed in their primary nest box. We excluded them from all analyses. Five out of 73 breeding males were polygynous, and their secondary nests were not considered in analyses of mating speed, clutch size, and egg size. So in total, 67 manipulated and breeding males were used for most of the analyses. The mating speed ranged from 0 to 37 days (see also Table 1). A linear model was run, where the response variable was mating speed and the predictors were the same as in the model for mating success.

We also tested whether females changed their early reproductive effort in respect to male secondary sexual traits, as is predicted by the theory of reproductive allocation (Sheldon 2000; Horváthová et al. 2012). In the first model, we looked at the number of eggs the female laid. A linear model was run on the clutch size, as it had a better fit than the alternate Poisson model, and residuals from the linear model were normally distributed. The response variable was clutch size, and the predictors were the same as in the model for mating success. Second, we looked at the volume of the eggs; a linear model was run with mean egg volume as the response variable and the same predictors as in the model for mating success.

We added sperm length among predictors of the models testing for paternity success. We used the male sperm length measured at arrival. For five males, we obtained sperm only for the feeding period. As we had the mean size at arrival and feeding for 28 males, we calculated the difference between the mean sperm size at arrival (mean ± SD; 96.7 ± 3.20 μm) and the mean size at feeding (98.2 ± 2.30 μm) and subtracted this difference from the size at feeding for those five males without arrival data. In this way, we extrapolated the size of the sperm at arrival for those males. The results would be very similar if these males were excluded from the analyses. For another five males, we did not obtain enough sperm either at arrival or during breeding, and therefore, we excluded them from this analysis that was consequently based on 62 males.

The total paternity success of a male can be separated into two parts: the within-pair paternity in the social nest and the extra-pair paternity obtained in other nests. We first looked at the within-pair paternity with a generalized linear model with a quasi-binomial distribution and event/trial syntax for the response variable. In consequence, the response variable was the number of within-pair young (event) according to the clutch size (trial). In addition to predictors used in the model for mating success, we added mean sperm length and its quadratic term to all three models testing for paternity success. We added the quadratic term into models to test for the possibility of stabilizing selection on sperm size (Lifjeld et al. 2010). The extra-pair paternity was analyzed by a generalized linear model with a quasi-Poisson distribution. The response was the number of extra-pair young that males sired in all other nests in the nest box population ($n = 119$ nests with genotyped offspring). The predictors were the same as in the preceding model. As in all other models except of that for mating success, we tested only success of males breeding in our nest boxes. For five identified polygynous males, we included the number of young they sired in their secondary nests to their extra-pair success. This was done to be equivalent to cases where polygynous males were not identified at all as they did not feed their secondary nests. By this method, extra-pair success was overestimated while within-pair success was underestimated for polygynous males. Nevertheless, the results would be closely similar if five identified polygynous males were excluded from this model (results not shown). Moreover, this slight inadequacy did not affect the model of male total paternity because in this model the two paternity components were summed together. The model for total paternity was the same as for extra-pair paternity except for the response variable that was the total paternity. Our estimates of male extra-pair and therefore also total paternity success only reflect the lower limits of the real values since focal males might also sire offspring in natural cavities, i.e., outside our genotyped nest box population.

The number of fledglings and recruits is reflective of male fitness, so we ran two other models with the number of male genetic offspring that fledged as a response in the first model and the number of genetic offspring that were recruited (in 2014–2015) in the second model. For both models, the predictors were the same as in the model for mating success.

Results

During the arrival period, 160 males were trapped and 153 were involved in the patch manipulation experiment (52 increased, 51 decreased, and 50 for control). Seventy-three of them were recaptured when they were feeding chicks. Five of them were polygynous. We excluded secondary nests of polygynous males from analyses of mating speed, clutch size, and egg size. We also excluded six males that were caught a long time after their arrival (see "Methods" section). Consequently, our sample size for most analyses was 67 breeding males. In all models testing for paternity success, our sample size was reduced to 62 males due to missing sperm samples from 5 males. In these 62 nests, 286 within-pair young were sired by social and 67 by extra-pair mates. These 62 social males also sired 93 offspring outside their primary nests.

The males involved in our treatment arrived on average on 112.9 ± 6.0 (mean ± SD) Julian day (April 23) and required about 9.7 ± 6.6 days to pair (see Table 1 for more details). Females laid on average 6.06 ± 0.95 (mean ± SD) eggs, and the mean volume of the eggs was 1623 ± 130 mm^3 (Table 1), with an average of 4.91 ± 2.60 chicks fledging from each nest (Table 1). We recaptured 83 of the nestlings in 2014 and 2015. The mean ± SD number of recruits per nest was 1.24 ± 1.26 (Table 1).

None of our main variables (arrival date, original size of male ornaments, and their experimental treatments) significantly affected male mating success (Table 2), although males with enlarged patches (21/52 = 40.4%) had a non-significantly lower mating success compared to the control group (25/50 = 50%) and males with reduced patches (27/51 = 52.9%). Similarly, males in the enlarged treatment had non-significantly lower mating speed than males in the other two treatments (Tables 1 and 2), and this seemed to be true mainly late in the season (Fig. 2), although the interaction between treatment and arrival date was marginally non-significant

Table 2 Models for mating success ($N = 153$) and the speed of mating ($N = 66$)

	Mating success			Mating speed		
	Estimate ± SE	F	P	Estimate ± SE	F	P
Intercept	−1.83 ± 3.52			−5.37 ± 19.5	0.08	0.784
Arrival date	0.001 ± 0.026	<0.01	0.954	0.079 ± 0.147	0.29	0.598
Forehead patch size	−0.0002 ± 0.0173	<0.01	0.990	−0.026 ± 0.098	0.07	0.791
Wing patch size	0.029 ± 0.202	2.04	0.155	0.094 ± 0.111	0.72	0.400
Age	0.906 ± 0.858	1.07	0.303	4.85 ± 4.64	1.10	0.299
Treatment		0.82	0.445		1.61	0.207
Treatment decreased	0.169 ± 0.410			2.38 ± 2.08		
Treatment enlarged	−0.344 ± 0.404			4.07 ± 2.28		

($p = 0.10$). We did not find any evidence for female pre-hatching differential allocation since neither egg size nor clutch size differed between treatments (Tables 1 and 3). Male success in sperm competition was not affected by their arrival date, size of original forehead patch, experimental treatment, or sperm size (Table 4, Fig. 3). Finally, we also did not find a significant effect of any predictor on male fitness as determined by the number of fledglings and recruits, although males in the enlarged treatment had somewhat poorer performance compared to those in reduced and especially control treatments (Tables 1 and 5).

Discussion

We found several lines of evidence suggesting that males in the enlarged treatment of forehead patch size might have inferior breeding performance compared to males in control and reduced treatments. They had lower mating success, it took them longer to pair, especially late in the season, and their fitness as measured by clutch size and number of fledglings and recruits was also lower than in the other two treatment groups. However, although these effects were visible in the difference between means (Table 1), they were also highly variable, which caused them to be statistically non-significant, despite the fact that we involved the whole nest box population in our experiment and thus had a sample size comparable to many previous studies.

The manipulation of male attractiveness is a common type of experiment when studying mate choice, female investment, and paternity (Mazuc et al. 2003; Grana et al. 2012; Horváthová et al. 2012). Manipulations of ornaments in the collared flycatchers were previously done in the isolated Swedish population on the island of Gotland (Qvarnström 1999; Qvarnström et al. 2000; de Heij et al. 2011). Here, we partly replicated the forehead patch size manipulation from the Qvarnström et al. (2000) study in a Czech population of collared flycatchers. Qvarnström et al. (2000) found that females only preferred males with enlarged patches late in the season while having no strong preferences early in the season. We did not find a statistically significant interaction between pairing latency and experimental treatment of the forehead patch. If anything, there was an opposite tendency. Females in our population did not show any preferences early in the season but tended to prefer control males and males with decreased patch sizes over enlarged ones later in the season. There are several potential explanations for these different results.

First, it may be due to the type of white markers used in the experiment. We used a white paint marker (Alteco) while

Table 3 Models for clutch size ($N = 67$) and egg volume ($N = 66$)

	Clutch size			Egg volume		
	Estimate ± SE	F	P	Estimate ± SE	F	P
Intercept	16.6 ± 2.31	51.8	<0.001	917 ± 356	6.63	0.012
Arrival date	−0.091 ± 0.017	27.4	<0.001	6.67 ± 2.68	6.20	0.015
Forehead patch size	−0.001 ± 0.011	0.03	0.871	−3.40 ± 1.82	3.50	0.066
Wing patch size	−0.0007 ± 0.013	<0.01	0.957	2.03 ± 2.07	0.96	0.331
Age	0.428 ± 0.552	0.60	0.440	71.6 ± 85.3	0.70	0.404
Treatment		1.11	0.335		2.35	0.104
Treatment decreased	−0.133 ± 0.247			76.5 ± 38.6		
Treatment enlarged	−0.391 ± 0.267			15.3 ± 41.8		

Table 4 Models for within-pair, extra-pair, and total paternity ($N = 62$)

	Within-pair paternity			Extra-pair paternity			Total paternity		
	Estimate ± SE	*F*	*P*	Estimate ± SE	*F*	*P*	Estimate ± SE	*F*	*P*
Intercept	−1.67 ± 1.48			0.870 ± 3.55			1.62 ± 1.38		
Arrival date	0.008 ± 0.011	0.60	0.441	−0.0003 ± 0.026	1.05	0.309	0.001 ± 0.010	0.03	0.870
Forehead patch size	0.001 ± 0.007	0.05	0.832	−0.016 ± 0.019	0.14	0.707	−0.001 ± 0.007	0.05	0.823
Wing patch size	0.006 ± 0.008	0.68	0.413	−0.005 ± 0.021	0.48	0.491	0.010 ± 0.008	1.41	0.240
Age	0.199 ± 0.334	0.35	0.554	−17.8 ± 1924	3.76	0.057	0.076 ± 0.358	0.05	0.978
Treatment		0.02	0.981		0.04	0.965		0.01	0.989
Treatment decreased	−0.004 ± 0.147			0.407 ± 0.417			−0.020 ± 0.153		
Treatment enlarged	0.024 ± 0.164			0.417 ± 0.449			−0.020 ± 0.172		
Sperm size		0.30	0.741		0.18	0.834		0.54	0.587
Sperm size: linear	−0.079 ± 0.512			−0.415 ± 1.35			0.045 ± 0.533		
Sperm size: quadratic	−0.393 ± 0.523			0.789 ± 1.25			−0.557 ± 0.547		

Qvarnström et al. (2000) used Tippex. However, this difference is unlikely to explain the opposite direction of our results as the shape of the reflectance curves of the two markers is very similar. In contrast, the shape of the reflectance curve of natural white is different from both artificial colorations (see Fig. 1). Consequently, it is possible that females can distinguish between natural and artificial white and consider only the natural one as attractive while the artificial one may be unattractive. If true, the different results could partially stem from a difference in the treatment of control groups. In our study, we did not color the control group at all, contrary to Qvarnström et al. (2000) who painted Tippex over the natural white in the same extent as was used to paint the enlarged patch group over their natural black. Consequently, females in our study might have perceived enlarged patch males as less attractive because they had the same extent of natural (and attractive) white as control males but, in addition, they had patches of artificial white that made them unattractive. In contrast, in the study of Qvarnström et al. (2000), both the enlarged patch and control groups had the same extent of artificial white, but the experimental group retained a larger extent of natural white making them more attractive.

Second, Qvarnström et al. (2000) kept the males caged for 1 day to break their dominance over their original territories. We released males immediately after patch size manipulation and thus allowed them to return to their territory without a need to fight for them once more. If nest-site competition was intense only late in the season due to the lack of unoccupied territories, then the pairing latency of large-patched males could be shorter only at the end of the season, as was found by Qvarnström et al. (2000), due to their ability to win the competition over territory (see Pärt and Qvarnström 1997). In contrast, pairing latency in our population should not be as strongly affected by male-male competition and thus directly represent female mate choice.

Finally, and most interestingly, differences in the role of ornaments in sexual selection may exist between populations (Scordato and Safran 2014). For example, it has been shown that forehead patch size is condition dependent (Gustafsson et al. 1995) and males with large forehead patches are preferred as social (Qvarnström et al. 2000) and extra-pair (Sheldon et al. 1997) partners in an isolated Swedish population. In contrast, wing patch size (Török et al. 2003) but not forehead patch size (Hegyi et al. 2002) is a condition-dependent signal important in male-male competition (Garamszegi et al. 2006) in a Hungarian population. Similarly to the Hungarian population, wing but not forehead patches played a role in extra-pair paternity in our Czech population (Edme et al. 2016). These similarities suggest a greater role of wing patches in

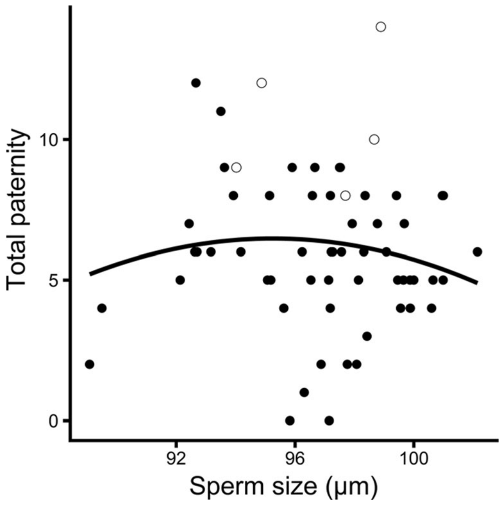

Fig. 3 Relationship between sperm size and total number of sired offspring (total paternity). *Solid circles* depict males that had only one social nest ($n = 57$). *Open circles* depict polygynous males ($n = 5$). *Fitted line* shows predicted quadratic regression

Table 5 Models for number of fledglings and recruits ($N = 67$)

	Fledgling			Recruit		
	Estimate ± SE	F	P	Estimate ± SE	F	P
Intercept	2.05 ± 1.54			1.73 ± 3.04		
Arrival date	−0.0004 ± 0.011	<0.01	0.966	−0.006 ± 0.023	0.09	0.766
Forehead patch size	−0.006 ± 0.007	0.60	0.439	−0.008 ± 0.014	0.35	0.558
Wing patch size	−0.0009 ± 0.008	0.01	0.917	−0.0007 ± 0.0176	<0.01	0.964
Age	−0.356 ± 0.393	0.83	0.365	−0.418 ± 0.815	0.27	0.605
Treatment		1.42	0.250		1.05	0.356
Treatment decreased	−0.011 ± 0.160			−0.426 ± 0.302		
Treatment enlarged	−0.269 ± 0.183			−0.407 ± 0.352		

Central Europe, the core of the distribution of the collared flycatcher. Nevertheless, females apparently paid attention to male foreheads in our population too, as they were less willing to mate with males with enlarged patches, and this was true especially late in the season. This change of mate preference with the season suggests an underlying change in costs and benefits of mating with large-patched males (Qvarnström 2001).

One explanation for plastic mate preferences may be the greater dependence of chicks on male paternal care late in the season. Consequently, females may be reluctant to pair with males that will not provide enough parental care during this difficult period of the breeding season. Highly ornamented males may invest resources into mating effort and provide less paternal care (Qvarnström 1997; Mazuc et al. 2003; Mitchell et al. 2007). Moreover, the size of the forehead patch may be used by females as an indicator of paternal care as this patch has been shown to decrease in the year following experimental increase of brood size (Gustafsson et al. 1995). Females living in populations with very limited resources may prefer males with smaller secondary sexual ornaments throughout the year (Griffith et al. 1999).

On the other hand, avoiding dominant males may also mean a loss on the side of indirect benefits if these males are genetically superior over subordinates. Therefore, females socially mated to high-quality fathers may increase the genetic component of offspring fitness by extra-pair copulation with superior males (Jennions and Petrie 2000). Extra-pair paternity is common in the collared flycatcher and is often related to secondary sexual plumage traits (Sheldon and Ellegren 1999; de Heij et al. 2011; Edme et al. 2016) as is also common in other species (Jennions and Petrie 2000; Akçay and Roughgarden 2007). However, extra-pair paternity is not determined solely by behavioral interactions among females and social and extra-pair males but also by the ability of sperm to fertilize ova, a process known as sperm competition. This area of research has been studied only recently and has yielded mixed results. Some studies have found a relationship between sperm traits and success in extra-pair paternity (Laskemoen et al. 2010; Bennison et al. 2015) while others have not supported this idea (Cramer et al. 2013).

Here, we found neither a linear nor a non-linear effect of sperm size on within-pair or extra-pair paternity. Thus, there was no evidence of either directional nor stabilizing selection on sperm size. Stabilizing selection for optimal sperm size is hypothesized to be linked to the intensity of sperm competition between species, with the strongest selection for optimal sperm phenotype in the most promiscuous species (Lifjeld et al. 2010). Sperm competition in our population is quite intense as roughly 20–25% of young are sired by extra-pair males (Krist et al. 2005; Krist and Munclinger 2011; this study). Therefore, at first sight, our results do not seem to support the hypothesis of Lifjeld et al. (2010). However, it is tremendously difficult to predict within-species effects from comparative studies. It could be that our population has already reached evolutionary equilibrium, when the sperm size of all males might be so close to the species' optimum that any subtle differences in sperm morphology play no role in their fertilizing abilities. Moreover, other sperm traits that we did not measure might be more relevant for success in sperm competition, for instance sperm viability (Smith 2012), speed of swimming (Birkhead et al. 1999), and number of sperm cells in the ejaculate (Laskemoen et al. 2010).

We partially replicated the study of Qvarnström et al. (2000) that manipulated forehead patch size in the collared flycatcher. Contrary to the Swedish population, we did not find any evidence for female preference of males with enlarged patches late in the season. Males with artificially enlarged patches seemed to be unattractive in the Czech population, and this was especially true late in the season. We also did not find any evidence that sperm size affects within-pair or extra-pair paternity and consequently male fitness. These findings call for replicated research both in well-established fields like female mate choice with respect to male ornaments and emerging ones like sperm variation and its effect on paternity and fitness.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethical committee of Palacky University and by the Ministry of Education, Youth and Sports (license number: MSMT-56147/2012-310) and complies with the current laws of the Czech Republic.

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Chapter 2: Female collared flycatchers choose neighboring and older extra-pair partners from the pool of males around their nests

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Female collared flycatchers choose neighbouring and older extra-pair partners from the pool of males around their nests

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Extra-pair copulation is common among passerine birds. Females might engage in this behavior to obtain direct or indirect benefits. They may choose extra-pair males with larger ornaments, especially if they are costly to produce. Here we studied extra-pair paternity in the collared flycatcher. Genetic analysis allowed us to identify the presence or absence of extra-pair young in the focal nests, and to identify extra-pair fathers. We also identified potential males available as extra-pair sires around the nests of females who had extra-pair young. First, we tested the relationship between paternity in own nest and ornament size (wing patch and/or forehead patch), morphological traits and age of social males and females. Second, we compared the same suite of traits among social mates, extra-pair males and all potential extra-pair mates. Finally, we investigated the effect of the size of ornaments on the distance between the social nest and that of nest the extra-pair father. Contrary to our prediction, males with larger ornaments and longer wings lost more paternity in their nests. We also found that early breeders lost less paternity in their nests. Extra-pair males were older and had longer wings than social and potential extra-pair males. Females mainly obtained extra-pair mates near their nests but the distance did not vary according to ornamentation. These results could potentially be explained by differences in mate guarding strategy as older males may be more experienced in guarding their mate and attract other females more easily. More data about mate guarding and prospecting are needed to increase our understanding of mechanisms underlying the extra-pair paternity in birds.

Many birds are socially monogamous, but extra-pair paternity is widespread (Griffith et al. 2002, Westneat and Stewart 2003). This is advantageous for extra-pair males, because they obtain more offspring without providing parental care to these extra young (Møller and Ninni 1998, Sheldon and Ellegren 1999). The advantage of extra-pair copulations for females is still debated and unclear. Females do not seem to obtain large direct benefits from mating with extra-pair males, as they do not provide food to the female or parental care to the extra-pair offspring (Arnqvist and Kirkpatrick 2005). On the contrary, it may be costly for female to engage in extra-pair copulation as males uncertain of their paternity may decrease parental care toward own nest (Dixon et al. 1994, Arnqvist and Kirkpatrick 2005, but see Schnitzer et al. 2013). However, one possibility of direct benefit for females is to obtain sperm from their extra-pair mates in case of total or partial infertility of their social partners (Sheldon 1994, Krist and Munclinger 2011). Females may also obtain indirect benefits which can include better or more compatible genes for their offspring (Sheldon et al. 1997, Jennions and Petrie 2000, Neff and Pitcher 2005, Akçay and Roughgarden 2007). The good genes hypothesis (reviewed by Akçay and Roughgarden 2007) states that females may obtain genes for viability or attractiveness from their extra-pair mates. The compatible genes hypothesis suggests that females may choose males whose genotypes are more compatible with their own and thus increase the fitness of the offspring (Tregenza and Wedell 2000, Neff and Pitcher 2005). Alternatively, female extra-pair behavior may not confer any benefits to females if it is caused by indirect selection on male behavior and cross-sex genetic correlation in this trait (Forstmeier et al. 2011, 2014).

If females strive to obtain good genes from their extra-pair mltes, these males are predicted to be superior to the social partner. Indeed, many studies show that extra-pair males possess larger ornaments than social mates and that social males with larger ornaments lose less paternity in their own nests (Kempenaers et al. 1992, Perreault et al. 1996, Richardson and Burke 1999, Johnsen et al. 2001, Bouwman et al. 2007, Albrecht et al. 2009, reviewed by Akçay and Roughgarden 2007). However, this observation is no general as opposite patterns are frequent (Johnson et al. 2002, Marshall et al. 2007). Similarly, females may also prefer extra-pair males which are older and thus signal good quality and survival ability (Akçay and Roughgarden 2007, Moreno et al. 2010, reviewed by Cleasby and Nakagawa 2012).

In addition to male traits, some environmental factors may influence the distribution of extra-pair paternity. For example, spatial and temporal availability of potential extra-pair partners should be considered (Schlicht et al. 2015).

Extra-pair males are often neighbors (Smith 1988, Neudorf et al. 1997, Mays and Ritchison 2004, Pedersen et al. 2006), but it is not always the case; extra-pair males can also come from distant territories (Woolfenden et al. 2005, Canal et al. 2012a). The factors that influence the distance between social and extra-pair nests have yet to be elucidated and need further investigation. For example, attractiveness may determine the distance a male is able to travel to obtain extra-pair copulation if he need to trade off within-pair and extra-pair paternity. Attractive males that have a lower risk of paternity loss in their own nest may spend more time outside own territory and thus visit more distant territories when searching for a possibility of extra-pair copulation. Consequently, distance between social and extra-pair nest could increase as a function of ornaments of extra-pair males. Similar pattern may occur if females, instead of males, actively seek extra-pair copulations (Double and Cockburn 2000, Tarof and Ratcliffe 2000, Pedersen et al. 2006). Such a foraying female might accept copulation with an extra-pair partner only if the male has larger ornaments than her social mate (Pedersen et al. 2006). Consequently, females that are mated to highly ornamented social partners will likely need to travel farther away to find even better extra-pair male. So in this case distance would increase with the size of ornaments of the social mate. As far as we know, these hypotheses linking male ornaments with the distance to extra-pair nest have not been tested yet.

In this study we tested which traits influenced the probability that a male was cuckolded, and which morphological traits and secondary sexual traits females use to choose extra-pair mates within an available pool of males. In many studies, social and extra-pair males have been compared, but the presence of other potential extra-pair males has rarely been taken into account. We also tested if the extra-pair males were neighbours and whether the traits of females, social or extra-pair males predicted the distance between the nests of cuckolded males and their cuckolders. We used collared flycatchers *Ficedula albicollis* as the study species due to frequent extra-pair paternity (Sheldon et al. 1997, Sheldon and Ellegren 1999), as was also found in our population (Krist et al. 2005, Krist and Munclinger 2011). Males of this species possess two secondary sexual ornaments that could play a role in sexual selection: a forehead patch (Sheldon and Ellegren 1999) and a wing patch (de Heij et al. 2011). Females can use the size of these ornaments to assess the quality of males. We predicted that 1) more ornamented males lose less paternity in their nests, 2) extra-pair males are more ornamented than social males and other males that did not sire any offspring in focal nests, 3) females choose males in their local neighborhood, and 4) the distances between the nests of social mates and extra-pair mates increase with the size of ornaments of the former group.

Material and methods

Study site and species

The study was conducted between 2006 and 2009 in Velky Kosir, Moravia, Czech Republic (49°32′N, 17°04′E, ca 350 m a.s.l). Approximately 350 nest boxes suitable for flycatchers were installed in an oak forest. Collared flycatchers *Ficedula albicollis* are small migratory passerine birds (13 g). Old males are black and white with large wing and forehead patches. Sub-adult males (i.e. those that are in the second year of their lives) have brownish primaries and their wing patches are smaller than those of adult males. Females are dull brown and white and their age cannot be reliably inferred from the plumage Males arrive earlier at the breeding site than females, in mid-April, to establish their territory. After pair-bond formation and nest building, females usually lay 4–8 eggs and start to incubate before the last eggs are laid, resulting in asynchronous hatching.

Field methods

All genotyped nests included in the present study were subject of cross-fostering for purposes of another study (Krist and Munclinger 2011). This sample constituted one quarter to one third of all initiated nests in the population per year. Consequently, we were unable to determine complete extra-pair paternity gains for males. However, as we strived to sample all males in the population, we were able to assign high proportion of extra-pair young to their genetic fathers (see Results) and compare traits of social and extra-pair fathers with those of other available males in the population.

The nests were visited daily when the onset of egg-laying was expected. The first day of egg-laying was noted (1st January = day 1). The laying order was written directly on the eggs, and we measured egg length and width with a digital caliper (± 0.01 mm). Each egg laid was replaced with a dummy egg. Mixed clutches with eggs from different nests were compiled. When the last eggs had been laid and incubation had started, the dummy eggs were replaced with a new clutch with the same size as the original (see Krist and Munclinger 2011 for more details). We refrained from checking the nests every day to avoid disturbance and desertion by females during the incubation period. The daily visits continued 10 d later, when hatching was expected. On day 6 after hatching, the chicks were marked with a numbered ring and weighed (nearest 0.25 g), and we took blood from the tarsal vein which was then stored in ethanol. On day 13, the chicks were measured (tarsus ± 0.01 mm and wing nearest 1 mm) and weighed again. Dead embryos and nestlings were collected and stored in ethanol.

During the feeding period, adults were caught and weighed, and we measured tarsal and wing length. The wing patch was measured with a ruler in the field and its size was calculated as the sum of the visible white patches on the primaries 3 to 8 from the tip of the coverts to the distal part of the wing (in mm). The forehead patch was photographed twice with a ruler aligned along it. The surface area was measured twice after being delimited to the nearest 0.1 mm^2 in imageJ software ver. 1.49, and the final surface area was defined as the mean of these measurements. A small amount of blood was taken from the tarsus. Male age was determined by wing plumage coloration and it was recorded as adult or sub-adult. As we wanted to assign extra-pair fathers during these field seasons, we strived to catch all males, including those on nests whose chicks were not genotyped, when they were feeding chicks. They were measured, and a blood sample was taken to allow for genetic analyses and to identify them as genetic sires.

Genotyping

Blood samples and tissue from chicks and dead embryos were used in DNA extraction with DNeasy® Blood and Tissue Kit (Qiagen). They were then genotyped at eight polymorphic microsatellite autosomal loci. A single multiplex PCR using a fluorescently-labelled primers and a Type-it® Microsatellite PCR kit (Qiagen) were used to amplify the microsatellites. The samples were treated for 5 min at 95°C, and then in 30 cycles of 30 s at the same temperature, 90 s at 65°C, 30 s at 72°C, and finally 30 min at 60°C. GeneScan™-500 LIZ® Size Standard (Applied Biosystems) was added to the PCR products, and analyzed with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). GeneMarker® ver. 1.9 was used to score the genotypes, and Cervus 3.0.3 (Kalinowski et al. 2007) was used to obtain loci characteristics based on allele frequencies. The eight loci were: Fhu2 (or PTC3) (Ellegren 1992) Cuμ04 (Gibbs et al. 1999) Fhy310, Fhy405, Fhy407, Fhy428, Fhy431, and Fhy452 (Leder et al. 2008). For the first parent, the combined non-exclusion probability for that group of loci was found to be 5.67×10^{-4}. Individuals that could not be genotyped at five or more loci were excluded and the genotypes for 1235 individuals were obtained.

Parentage assignment

Due to the nature of the cross fostering design (Krist and Munclinger 2011), maternal identity was not known and had to be assigned with the use of microsatellite markers. Since the exclusion power was already high for the first parent, and each chick had 8 candidate mothers at most, this maternal assignment proved to be relatively easy and reliable (Krist and Munclinger 2011).

Seven loci were used (Fhy 310 was excluded because of the risk of null alleles) for a comparison of the chicks' genotypes and the genotype of the male paired with the genetic mother. Likelihood approach in Cervus 3.0.3 (Kalinowski et al. 2007) was used to calculate confidence of assignment based on the difference in likelihood scores between the most likely and the second most likely parent. When males matched with all or all but one locus, they were considered to be the genetic fathers. In a few cases that had two mismatches, the chick was indicated as sired by the social father with 95% confidence and we considered it as such.

In other cases, the nestlings were classified as extra-pair young. For these, we compared the offspring's genotype with all male genotypes in the population to determine extra-pair sires. We did this paternity assignment only for years 2007–2009 since in 2006 we blood-sampled only a small proportion of potential sires. All paternities were determined with a 95% maximum likelihood in Cervus Software 3.0.3 (Kalinowski et al. 2007), based on our conservative estimate that we sampled half of the male population. Extra-pair chicks in one nest were usually sired by one extra-pair male, but in eight cases clutch extra-pair paternity was shared by two or more extra-pair males.

To compare traits of social and extra-pair males, we formed groups called trios which were unique combinations of ring numbers of the social male, extra-pair male, and female (Kalinowski et al. 2007). In cases where one female had extra-pair young with two or more different extra-pair males, we considered her to be a part of two separate trios. In total, 59 trio combinations were used for the comparison of traits.

Spatial analyses and potential males

The 350 nest boxes were distributed among four different plots in the study area and we were interested in whether females chose extra-pair males randomly from all available males or if they preferred males from their own neighborhood. To precisely determine the distance between the social and extra-pair nest and social and all other available nests, a linear distance matrix was designed using GIS software Qgis 2.4.0 (<http://qgis.org>). All coordinates were presented in degrees, minutes, seconds, and a WGS 84 projection was used. Analyses were conducted separately for each year to avoid superimposition of occupied nests. We kept records of every nest occupied by collared flycatchers from each breeding season even if information about a male was missing. This information may have been missing because nests were deserted or predated, or we were unable to capture males but they were still available as potential extra-pair males. We compared the distances between the female's nest and the nest of the extra-pair father with the mean distance to all nests occupied by collared flycatchers either in the whole study area or only within the same plot. In the latter case, we excluded nine cases where the extra-pair father was in fact found on another plot. We performed the second analysis because most extra-pair sires were found within the same plot where the female bred (see Results).

We wanted to identify all potential extra-pair males available for the females who obtained extra-pair young, and compare those males with the social and actual extra-pair fathers. As the actual extra-pair father was often a close neighbor (see Results), we considered as potential extra-pair fathers only those individuals that bred as close as or closer to the focal nest than the actual extra-pair father. We draw a buffer zone around each social nest with a radius equal to the distance between the nest and the extra-pair father. We added 20 m (the mean of half the distance between two adjacent nest-boxes) to the real distance to include nests with approximately the same distances from the social to the extra-pair nests. Using the plug-in 'spatial queries ver. 0.1' in QGIS, we identified every occupied nest that lay within the focal buffer and obtained the list of potential males for a focal nest.

Moreover, we repeated this spatial analysis when we considered the temporal availability of potential extra-pair sires. Not all males breeding in the vicinity around the focal nest were indeed available as extra-pair sires since some of them arrived after the fertile period of the focal female ended. Therefore, in the second analysis we included among potential sires only males that bred at the same time or earlier as the focal female.

Statistical analyses

To analyze the relationship between social parents traits and the occurrence of extra-pair offspring in the nest (prediction 1), we used a generalized linear mixed model (GLMM) with a binomial distribution (presence/absence of extra-pair offspring in the social father's nest) and a logit function as link. We ran this analysis with the glmer function from package lme4 ver. 1.1-6 (Bates et al. 2015) in R studio ver. 0.98.501 (R Core Team, <www.R-project.org/>). The presence or absence of extra-pair young in the nest was the model's response variable. The predictors included two morphological characteristics for social males and females (tarsal length and wing length), wing patch size for both sexes, and the forehead patch size and age of males (adults or sub-adults). We were unable to include female age among variables, despite its potential relevance for extra-pair paternity (Moreno et al. 2015), since females have no delayed plumage maturation. The year of study and the ring numbers of both males and females were used as random factors. The p-values were obtained by comparing the deviance between the full model and the model without the considered factor. The difference between the two deviances was compared with a chi-square distribution (Crawley 2007).

To compare the traits of males according to their status (social, extra-pair, potential; prediction 2) separate linear mixed models were run. Father status was used as a predictor in all models, and response variables were tarsus, wing length, wing patches, forehead patch size, or the female laying date for each respective model. For potential males, these traits were averaged within trios and consequently the number of potential males equalled the number of trios. The lmer function with the package lme4 (R package ver. 1.1-8, Bates et al. 2015) and lmerTest (R package ver. 2.0-29, Kuznetsova et al. 2015) were used. Age is a binary type of data so we did not compute averages for potential males and we used the glmer function with a logit link function instead of the lmer). The random factor was the trio identity for all models.

To test whether females chose males randomly with respect to the distance to their nests (prediction 3), we compared the distance between females' nest and the nest of extra-pair sires with the mean distance to all available nests (including the distance between the social and extra-pair nest). We used a paired t-test assuming unequal variance between groups. This analysis was run twice: firstly for all trios and secondly for only trios where the extra-pair father was from the same plot. We also used three separate linear mixed models to test whether morphological characteristics of social males, extra-pair males and females predict the distance between the social and extra-pair nest (prediction 4).

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.043tn> (Edme et al. 2016).

Results

Distribution of extra-pair paternity

In total, 160 nests were used for the cross-fostering experiments between 2006 and 2009. Females from 143 nests were genotyped while social males were genotyped from 135 of them. In total 941 offspring were genotyped and for 800 of them the social father was known. Extra-pair fathers sired 214 of these 800 offspring (26.8%). Three social males were hybrids with the pied flycatcher and their nests were excluded from all analyses. Consequently, sample size when testing prediction 1 was 132 nests. We also did not use data from year 2006 for paternity assignment. Our sample size for paternity assignment was then 181 extra-pair young from 2007–2009. We were able to assign genetic fathers to 114 of them (63.0%). These 114 extra-pair young formed 59 trios (unique combination of social male, female and extra-pair male). Those 59 trios represent our basic sample size for testing predictions 2, 3 and 4.

Paternity loss and adults' ornamentation

We checked for collinearity among the predictors of the model. Variance inflation factor (VIF) revealed that age and wing patches were collinear ($\mathrm{VIF} > 2$) (Graham 2003) (Table 1). Therefore, we re-ran the model once without

Table 1. A test whether traits of social male and female influence the probability of having extra-pair young in a nest (prediction 1, n = 132 nests). GLMM model with presence/absence of extra-pair young in the nest (response variable) and males and females traits (predictors). Random effect was females and males ring and year. Positive estimates indicate a high probability of having extra-pair young in the nest (SE = standard error, DF = degree of freedom, VIF = variance inflation factor).

Random effects	Variance				
Male ring	0.000				
Female ring	0.618				
Year	0.008				
Residual	0.902				
Fixed effects	Estimate ± SE	F-value	DF	p-values	VIF
Intercept	–65.17 ± 23.57				
Julian day	0.216 ± 0.070	9.42	1,126	< 0.001	1.26
Male age	2.38 ± 1.08	4.93	1,126	0.020	3.78
Male forehead patch	0.002 ± 0.002	1.35	1,126	0.208	1.05
Male wing patch	0.090 ± 0.028	4.92	1,126	< 0.001	3.55
Male wing length	3.59 ± 1.68	0.74	1,126	< 0.001	1.50
Male tarsus length	–0.066 ± 0.514	0.06	1,126	0.899	1.18
Female wing patch	–0.011 ± 0.029	0.04	1,126	0.670	1.08
Female wing length	1.61 ± 1.47	0.06	1,126	0.245	1.21
Female tarsus length	–0.432 ± 0.453	0.52	1,126	0.303	1.09

wing patches, and for a second time with patch size but without age. Wing patch was a significant predictor of loss of paternity ($F_{1,125} = 5.57$, $p < 0.001$) when tested in isolation, but age was not ($F_{1,106} = 0.12$, $p = 0.417$). We found that wing patches were larger in cuckolded males than in non-cuckolded ones (all males: 54.37 ± 13.76 mm^2, $n = 73$, and 46.58 ± 14.68 mm^2, $n = 59$, adults only: 58.77 ± 8.25 mm^2, $n = 62$, and 53.1 ± 8.59 mm^2, $n = 45$). Males who lost paternity had longer wings than non-cuckolded males (8.32 ± 0.18 cm and 8.25 ± 0.16 cm respectively) (Table 1) and the size of the forehead patch area did not significantly differ (mean cuckold vs non-cuckold 53.35 ± 9.32 mm^2 and 50.18 ± 9.26 mm^2) (Table 1). Tarsal length was not different between the two types of males. The females who cuckolded males showed no differences in any of the traits from those who did not (Table 1). There was also a higher probability to have extra-pair young in the focal nest as the breeding season progressed (Table 1).

Comparison between social mates, extra-pair mates and potential mates

We identified 46 extra-pair males and 45 social males and females in these 59 trios. For several reasons, the number of males does not need to equal the number of trios. For example, some extra-pair mates had extra-pair young in more than one nest (range 1–4 nests). Other individuals bred and were cuckolded in more than one year. The number of potential mates (the actual extra-pair mates were excluded from the list of potential mates) in the buffer zone around the focal nest ranged from 0 to 127 males (all plots: mean ± SD = 21 ± 27 males; within plots: mean ± SD = 8.6 ± 7.4 males) or from 0 to 71 if only nests with the same or earlier laying date were considered to provide potential sires for focal extra-pair young (all plots: 10.83 ± 14.89, within plots: 5.31 ± 7.18).

Forehead patch, tarsus length and date of laying did not differ in the three categories of males (social, extra-pair, and potential mates) (Fig. 1, Table 2). However the wing

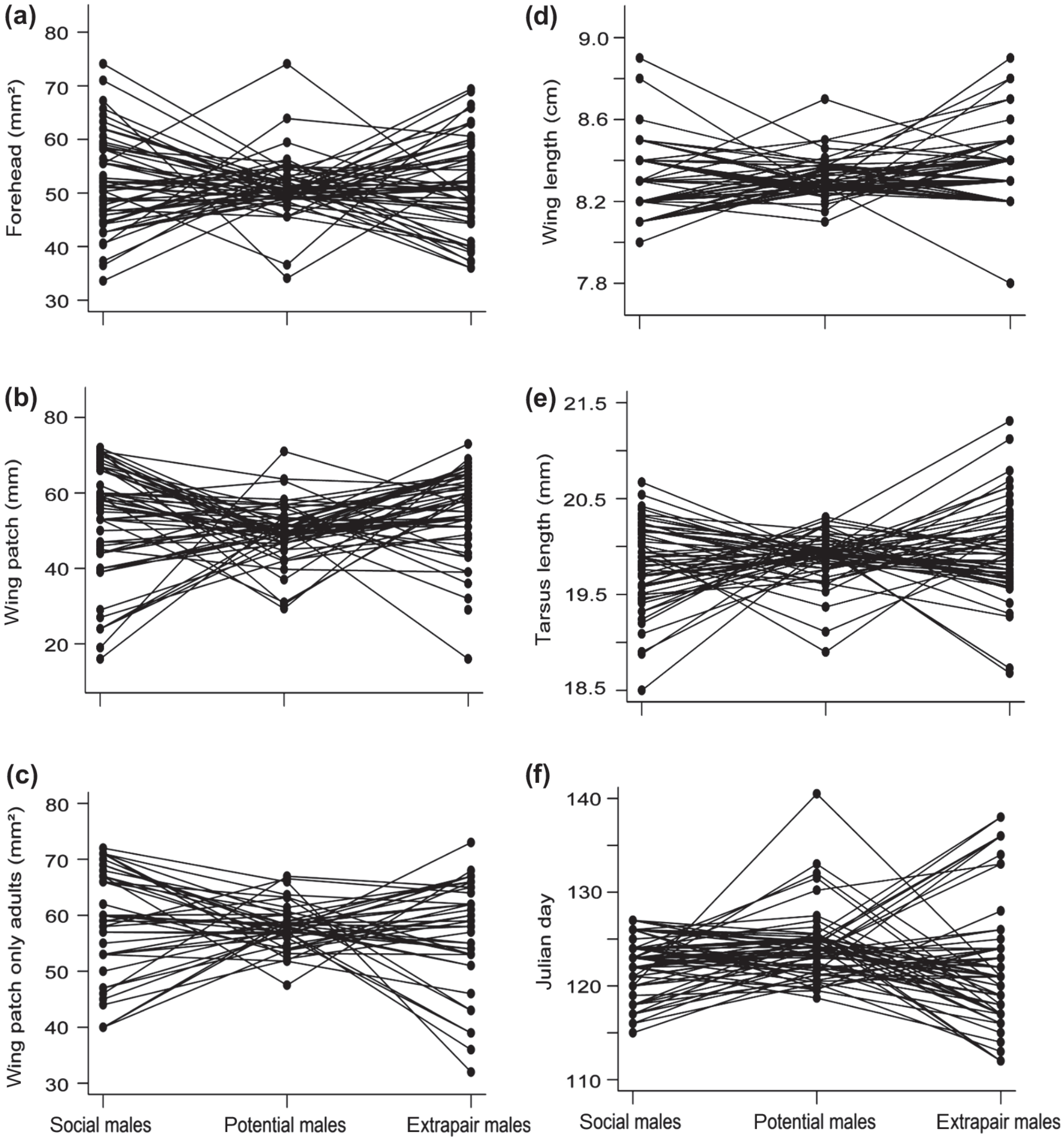

Figure 1. Comparisons of morphological and life-history traits between social, potential and extra-pair fathers ($n = 59$). Whereas values for social and extra-pair males are individual data points, each point for potential fathers represents the mean of all potential fathers for that social nest. Lines connect values related to the same focal nest.

Table 2. Comparison between traits of social, extra-pair and potential males (prediction 2, n = 59 trios). All males breeding in the zone around social nest were considered as potential sires. Each line shows results of separate model with male status as the predictor and morphological or life-history trait as a response variable. DF = degree of freedom. Trio identity was fitted as a random effect.

	Random effects		Fixed effects			Means ± SD		
	Trio (variance)	Residual	F test	DF	p-values	Social	Extra-pair	Potential
Forehead (mm^2)	0.000	6279	0.491	2,168	0.612	52.54 ± 9.08	51.51 ± 8.64	50.40 ± 9.34
Wing patch (mm^2)	0.000	129.3	4.439	2,170	0.013	52.91 ± 14.42	55.96 ± 10.91	48.90 ± 18.32
Wing patch (adults only) (mm^2)	0.00	67.5	0.523	2,120	0.593	58.70 ± 9.62	56.85 ± 9.65	57.87 ± 4.05
Wing length (cm)	0.004	0.025	4.180	2,104.5	0.017	8.31 ± 0.19	8.39 ± 0.02	8.29 ± 0.17
Tarsus (mm)	0.000	0.170	1.648	2,170	0.195	19.82 ± 0.3	19.96 ± 0.49	19.94.00 ± 0.52
Julian day	1.422	21.7	3.366	2,115.5	0.037	122.25 ± 3.07	122.03 ± 6.81	123.90 ± 6.68
Age	0.000	1.09	6.282	2	< 0.001	Adults: 48 sub-adults: 11	Adult: 57 sub-adults: 2	Adults: 683 sub-adults: 255

characteristics differed among males. The wings of extra-pair mates were longer than those of social and potential mates (Fig. 1, Table 2). Wing patch size was also larger in extra-pair males than in potential mates (Fig. 1, Table 2), but there was no difference between social and extra-pair mates. When we controlled for age (adults have larger patches than sub-adults), wing patch size was no longer different (Fig. 1, Table 2). Extra-pair fathers were older than social mates (estimate ± SE = 1.876 ± 0.792, z-value = –2.369, p = 0.017) and potential extra-pair mates (estimate ± SE = 2.366 ± 0.722, z-value = –3.277, p < 0.001). There was no age difference between social mates and potential mates. Social males and extra-pair males had females who bred at the same time, but potential males had females who laid their eggs later (Fig. 1, Table 2). The results are closely similar when only males breeding at the same time or earlier are considered as potential sires of extra-pair chicks (see Methods). The only natural exception is then the breeding date that is by definition earlier for this restricted set of potential males (Supplementary material Appendix 1).

Distance of extra-pair sires

The selection of extra-pair mates was not random, and females mostly chose an extra-pair mate close to their nest. In 50 of 59 cases, males bred within the same plot as females (chi-square = 28.491, DF = 1, p-value < 0.001). A comparison was done between the actual distance between social and extra-pair nests and that between social nests and the mean distance to all other available nests of collared flycatchers (prediction 3). We carried out this analysis twice. The first time we took into account only the trios with the extra-pair mate within the same plot as the social nest. The second time, we ran the analysis with all 59 trios which also included extra-pair sires breeding on other plots. The actual distances were shorter than the mean distances to all available nests

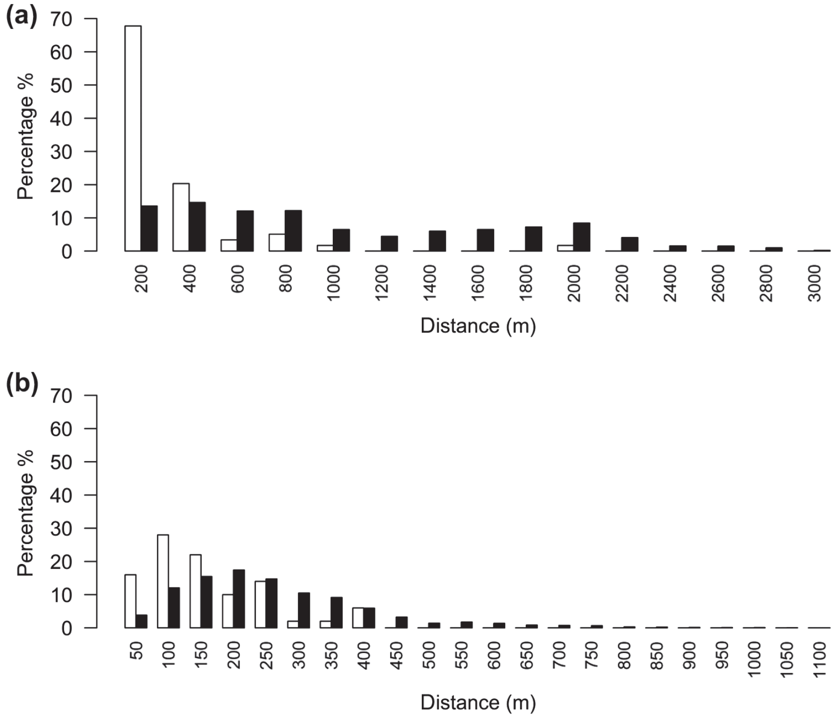

Figure 2. Distribution of distances between social nests and extra-pair nests (open bars) or social nests and all available nests (filled bars). Panel (a) considers nests in all plots, panel (b) considers only extra-pair nests and all available nests that were located on the same plot as the social nest.

in both cases (within plot comparison: 136.5 ± 90.2 vs 239.7 ± 64.7 m, t = –7.24, DF = 49, p < 0.001; all data: 222.6 ± 291.7 vs 962.8 ± 246.0 m, t = –15.9, DF = 58, p < 0.001, Fig. 2). In the test of our prediction 4 we found that neither female traits nor the extra-pair male traits influenced the actual distances between social nest and that of extra-pair father (Table 3, 4). However, social males with longer wings had their extra-pair rival closer than was the case of social males with shorter wings (Table 5).

Discussion

The aim of this study was to determine which traits influenced the distribution of extra-pair paternity in the collared flycatcher. We only genotyped nestlings in a fraction of nests but sampled the whole population of breeding males. Consequently we were able to assign genetic fathers to a high proportion of genotyped chicks which enabled us to compare traits of social and extra-pair fathers on solid sample size that was gathered over three to four years. We found that more ornamented males had more extra-pair young in their nest than less ornamented males. This contradicts with our first prediction that more ornamented males lose less paternity. Our second prediction about female preference for more ornamented extra-pair males was only partly supported since there was no difference in ornament size between the social, potential and extra-pair mates besides that caused by age. However, extra-pair males were older and had longer wings than both social and potential mates. In support of our third prediction we found that females choose extra-pair mates among close neighbors. In contrast, we found little evidence for our last prediction as distance between social and extra-pair nest is not affected by traits of either males or females.

More ornamented males are more cuckolded

Considering the male traits, only wing length and wing patches appeared to play a role in the probability of being cuckolded. However, our results are in contradiction with our prediction 1. In fact, males with longer wings and larger patches had more extra-pair offspring in their nests. In many previous studies it has been shown that more ornamented males lose less paternity (Møller and Ninni 1998, but see Sundberg and Dixon 1996, Bouwman et al. 2007) and increase their total reproductive success (Sheldon and Ellegren 1999). We need to stress, however, that the presence of extra-pair young in the nests of more ornamented males does not mean that their total reproductive success is lower than that of non-cuckolded males. Males who lost paternity in their own nests may have gained extra-pair paternity in other nests to compensate for the potential loss of paternity in their own. Our finding that males with longer wings lost more paternity in own nest but were favorite extra-pair fathers highlights that the same trait that causes loss of within-pair paternity may lead to the gain of extra-pair paternity. Unfortunately, we were unable to determine the total extra-pair paternity per male in this study, since we took DNA samples only from a subsample of nests that were involved in the cross-fostering experiment.

Table 3. Distance between social and extra-pair nest as a function of female traits (n = 59). Linear mixed model with distance as response variable and female characteristics as predictors. (SE = standard error, DF = degree of freedom). A negative estimate indicates a decrease in distance.

Random effects	Variance			
Female ring	< 0.001			
Residual	89814			
Fixed effects	**Estimate ± SE**	**DF**	**t-value**	**p-values**
Intercept	2914 ± 3231	54	0.902	0.371
Female wing patch	–1.279 ± 5.158	54	–0.248	0.805
Female wing length	–220.9 ± 286.7	54	–0.771	0.444
Female tarsus length	–15.4 ± 80.9	54	–0.191	0.785
Julian day	–4.38 ± 13.42	54	–0.334	0.740

Table 4. Distance between social and extra-pair nest as a function of traits of the extra-pair male (n = 59). Linear mixed model with distance as response variable and extra-pair male characteristics as predictors (SE = standard error, DF = degree of freedom). A negative estimate indicates a decrease in distance.

Random effects	Variance			
Extra pair male ring	5980			
Residual	88654			
Fixed effects	**Estimate ± SE**	**DF**	**t-value**	**p-values**
Intercept	1072 ± 2071	42.5	0.548	0.607
Forehead patch	0.539 ± 0.568	43.6	0.948	0.348
Age	130.0 ± 281.7	46.6	0.462	0.646
Wing patch	6.15 ± 5.22	41.5	1.179	0.245
Wing length	–159.5 ± 311.7	45.7	–0.512	0.611
Tarsus length	–14.28 ± 102.20	44.3	–0.140	0.890
Julian day	1.24 ± 7.16	40.8	0.173	0.863

Although our results are contrary to what we predicted, they could be explained by different mate guarding strategies among males. Males with larger patches might guard their females less and instead spend time outside their territories in trials to attract other females and sire extra-pair young. On the other hand, males of a lower quality may guard their mates more intensively to avoid losing paternity (Kokko and Morrell 2005). Regardless of this, the loss of paternity depends on both male and female strategies (Kokko and Morrell 2005), and it seems difficult for males to efficiently guard their females and obtain extra-pair copulations for

Table 5. Distance between social and extra-pair nest as a function of traits of the social male (n = 59). Linear mixed model with distance as response variable and social males characteristics as predictors (SE = standard error, DF = degree of freedom). A negative estimate indicates a decrease in distance.

Random effects	Variance			
Social male ring	0.000			
Residual	8727			
Fixed effects	**Estimate ± SE**	**DF**	**t-value**	**p-values**
Intercept	5386 ± 3097	50.6	1.739	0.088
Age	–68.1 ± 165.6	50.6	–0.411	0.682
Forehead patch	–0.364 ± 0.461	50.6	–0.789	0.433
Julian day	–13.0 ± 16.7	50.6	–0.779	0.439
Wing patch	0.229 ± 4.56	50.6	0.050	0.96
Wing length	–515.5 ± 231.0	50.6	–2.231	0.030
Tarsus length	45.9 ± 99.5	50.6	0.462	0.645

themselves at the same time. However, mate guarding may be inefficient when females are highly motivated to escape their partners guard (Kokko and Morrell 2005). This might be the case in blue tits *Cyanistes caeruleus*, where mate guarding did not protect paternity of social males, (Kempenaers et al. 1992).

Moreover, a further trade-off may exist between mate guarding and other male activities such as territory defence and the solution of this trade-off may depend on male age and experience. It has been found that young collared flycatchers males defend their territories more aggressively than old males (Garamszegi et al. 2006) and thus may be more exposed to loss of paternity because they provide more opportunities for females to seek out extra-pair copulation. Studies on the role of male age in the loss or gain of paternity are contradictory (Kempenaers et al. 1992, Moreno et al. 2010, Bowers et al. 2015, reviewed by Cleasby and Nakagawa 2012) but this often seems to be an important factor. In our case, age does not appear to be an important factor by itself for the loss of paternity, but only becomes significant when linked to the size of the wing patch.

Similar to Canal et al. (2012b), we also found that males that paired with females laying earlier in the breeding season, had fewer extra-pair young. Males arrive before females to establish territories (Harnos et al. 2015), and better territories (quality of the nest for example) are occupied by more experienced males (Askenmo 1984, see also Pärt and Qvarnström 1997). It is possible that because they arrived earlier, the males who paired first are better able to secure their females from extra-pair copulation. In some species, females choose a mate based on his traits and the quality of the territory (Alatalo et al. 1986). Females may be less motivated to cuckold their mates if these are good males who arrived first at the breeding site (Potti 1998). To arrive and breed earlier could also be advantageous because when their females finish laying, those males could look for extra-pair copulations without the danger of losing paternity. On the other hand, late breeding can represent a double cost for males. First, late breeders are less able to successfully raise healthy offspring. Secondly, it may encourage females to look for higher quality extra-pair males to provide a genetic advantage to the offspring (Qvarnström et al. 2000).

Extra-pair males are older and have longer wings than other potential partners

Extra-pair partners were older males with longer wings than the mean for all available males which indicates that either females assess the quality of the males around their nests or males seek extra-pair copulations based on their phenotype. As we lack information about movements during pairing, we do not know if male or female collared flycatchers visit many different territories or how often they may engage in this behavior. We can consider several possible scenarios for extra-pair mate choice. Firstly, females could eavesdrop on male–male singing contests (Mennill et al. 2002). If males' songs provide information about male quality or territory, females may listen to them and choose to visit males who seem likely to be good extra-pair males. Secondly, females could be more prospective and do extra-territorial forays to directly obtain information on potential extra-pair mates (Double and Cockburn 2000). In line with these two scenarios it has been found that captive females of the pied flycatchers often actively solicit extra-pair copulations (Drevon and Slagsvold 2005). Thirdly, females may stay in their territories and accept copulation from other males who intrude into her partner's territory (Hung et al. 2009, see also Moreno et al. 2015). Lastly, both females and males might leave their territories to find suitable extra-pair partners (Mays and Ritchison 2004). Unfortunately, little is known about the behavior of *Ficedula* flycatchers when seeking extra-pair copulations in the wild. Such behavioral data is badly needed but difficult to obtain. Employment of new technologies like automated radio telemetry can shed light on such secret behaviors as extraterritorial forays (Ward et al. 2013, 2014).

Females may obtain direct or indirect benefits when seeking extra-pair copulation. To obtain genetic benefits, they may copulate with extra-pair males superior to their social partner with respect to good or compatible genes (reviewed by Hsu et al. 2015). In our study, females did not choose males with larger secondary sexual ornaments that may be indicative of good genes (Neff and Pitcher 2005). There was no difference in the wing patches or forehead patches between social and extra-pair males. Moreover, we did not find evidence of superiority of extra-pair chicks in a previous study which also suggested that females did not obtain good or compatible genes for their offspring from extra-pair copulation (Krist and Munclinger 2011). However, that study was aimed mainly on short-term fitness benefits and we were unable to exclude superiority of extra-pair young in adult phase of life (Krist and Munclinger 2011). Here we found that extra-pair partners are older than social mates. By choosing older partners, females might obtain genes for offspring longevity. This possibility remains to be tested.

Besides good or compatible genes females might obtain direct benefits from extra-pair copulations. For example, female red-winged blackbirds *Agelaius phoeniceus* were found to forage in territories of their extra-pair partners (Gray 1997). But this type of direct benefit may be rare, and probably does not play a major role in collared flycatchers since this species does not defend feeding territories (Cramp and Perrins 1993). More general direct benefits from extra-pair copulation may be the insurance of the clutch against social mate infertility (Sheldon 1994), since sperm depletion or infertility may devalue the reproductive investment of females. In theory, females may be able to detect infertility or the poor sperm quality of their partners and choose extra-pair mates who possess traits indicative of good quality sperm (Sheldon 1994). However this phenotype-linked fertility hypothesis has weak empirical support since ejaculate or sperm traits are usually uncorrelated to male phenotypes (Mautz et al. 2013). In line with this we also found no difference in ornament size of extra-pair and social males despite our previous study tentatively suggested that females might engage in extra-pair copulations to insure clutch fertility (Krist and Munclinger 2011). Useful steps would be to take into consideration sperm traits to test if they are linked to male phenotype and have effect on paternity in the collared flycatcher. In the sister species, the pied flycatcher, support for the linkage between sperm traits and phenotype is mixed (Calhim et al. 2009, Lifjeld et al. 2012) while data on effect of sperm traits on paternity are needed (Cramer et al. 2013).

Copulation with neighboring males

Some previous studies have found that extra-pair males are close neighbors (Kempenaers et al. 1992, 1995, Perreault et al. 1996, Valera et al. 2003). In agreement with our prediction, almost all of the extra-pair males bred on the same plot as the females, with a mean distance of about 136 m. There are several explanations for why extra-pair sires should be close neighbors. First, males can allow their extra-pair partners to forage in their territories (Gray 1997). Another potential benefit may be a decreased risk of predation since the presence of other males means better vigilance for predators (Gray 1997, Eliassen and Jørgensen 2014). Third, if females only need sperm to be sure of fertilizing their eggs, they may copulate with a neighbor without considering the male traits. Here also mate guarding by social males may play a role since females probably have more opportunities to meet with neighbors and such meetings may take less time then travelling to distant territories and thus have greater chance of being hidden to the guarding mate. In line with this 'opportunity' or 'random pairing' hypothesis we found little evidence for the effect of female or male traits on the distance between social and extra-pair nest. The only pattern we found was a decrease of the distance with increase of the wing length of the social male. Currently we have no explanation of this finding. Surely more studies are needed to elucidate if male and female morphological and ornamental traits generally affect spatial distribution of extra-pair paternity.

Conclusions

In summary, males with longer wings and larger patches lost more paternity in their nests while older males with longer wings were more successful in gaining paternity in other nests. This finding suggests either that male effort in seeking of extra-pair mating opportunities depend on their phenotype or that females assess the quality of potential males around their nests when choosing extra-pair mates. On the other hand, females usually choose close neighbors as extra-pair partners and the distance to their nests is not dependent on their traits. This suggests, on the contrary, that females are not very choosy when selecting extra-pair mate. This is expected if they engage in extra-pair copulation to insure against potential infertility of their mate. Further research should strive to determine the factors that influence the distance between social and extra-pair nests and the behavior of all involved participants. On the one hand, in many species it is not well known if females or extra-pair males usually travel to obtain extra-pair copulations. On the other hand, the intensity of mate guarding by social mates is also likely to vary, but similarly to extraterritorial forays has rarely been quantified (but see Double and Cockburn 2000, Stutchbury et al. 2005, Woolfenden et al. 2005). Focus on sperm traits should also provide useful information since we lack general knowledge on the linkage between sperm morphology, male attractiveness and success in sperm competition.

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Supplementary material (Appendix JAV-00839 at <www.avianbiology.org/appendix/jav-00839>). Appendix 1.

Chapter 3: Moderate heritability and pronounced seasonal plasticity of sperm morphology in a species with high risk of sperm competition, the collared flycatcher *Ficedula albicollis*

Moderate heritability and pronounced seasonal plasticity of sperm morphology in a species with high risk of sperm competition, the collared flycatcher *Ficedula albicollis*

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Running head: Environmental *versus* genetic variation in avian sperm size

Key words: additive genetic variance, environmental variance, heritability, phenotypic plasticity, season, sperm competition, sperm size, wild population

Abstract

Spermatozoa represent the morphologically most diverse type of animal cells and show remarkable variation in size across but also within species, including birds. Here we applied quantitative genetic methods to a pedigreed multigenerational data set of the Collared Flycatcher *Ficedula albicollis*, a passerine bird with high levels of extra-pair paternity, to partition environmental and genetic sources of phenotypic variation in sperm dimensions in a natural bird population. We found a significant increase in sperm total length within individual males between the arrival and nestling period, demonstrating substantial seasonal phenotypic plasticity in sperm size. This seasonal variation may reflect constraints in the production of fully elongated spermatozoa shortly after arrival on the breeding grounds. There was no evidence for effects of male age on sperm dimensions. Narrow-sense heritability (h^2) of sperm total length amounted to 0.45 (± 0.14 SE). This provides a contrast to previous higher heritability estimates ($h^2 = 0.63 \pm 0.11$ SE) from laboratory populations of the zebra finch *Taeniopygia guttata*, a species with a low intensity of sperm competition but unusual genetic architecture where a 'super gene' caused by a chromosome inversion strongly contributes to the variance in sperm morphology.

Introduction

Across the animal kingdom, the function of spermatozoa is to fertilize ova and hence its form must have been selected to maximize fertilization success. Despite this common utility, sperm cells display a uniquely large variation in size, shape, and motility (Birkhead et al. 2009). Sperm trait variation exists between species (Birkhead et al. 2009, Simpson et al. 2014) (Aranea: Alberti 1995, Insects: Jamieson 1987, 1999; Fish: Jamieson 1991a) but also between populations of the same species (e.g. Hettyey and Roberts 2006; Minoretti and Baur 2006; Schmoll and Kleven 2011; Lifjeld et al. 2012; Laskemoen et al. 2013). Furthermore, substantial variation has been reported between individuals within populations (e.g. Ward 1998; Morrow and Gage 2001b; Helfenstein et al. 2008; Cramer et al. 2013).

Part of both between-species and between-male variation may be explained by sexual selection. In avian species where males are under intense post-copulatory sexual selection, as measured by the proportion of extra-pair young in their nests, spermatozoa were longer (Lifjeld et al. 2010) and also less variable in length between males (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010), as compared to species with a lower intensity of sexual selection. Similar decrease of sperm variation between males has also been found in polyandrous ants and bees (Fitzpatrick and Baer 2011). Low between-male variation in species with intense sexual selection is likely caused by strong stabilizing selection that depleted genetic variance in sperm length to favor the same optimal genotype and consequently phenotype (Lifjeld et al. 2010). Here the optimal genotype will be the one producing homogeneous spermatozoa with the optimal morphology to successfully fertilize ova. Therefore, we expect low additive genetic variance and hence heritability of sperm length in promiscuous species, while these quantitative genetic parameters are expected to be higher in species with low risk of sperm competition.

The genetic sources of phenotypic variation in sperm traits have rarely been studied. Moreover, most of these studies have focused on domesticated farm animals (Table 10.1 in Birkhead et al. 2009) or on captive populations of mammals (Woolley and Beatty 1967; Woolley 1971), birds (Birkhead et al. 2005; Mossman et al. 2009) and insects (Ward 2000; Morrow and Gage 2001a; Baer et al. 2006). Heritabilities of spermatozoa size obtained from captive insect populations were high: $h^2 = 0.52$ (± 0.06 SE) in *Gryllus bimaculatus* (Morrow and Gage 2001a), 0.69 (± 0.23 SE) in *Scathophaga stercoraria* (Ward 2000) and 0.43 (± 0.154 SE) in *Bombus terrestris* (Baer et al. 2006). Similarly, sperm total length as well as the length of individual sperm sections were highly heritable in captive populations of the zebra finch *Taeniopygia guttata* ($h^2 = 0.48 \pm 0.03$ SE, 0.46 ± 0.09 SE and 0.60 ± 0.12 SE for head, midpiece and flagellum length respectively in Birkhead et al. 2005, and 0.63 ± 0.11 SE for total sperm length in Mossman et al. 2009). In the zebra finch, relatively high heritability of sperm morphology may be expected for at least three reasons.

First, a standardized laboratory environment is likely to be much less variable than that in the wild and heritability may thus be higher due to lower environmental variance (Charmantier and Garant 2005). Second, the zebra finch is a species with naturally a low degree of extra-pair paternity (Birkhead et al. 1990; Griffith et al. 2010) and therefore probably a relatively low intensity of post-copulatory sexual selection in most of its evolutionary past. Their populations might therefore not be under strong stabilizing selection for an optimal sperm genotype and phenotype (Lifjeld et al. 2010) which might allow for higher additive genetic variance and thus heritability. Third, zebra finch might have a very specific genetic system with heritability of sperm morphology maintained by heterozygote advantage. Recent zebra finch genetic studies found that genes underlying sperm morphology are linked on Z chromosome that has low recombination rate due to large chromosome inversion in some haplotypes (Kim et al. 2017; Knief et al. 2017). Consequently, these genes act as one “supergene” that is inherited in a Mendelian way with heterozygotes (one normal and one inverted Z chromosome) having the fastest sperm and greatest fitness (Kim et al. 2017, Knief et al. 2017). Therefore, we need studies on other species that may have no Z chromosome inversion to see if heritability of sperm traits is indeed lower in those with high risk of sperm competition (sensu Engqvist and Reinhold 2005) , as is predicted by the evolutionary theory.

Apart from genetic effects, sperm traits have been found to vary with certain environmental and ontogenetic factors. For example, sperm and ejaculate traits can change with male age. Older males in some species have longer, faster and more spermatozoa (Green 2003; Gasparini et al. 2010; Laskemoen et al. 2010). Furthermore, sperm morphology was found to vary with the advancement of the breeding season (e.g. Calhim et al. 2009; Lüpold et al. 2012; Cramer et al. 2013, Schmoll, Kleven and Rusche, submitted). However, it is often not clear whether the observed environmental variation was caused by the phenotypic plasticity within individual males or by selective sampling of different males, because mostly only one sperm sample was taken per individual male. Repeated sperm sampling of the same individual at different time points during the breeding season and at different ages is essential for disentangling these two sources of variation in sperm traits (i.e. within-individual vs. between-individual variation).

In this study, we focus on a wild population of the collared flycatcher *Ficedula albicollis*, a species with high risk of sperm competition, to partition environmental and genetic sources of phenotypic variation in sperm morphological traits using quantitative genetic methods based on a large multigenerational pedigreed data set. We demonstrate moderate heritabilities and in parallel pronounced within-individual seasonal phenotypic plasticity of sperm dimensions.

Methods

STUDY POPULATION AND FIELD METHODS

This study was done in 1998-2015 (breeding data for construction of the pedigree) and 2012–2016 (sperm sampling). The study area is situated in Velký Kosíř (49° 32’N, 17° 04’E) in Moravia, Czech Republic. Five study plots are located on a hill (rising 300–400 m above sea level) covered by an oak forest (*Quercus petraea*). In total, the study area harbours around 350 nest boxes (diameter of entrance 32 mm, inner dimensions 12 cm x 11 cm x 24 cm).

The collared flycatcher is a 13 g migratory passerine wintering in Southern Africa and arriving around mid-April on the breeding sites in the Czech Republic (Briedis et al. 2016). In our population, sperm competition is intense as approximately 25% of all chicks are sired by an extra-pair male (Krist et al. 2005; Krist and Munclinger 2011; Edme et al. 2017). Females lay usually 5-7 eggs in the first clutch of the season. Replacement clutches occur in case of clutch failure, but there are no true second clutches.

We caught males twice during the breeding season. First, we trapped them at the time of their arrival with small traps when they entered the nest-boxes when searching for potential breeding sites. These traps were activated in all nest-boxes simultaneously at variable intervals (1-day to 1-week) from mid-April to mid-May depending on the number of newly arrived males on a previous catching day. If many new males appeared, we trapped the subsequent day too, if only few of them appeared, the interval to next trapping was prolonged. We consider the date of first trapping of an individual in any given year as the date of its arrival on the study site, which was validated by data from geolocators carried by 16 males (for details see Edme et al. 2017). Second, we trapped males when they were feeding nestlings. We determined male age by plumage characteristics as either yearling (brownish primaries) or older (black primaries) (Cramp and Perrins 1993). When males were not ringed, we assigned them a unique ring number (Praha ringing center, Czech Republic). All adults and also nestlings were blood-sampled (1–5 µl) from the tarsal vein (males during the arrival and nestling feeding periods, females during the nestling feeding period only).

Sperm samples were collected by a cloacal massage (Quay 1986) of males captured during both the arrival and the nestling feeding periods in 2013-2015. Males were only sampled during the feeding period and only during the arrival period in 2012 and 2016, respectively. All sperm samples were stored in 4% formaldehyde at room temperature or at 8°C in a refrigerator until processing. Such storage does not affect the size measurements of spermatozoa (Schmoll et al. 2016).

SPERM MORPHOMETRY

Microscopic slides were prepared after each breeding season by spreading out seven µl of the sperm solution, let it dry, rinse it with distilled water, and let it dry again. To obtain a sufficient number of spermatozoa for our measurements, we prepared up to three slides per sperm sample in case there were no 20 suitable spermatozoa present in the first slide. A total of 860 sperm samples were collected in the field but 20 or more spermatozoa were found in only 600 of them. These 600 samples belonged to 367 different males (see Table 1). We took photos of 20 morphologically normal looking spermatozoa per sample.

Spermatozoa sampled in 2012 and 2013 were photographed using an Olympus BX51 microscope and an Olympus DP71 microscope digital camera, and those sampled from 2014 to 2016 using an Olympus CX41 microscope equipped with an Infinity 2 digital camera. In both cases a 400x magnification under light-field conditions was used. We included microscope type as a fixed factor in all analyses to control for potential minor differences in set-up/magnification between the two microscopes. For each spermatozoon sperm head, midpiece and tail were measured using ImageJ software 1.49v (Schneider et al. 2012). Sperm total length was calculated by summing up the three sperm sections and flagellum length is the sum of midpiece and tail length. All measurements were made by the same person (PZ) to avoid measurement error resulting from inter-observer differences. As we were mainly interested in between-sample and between-male variation in sperm morphometry and strived to minimize the impact of measurement error, we disregarded within-sample variation (see Table 2 for within-sample CVs) and calculated mean sperm dimensions from 20 individual sperms in each sample. All analyses, except of the estimation of intra-observer measurement error (see below), are based on the mean sperm length per sample. We discuss how usage of dimensions of individual sperms could affect our estimates of heritabilities (see Discussion).

Repeatability and thereby intra-observer measurement error of sperm length was assessed by taking measurements of the same twenty spermatozoa twice for thirty randomly selected sperm samples (but only one sample per male). The thirty sperm samples (i.e. 600 sperm cells) were measured twice in the same order. A linear model was fitted to assess repeatability (response variable: length of sperm section, random factor: sperm identity). The repeatability of the measurements of the length of the different sperm sections ranged from 80.0% to 96.0% (Table 2), as calculated with the R package rptR (Stoffel et al. 2017).

PEDIGREE

The breeding ecology of the collared flycatcher on the study site has been recorded since 1998 and the population is highly philopatric (Krist 2009). Therefore extensive social pedigree information is available. We constructed the pedigree for the years 1998–2015 according to observations in the field where adults were considered the social parents of a brood when providing parental care in the form of nestling provisioning. We pruned the pedigree with respect to information about sperm size using the package pedantics in R (Morrissey 2014). Consequently, the pruned pedigree contained 781 informative individuals and 265 pair-wise paternal-sib relationships, from which 127 were full-sibs and 138 paternal half-sibs. The maximum depth of the pruned pedigree was 10 generations.

We conducted paternity analyses for part of the population in 2001–2002 (Krist et al. 2005), 2006–2009 (Krist and Munclinger 2011) and of the whole population in 2013 (Edme et al. 2017). We did not find any case of intra-specific brood parasitism. Therefore, social mothers were considered genetic mothers in all cases. We determined offspring genetic paternity for 1396 of 7700 offspring in the complete pedigree. 978 of them were sired by social males and 328 by extra-pair males, which constitute a 25.1% rate of extra-pair paternity. The identity of extra-pair fathers was determined for 207 extra-pair offspring. In addition, we also assigned genetic sires for 90 offspring with unknown social fathers. We corrected the social pedigree with all the available genetic information. As we were able to do this only for about 18% of offspring (1396/7700), our estimate of heritability may be lower than the true value. However, for rates of extra-pair paternity up to 40% a simulation study has shown that heritability was underestimated only by 20% as a maximum (Charmantier and Réale 2005) or by 15% as a maximum under non-random extra-pair mating but lower rates of extra-pair paternity (12.5%: Firth et al. 2015).

STATISTICAL ANALYSIS

All analyses were performed in R studio 3.3.1 (R Core Team 2016). In order to investigate environmental *versus* genetic sources of variation in sperm dimensions we first used linear mixed effects models (R package *lme4* version 1.1-12 (Bates et al. 2015) to assess the significance of various fixed effects on sperm phenotype. Based on this phenotypic model we subsequently tested for the contribution of additive genetic effects using a quantitative genetic 'animal model*'* (Kruuk 2004).

As fixed effects we included microscope type (categorical), male age (categorical: yearling/older) and sampling period (categorical: arrival/provisioning). We applied within-subject centering to male age and sampling period in order to allow distinguishing within-individual from between-individual effects for these fixed effects (see van de Pol and Wright 2009). The between-individual predictor is then represented by the mean per individual, while the within-individual predictor is the difference of the mean per individual and the individual measurement (van de Pol and Wright 2009). As both the variables were categorical, we transformed them to assume the values 0 and 1 before applying within-subject centering of the data.

As random effects we included year and male identity to account for the non-independence of spermatozoa from the same year and the same male, respectively, and to calculate repeatabilities of sperm dimensions within males. Repeatability is defined as the proportion of the total variance accounted for by differences between groups. Based on the phenotypic models we calculated the repeatabilities of sperm dimensions for the grouping levels male identity and year using the R package rptR (Stoffel et al. 2017).

We used an animal model to estimate additive genetic variances for the different sperm dimensions. The animal model is a specific mixed-effect model that allows partitioning of genetic *versus* environmental components of the total phenotypic variance of a trait (Kruuk 2004; Wilson et al. 2010). The advantage of the animal model compared to other ways for estimating quantitative genetic parameters (like parent-offspring regression) is the use of multi-generational pedigree information even if this information is incomplete or complex. Our animal model was based on the phenotypic model described above (i.e. year and male identity as random effects and male age, period of sampling and microscope type as fixed effects) with the addition of the pedigree as another random effect. Such model allows separation of permanent environmental effect (V_{pe}) from additive genetic effects (V_a) and thus estimation of the narrow-sense heritability (h^2) of the trait which is defined as the proportion of phenotypic variance (V_p) explained by additive genetic effects: $h^2 = V_a/V_p$. Moreover, in bivariate animal models we also determined covariance between sperm sections to test if they can evolve independently of one another. We calculated genetic correlation between traits x and y as: $r_a = COV_{a(xy)} / \sqrt{} [V_{a(x)} \times V_{a(y)}]$, where COV_a is the additive genetic covariance between the two traits and V_a is their additive genetic variance (Falconer 1989, p. 317). All quantitative genetic analyses were done in the package ASReml-R (Butler 2009).

Results

SEASONAL AND AGE EFFECTS ON SPERM DIMENSIONS

While sperm dimensions did not differ between yearlings and older males, they varied significantly with sampling period (Tables 3–5). Specifically, head size and midpiece increased in length during the season, while tail demonstrated an opposite change (Table 5, Figure 3). Consequently, length of midpiece and tail were strongly correlated on the phenotypic level ($r = -0.558$, $n = 600$, $p < 0.001$) while the other two remaining relationships were much weaker (head – midpiece: $r = 0.105$, $p = 0.010$; head – tail: $r = -0.091$, $p = 0.026$). In absolute terms, the seasonal change was largest for the longest section, midpiece, which led to longer flagella and whole spermatozoa in the feeding period (Tables 4–5, Figure 3). The effects within individuals and between individuals were in the same direction. Both these effects were also significant in most models (Tables 4–5). The within-individual effect demonstrates within-male changes over the breeding season, representing individual phenotypic plasticity in sperm components and total length. The presence of a between-individual effect suggests that in the pre-breeding stage there were both males with short and long sperm in the population while only those with longer sperm were later found breeding (see Table 1, Figure 3).

QUANTITATIVE GENETICS OF SPERM DIMENSIONS

Based on the phenotypic model, we found significant within- male repeatability, which was higher for flagellum and total length and lower but still significant for individual sperm sections (Table 2). Head and tail length were also significantly repeatable within a year (Table 2). Similarly as for repeatability, heritability of total sperm length and flagellum were higher ($h^2 = 0.45 \pm 0.14$ SE and 0.41 ± 0.14 SE, respectively) and significant (Table 6, Figure 1), compared to smaller heritabilities for separate sperm sections ($h^2 = 0.11–0.21$), the latter of which were not significant (Table 6, Figure 1). This contrast was caused by the relatively smaller additive genetic variances for the separate sperm sections as compared to larger permanent environmental variation (midpiece and tail) or annual variation (head) as well as increased unexplained, residual variance (Table 6, Figure 2).

Additive genetic covariance and correlation between length of midpiece and tail was small and positive which is in contrast to large, negative residual covariance of these two components (Table 6). Thus the strong negative phenotypic correlation between midpiece and tail (see above) is solely environmental in origin. In some contrast, additive genetic covariance between head and tail was relatively large and negative resulting in high estimate of genetic correlation (Table 6) which might suggest evolutionary constraint imposed on these two sperm sections. However, these results must be treated with a caution since all estimates of genetic covariance and correlations had wide standard errors (Table 6) suggesting that our dataset had only limited power in this respect.

Discussion

GENETIC SOURCES OF VARIATION

In order to partition environmental and genetic sources of variation in avian sperm size in a wild bird population, we have estimated repeatabilities and heritabilities of sperm dimensions in the promiscuous collared flycatcher. We found consistent between-male variation in sperm dimensions as evidenced by within-male repeatabilities ranging from 0.26 to 0.61. In line with these within-male repeatabilities, also heritabilities of sperm size were moderate in our population. Heritability of sperm total length amounted to 0.45 ± 0.14 SE and heritabilities for sperm sections ranged from 0.11 to 0.21 and were statistically insignificant.

Moreover, these values might be inflated because we did not systematically conduct partial cross-fostering experiments that would enable us to separate maternal or common nest effects from additive genetic effects with a high statistical power. On the other hand, our heritability estimates are largely uncontrolled for paternity errors in the pedigree (see Methods). As these two sources of inaccuracy bias our heritability estimates in opposite directions (unexplained environmental effects up, paternity errors down), they may partially cancel one another and our heritability estimate may be close to real value. However, it is also important to realize that heritability estimates are conditioned on the structure of the statistical model used to calculate them (Wilson 2008). For example, if we considered also within-sample variation in sperm length and used individual sperm cells instead of the sample mean for calculation of heritabilities, these would be much lower due to larger unexplained, residual variation. Heritability of total sperm length would decrease to 0.20 ± 0.06 SE in such a model (results not shown). Similarly, heritability would also decrease to 0.36 ± 0.13 SE if we used mean sperm length per sample but did not fit any fixed effects that decrease amount of residual variation (results not shown). Taken together, although we used the model that decreased residual variation and provided relatively high heritabilities of sperm dimensions in the collared flycatcher, these were still substantially lower than those reported by Birkhead et al. (2005: h^2=0.45–0.68) and Mossman et al. (2009: h^2=0.63) for captive populations of zebra finches. There are several potential explanations for the differences between the two species.

First, sperm competition is less intense in the zebra finch [rate of extra-pair paternity (EPP): 2% in wild populations (Birkhead et al. 1990; Griffith et al. 2010)] compared to our study population with 25% of EPP. Stabilizing selection on sperm length was apparently intense in the evolutionary past of species with high risk of sperm competition, such as the collared flycatcher (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010) but rather relaxed in species with low risk of sperm competition, such are wild populations of zebra finch, although the selection pressure due to sperm competition may be higher in captive populations (see Forstmeier et al. 2011). Different evolutionary histories might have led to low additive genetic variances in sperm morphology in the species with high risk of EPP but to larger genetic variation in species with low risk of EPP, similarly as traits related closely to fitness often show lower heritabilities than those less closely related to fitness (Houle 1992; Kruuk et al. 2000; Teplitsky et al. 2009). Consequently, the collared flycatcher may have decreased genetic variance for sperm morphology due to intense selection in its evolutionary past despite this selection does not appear to work at present as evidenced by insignificance of sperm length for paternity success (see Edme et al. 2017).

Second, captivity and its highly controlled environments in the study by Birkhead et al. (2005) may inflate the heritability compared to wild populations where any (unmeasured) environmental effects boost residual variance and hence decrease the heritability that can be realized in the natural environment where sperm traits have, and are being, evolved. Furthermore, additive genetic variances have a tendency to be higher in favorable compared to stressful environment (Charmantier and Garant 2005). Consequently, heritability may be lower in the wild compared to laboratory conditions because of both increased environmental and decreased additive genetic variation. The distinction between these two scenarios is usually very challenging (Teplitsky et al. 2009).

Third, zebra finch might in fact have a very specific architecture of genes controlling sperm morphology. Two recent studies found these genes are located on Z chromosome and due to strong linkage disequilibrium effectively work as one “supergene” (Kim et al. 2017; Knief et al. 2017). The linkage disequilibrium is caused by the inversion of large central part of Z chromosome that limits recombination between inverted and normal haplotypes. Haplotypes are then inherited in a Mendelian way and the phenotype largely depends on whether the animal is a homozygote or a heterozygote for normal and inverted haplotype (Kim et al. 2017; Knief et al. 2017) which explains high heritability of sperm morphology as well as negative genetic correlation between some sperm sections (Birkhead et al. 2005). As heterozygotes have an advantage in the form of longest and fastest sperm (Kim et al. 2017; Knief et al. 2017), both haplotypes are maintained in the population.

Lower heritabilities of sperm morphology in the collared flycatcher suggest more conventional, polygenic, inheritance of these quantitative traits as has also been found for other morphological traits in this species (Silva et al. 2017). This view is also supported by a genomic study which found high recombination rate within flycatcher Z chromosome which is in contrast to the low recombination rate in the central part of Z chromosome in the zebra finch (Kawakami et al. 2014). Furthermore, in line with this difference in the strength of linkage disequilibrium, strong negative genetic correlation was found between length of midpiece and flagellum (and therefore tail) in the zebra finch (Birkhead et al. 2005) but the negative phenotypic correlation between the same components that was also found in the collared flycatcher seems to be entirely of environmental origin.

Although the genome of the collared flycatcher is also sequenced (Ellegren et al. 2012; Kawakami et al. 2014), we still do not know which genes are responsible for sperm morphology in this species. Kim et al. (2017) identified several genes explaining variance in sperm morphology in zebra finches. Evaluation of the role of these candidate genes for sperm morphology in the collared flycatcher and other species would be useful next step to our understanding of genetic base of sperm competition in birds.

ENVIRONMENTAL SOURCES OF VARIATION

As in some other studies on birds (Møller et al. 2009; Laskemoen et al. 2010; Rowe et al. 2010) we found no effect of male age on sperm size. This is in contrast to other taxa such as insects and fish where sperm size increased with male age (Green 2003; Gasparini et al. 2010). Reasons for the different sperm size dynamics over male ontogeny in different taxa are not clear at present. Surely more studies on the same and other taxa are needed to confirm the generality of these patterns and to search for their explanations.

On the other hand, our results clearly show that sperm size changed with the advancement of the breeding season. Indeed, spermatozoa were longer during the nestling period compared to the time when males arrived at the breeding site. Interestingly, the observed changes were not uniform as head, midpiece and flagellum increased in size while the tail became smaller with the season. These results are in agreement with previous studies (Lüpold et al. 2012; Cramer et al. 2013b) where length of spermatozoa or their sections also increased in the course of the season. As we sampled the same males repeatedly, we were also able to determine whether seasonal variation in sperm size is due to within- or between-individual effects. Significant between-male effects suggested that males with longer sperm have greater chance to breed and therefore be caught at the nestling stage while those with shorter sperms appeared in the population only in the pre-breeding stage. However, observed seasonal changes were also present within individual males, also demonstrating substantial phenotypic plasticity in sperm size in response to advancing season. This may either reflect adaptive phenotypic adjustment or constraints imposed on sperm production.

If males had made a strategic allocation of resources into sperm production, they would probably invest in sperm with higher competitive ability early in the breeding season. Later in the season the number of receptive females drops and males are expected to invest more in parental care and molt than in sperm production. According to this hypothesis, sperm should be longer early in the season (Calhim et al. 2009) as longer sperm may swim faster and may be more efficient in sperm competition (Bennison et al. 2015). However, this was not the case in our study, as sperm were shorter at the start of the season. Moreover, we also did not find any evidence for greater success of longer sperm in sperm competition in the study population (Edme et al. 2017).

Consequently, seasonal changes in sperm length may rather be the result of a time constraint imposed on this migratory species instead of representing male strategic allocation. Although males start to produce sperm already during migration (Bauchinger et al. 2007), their spermatozoa may not have reached full maturity just after arrival, as spermatogenesis until full maturation needs about two weeks (Aire 2007). Another possibility is that males copulate a lot at the beginning of the breeding season and therefore, on average, less mature, and thus shorter spermatozoa are obtained in sperm samples early in the year. Indeed, spermatozoa tend to be shorter when ejaculates are sampled from the same male successively (Harris et al. 2007; Crapa et al. unpublished data). This may reflect a trade-off between awaiting the availability of longer sperm (longer midpiece and thus flagellum) which allows sperm to swim faster and may increase competitive fertilization success (LaMunyon and Ward 1998; Bennison et al. 2015 but see

Cramer et al. 2013a; Edme et al. 2017) and copulating at a higher rate using shorter sperm that are immediately available. The latter strategy may be adaptive if shorter sperm can be produced at a higher rate during the period with highest risk of sperm competition (Immler et al. 2011).

CONCLUSIONS

In this study, we demonstrated moderate repeatability and heritability of sperm morphology in a species with high risk of sperm competition which is in contrast to higher repeatability and heritability of these traits in the zebra finch. The lower heritability in the collared flycatcher can be caused by high rate of extra-pair paternity that raised stabilizing selection on optimal sperm phenotype in the collared flycatcher. Alternatively, zebra finch might have sperm size more heritable due to the specific genetic architecture that causes sperm morphology to vary in a Mendelian way. Sperm morphology was also plastic and changed during the breeding season in the collared flycatcher. Spermatozoa had longer heads and midpieces but shorter tails in later stages of the season. This may reflect the time constraint imposed on males by their need to copulate at a high rate shortly after arrival to the breeding grounds when sperm maturation is still not complete.

Quantitative genetic studies of sperm morphology in other species would be useful to test if heritability of sperm morphology is indeed dependent on the risk of sperm competition or on the genetic architecture. The latter might be very specific in the zebra finch but more representative in the collared flycatcher where recombination of involved genes is probably common and thus sperm morphology is inherited like a polygenic character. It would also be interesting to test if seasonal changes in sperm morphology are evident only in migratory species which could reflect time constraint to breed early or are paralleled in sedentary species too which would suggest other constraint or, by contrast, adaptiveness of phenotypic plasticity of sperm traits.

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Table 1: Number of sperm samples with 20 spermatozoa, collected from collared flycatchers either during the period of spring arrival (median date 24th April) or during the period of nestling provisioning (median date 27th May), including the number of males that were sampled in both periods.

Year	Arrival	Nestling provisioning	Total	Males sampled twice
2012	-	30	30	-
2013	130	38	168	28
2014	110	59	169	40
2015	107	38	145	29
2016	78	10	88	-

Table 2: Within-sample coefficient of variation (CV) and repeatabilities (with 95% CI) of sperm dimensions calculated on various levels. Within-sample CV was calculated as the mean ± standard deviation (SD) of 600 individual within-sample CVs, each based on 20 sperm cells. Repeatability of measurement of the same spermatozoa measured twice by the same person and repeatabilities of mean sperm size (means from 20 individual sperms of the sample) within individual males and years of sampling controlled for the fixed effects (male age, period of sampling and microscope type) in the phenotypic model.

	Within-sample CV	Repeatability		
	(N=600 samples)	Measurements (N=600 sperm cells measured twice)	Male identity (N=600 samples from 367 males)	Year (N=600 samples from 5 years)
Total sperm length	3.14±1.35	0.95 [0.95–0.96]	0.60 [0.53–0.66]	0.006 [0–0.03]
Flagellum length	3.45±1.51	0.96 [0.95–0.96]	0.59 [0.52–0.67]	0.03 [0–0.08]
Head length	7.09±1.88	0.80 [0.76–0.82]	0.26 [0.16–0.39]	0.20 [0.007–0.44]
Midpiece length	5.71±3.91	0.96 [0.95–0.96]	0.40 [0.32–0.49]	0.01 [0 –0.04]
Tail length	20.84±9.63	0.91 [0.90–0.92]	0.48 [0.40–0.58]	0.02 [0.01–0.07]

Table 3: Mean length ± standard deviation (SD) (µm) of sperm sections for arrival (median date 24th April, n=8500 sperm cells) and nestling provisioning (median date 27th May, n=3500 sperm cells) periods.

	Arrival	**Provisioning**
Total sperm length	97.99±4.68	99.47±4.00
Flagellum length	86.99±4.56	88.27±3.90
Head length	11.00±1.04	11.19±0.97
Midpiece length	68.88±5.98	71.03±4.72
Tail length	18.10±5.34	17.24±4.06

Table 4: Phenotypic models for sperm total length and flagellum length. Results of a linear mixed effects model where fixed effects of male age and sampling period were within-male centered and male identity and year were included as random effects. Positive estimates mean longer sperm for old males and in the provisioning period.

	a) Total sperm length				**b) Flagellum length**			
Random effects	Variance				Variance			
Male identity	5.368				5.396			
Year	0.049				0.318			
Residual	3.440				3.294			
Fixed effects	Estimate ± S.E.	df	t	P	Estimate ± S.E.	df	t	P
Intercept	92.727±1.095	16.2	84.6	<0.001	82.778±1.402	11.2	59.1	<0.001
Age (between males)	-0.269±0.358	418.5	-0.7	0.425	-0.336±0.356	422.9	-0.9	0.346
Age (within males)	0.025±0.459	169.2	0.1	0.956	-0.043±0.462	253.9	-0.1	0.924
Microscope	2.270±0.338	2.1	6.7	0.017	1.712±0.597	3.2	2.9	0.058
Period (between males)	1.753±0.460	294.5	3.8	<0.001	1.627±0.467	406.3	3.5	<0.001
Period (within males)	1.544±0.225	222.1	6.9	<0.001	1.214±0.223	239	5.4	<0.001

Table 5: Phenotypic models for sperm components. Results of a linear mixed effects model where fixed effects of male age and sampling period were within-male centered and male identity and year were included as random effects. Positive estimates mean longer sperm for old males and in the provisioning period.

	(a) Head length				(b) Midpiece length				(c) Tail length			
Random effects	Variance				Variance				Variance			
Male identity	0.091				4.816				3.415			
Year	0.072				0.128				0.126			
Residual	0.187				6.829				3.578			
Fixed effects	Estimate ± S.E.	df	t	P	Estimate ± S.E.	df	t	P	Estimate ± S.E.	df	t	P
Intercept	9.755±0.462	4.4	21.1	<0.001	64.848±1.369	14.2	47.3	<0.001	17.939±1.128	18.7	15.9	<0.001
Age (between males)	0.048±0.062	462.5	0.78	0.440	-0.419±0.406	446.7	-1.0	0.303	0.187±0.317	447.1	0.6	0.556
Age (within males)	0.092±0.109	290.4	0.9	0.396	0.195±0.647	224.7	0.3	0.763	-0.334±0.473	252.9	-0.7	0.480
Microscope	0.644±0.253	3.1	2.5	0.080	1.529±0.477	2.6	3.2	0.060	0.119±0.425	4.0	0.3	0.793
Period (between males)	0.165±0.083	475.0	1.9	0.047	2.133±0.523	306.8	4.1	<0.001	-0.527±0.412	379.1	-1.3	0.201
Period (within males)	0.345±0.053	273.3	6.5	<0.001	2.411±0.317	243.6	7.6	<0.001	-1.206±0.231	235.1	-5.2	<0.001

Table 6: Variance components from univariate and covariance parameters from bivariate animal models for sperm total length and the sperm sections head, midpiece, tail and flagellum (midpiece and tail combined). Subscripts a, pe, year and r refer to additive genetic, permanent environmental, annual and residual variance respectively. Heritabilities and genetic correlations (r_a) were calculated using equations given in Methods. Standard errors (SE) are provided with all estimates.

	V_a	V_{pe}	V_{year}	V_r	Heritability
Total length	3.983±1.260	1.456±1.101	0.027±0.077	3.438±0.318	0.447±0.143
Flagellum length	3.724±1.224	1.728±1.083	0.277±0.286	3.284±0.303	0.413±0.136
Head length	0.065±0.032	0.030±0.031	0.071±0.060	0.184±0.018	0.186±0.096
Midpiece length	2.466±1.325	2.388±1.347	0.102±0.176	6.799±0.611	0.209±0.136
Tail length	0.798±0.853	2.632±0.910	0.125±0.145	3.572±0.321	0.112±0.120
	COV_a	COV_{pe}	COV_{year}	COV_r	r_a
Head – Midpiece	0.115±0.147	-0.197±0.147	-0.061±0.091	0.005±0.0773	0.278
Head – Tail	-0.173±0.117	0.172±0.120	-0.074±0.078	-0.056±0.056	-0.828
Midpiece – Tail	0.187±0.809	-1.24±0.874	-0.169±0.212	-4.32±0.442	0.121

Figure Legends

Figure 1: Heritability (mean ± SE) of total sperm length and sperm components from the animal model.

Figure 2: Relative contribution of particular variance components to the total phenotypic variance of sperm components as decomposed by the animal model. V_a: additive genetic variance, V_{pe}: permanent environmental variance, V_{year}: annual variance; V_r: residual variance. Note that the height of V_a corresponds to heritability while the height of V_a+V_{pe} corresponds to repeatability.

Figure 3: Variation of spermatozoa length (black dots are means per male) according to the period of sampling (arrival at the breeding site and provisioning of the chicks). Thin black lines connect values of the same male sampled in the two periods and thus represent within-male variation while blue lines show the best fit of the between-male variation.

Figure 1

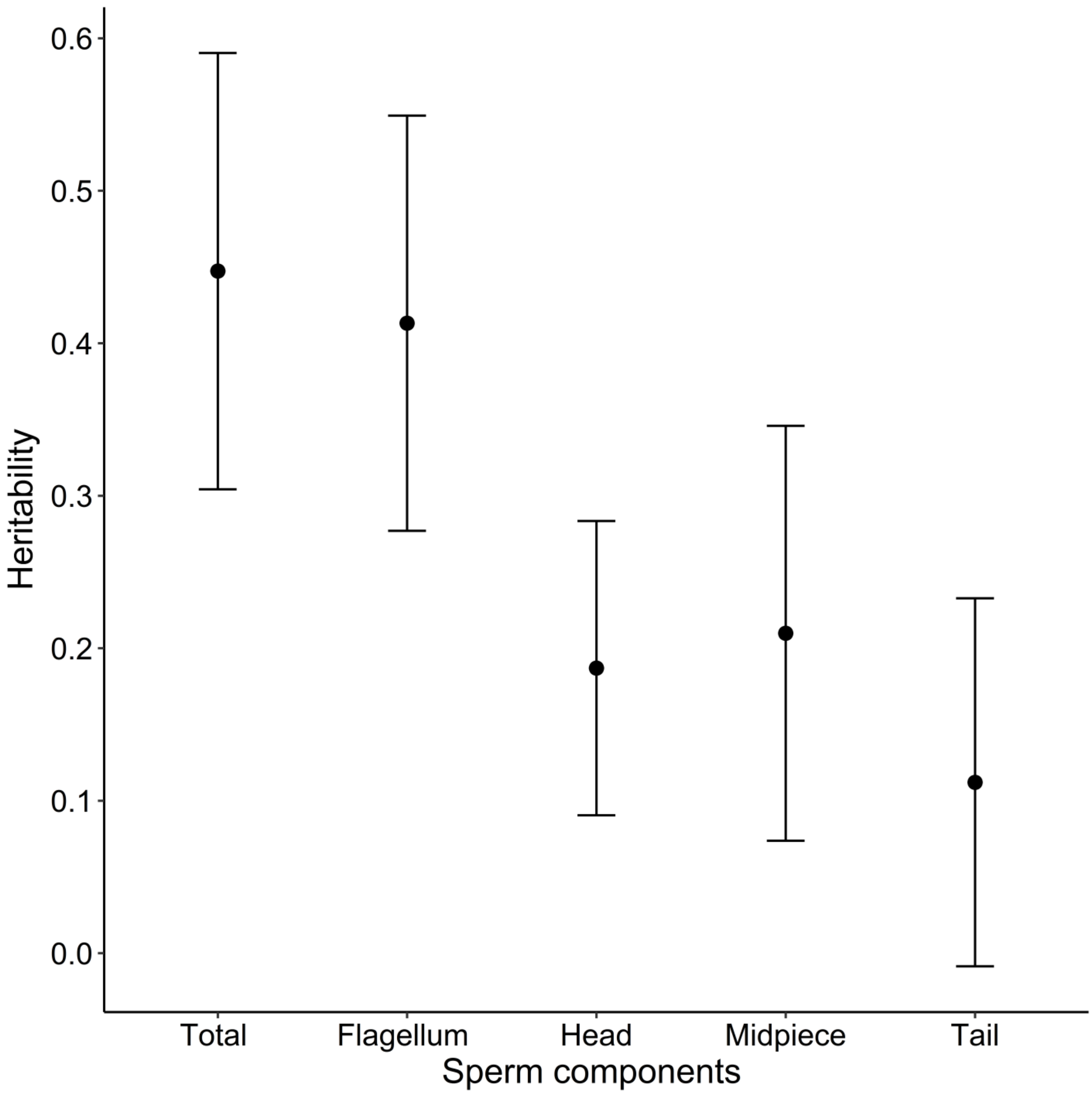

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Heritability
Total
Flagellum
Head
Midpiece
Tail
Sperm components

Figure 2

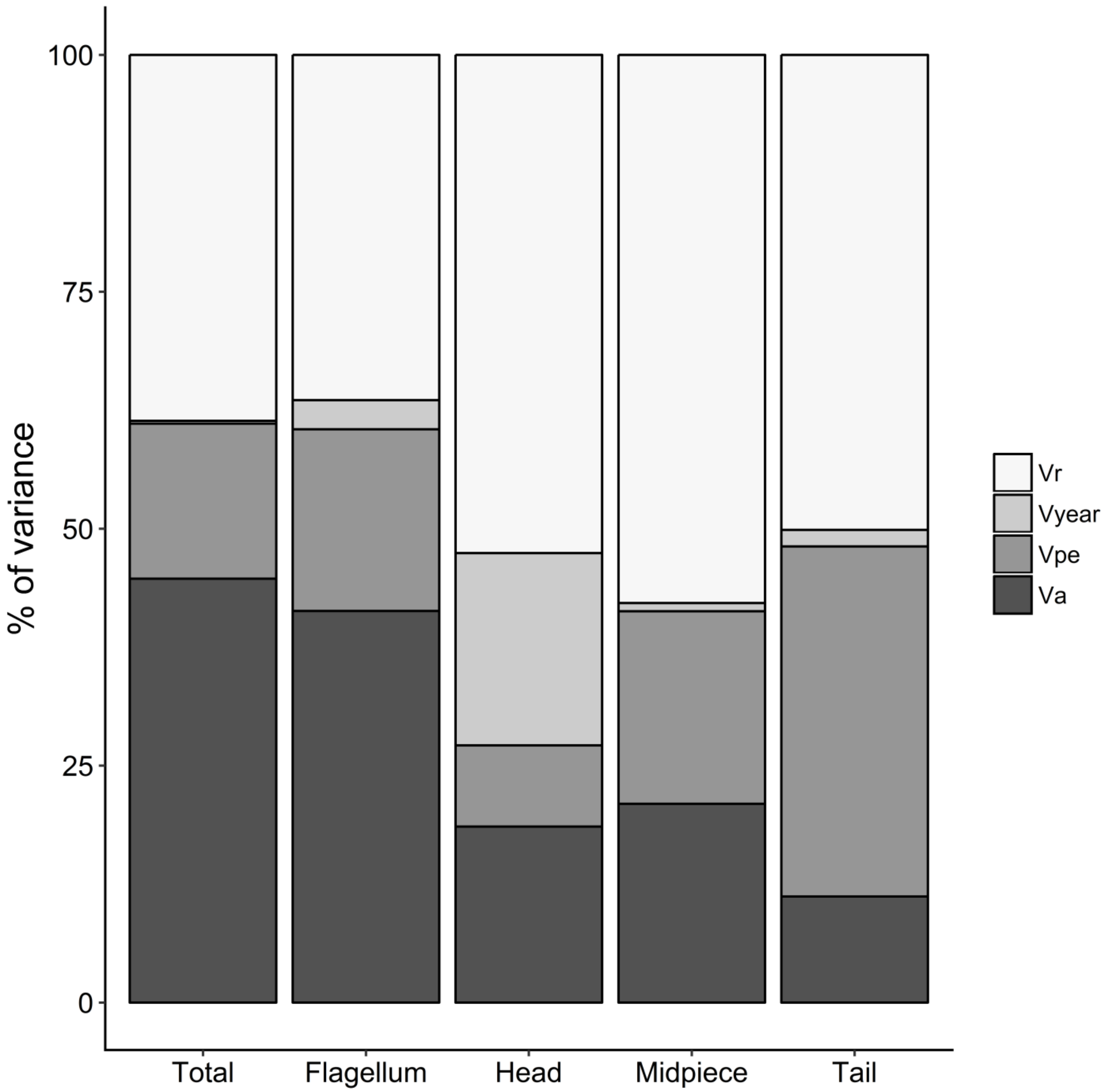
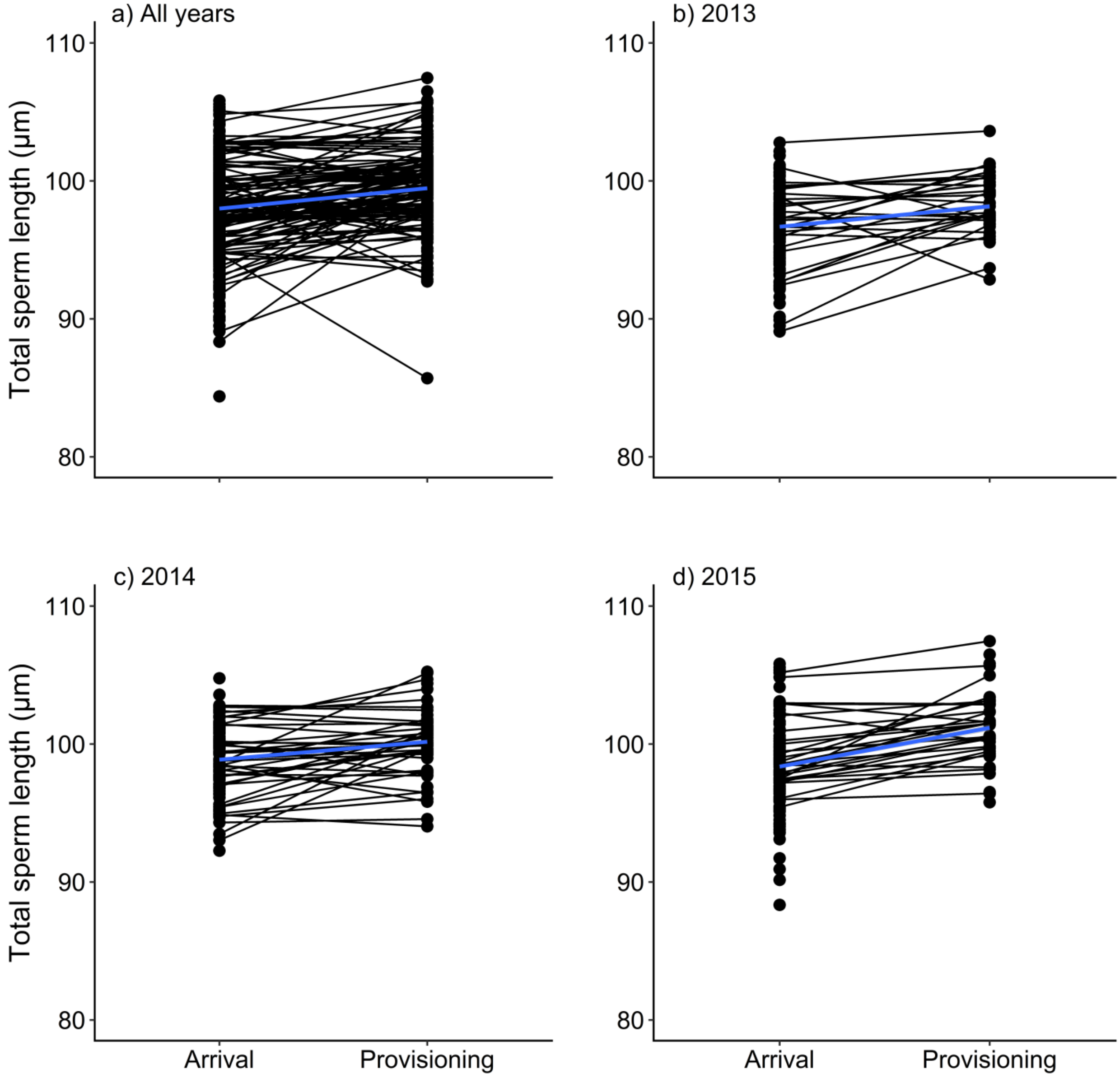

% of variance
100
75
50
25
0
Total
Flagellum
Head
Midpiece
Tail
Vr
Vyear
Vpe
Va

Figure 3


a) All years
b) 2013
c) 2014
d) 2015
Total sperm length (μm)
110
100
90
80
Arrival
Provisioning

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[In French below because my family and most of my friends do not understand Shakespeare's language]

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[*In French, for English reader dummies*]

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Palacký University Olomouc

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Pre- and post-copulatory sexual selection in the collared flycatcher (*Ficedula albicollis*)

Pre- a post-kopulační pohlavní výběr u lejska bělokrkého (*Ficedula albicollis*)

Summary of the Ph.D. thesis / Autoreferát disertační práce

Anaïs Edme

Supervisor: Mgr. Miloš Krist, Ph.D.

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Abstract

Sexual selection is one of the forces that lead evolution and may happen at different times during the reproductive period. Mate choice and extra-pair mate choice are forms of pre-copulatory selection. Secondary sexual traits may be indicators of male quality and females should express preferences for those traits. Males with larger ornaments may be able to obtain more partners and increase their number of offspring. Females may obtain direct benefits and/or indirect genetic benefits from their social mate. But when mated to non-preferred males, females may choose an extra-pair mate with superior traits than their social mate, since females usually do not receive direct benefits from extra-pair mates and are therefore expecting to receive indirect benefits. As females copulate with many males, sperm from different males compete to fertilize the set of ova. Sperm length should influence the outcome of sperm competition and the ability to produce more offspring. Under strong sexual selection (high rate of extra-pair paternity), stabilizing selection should decrease the genetic additive variance of sperm length to allow an optimum sperm morphology to outcompete sperm from other males.

This thesis focuses on the pre- and post-copulatory steps of sexual selection in the collared flycatcher (*Ficedula albicollis*), a migratory bird with a high rate of extra-pair paternity. Secondary sexual traits were manipulated to determine which traits females used to select mates. Genetic analyses allowed the assignment of offspring to their genetic fathers and determined the rate of extra-paternity. Traits of males that shared paternity were compared. Arrival date and proximity of males were determined as they may influence the outcome of mate choice and paternity. Sperm were sampled at different periods of the breeding season to estimate the dynamics of sperm length variation. Long-term pedigree data and a quantitative model helped to separate the genetic and environmental variances that influence sperm length.

Females did not demonstrate a consistent preference for more ornamented males as social mates. Males with large ornaments were less attractive to females late in the season and lost more within-pair paternity. Extra-pair males were not more ornamented than the males they cuckolded but both social and extra-pair males were superior to males around the females' nests. Early arrival at the breeding site allowed males to protect their within-pair paternity. Males did not win more extra-pair offspring when they were more ornamented and sperm length did not influence fertilization success. Sperm length demonstrated phenotypic plasticity throughout the breeding season and had moderate heritability.

Future research should focus on the environment in which sperm competition takes place. Indeed, studies trying to identify sperm traits that make sperm successful in fertilization focus on sperm traits before the bottleneck of cryptic female choice. More studies on the heritability of sperm traits in wild populations and especially on the effect of the environment on sperm length are needed to understand how it is influenced by the pressure of selection. The genomes of many species have been sequenced, but the genes which code for sperm morphology have largely been ignored. More knowledge about the genes coding for these traits will help in understanding the genetic process acting on sperm diversity.

Abstrakt

Pohlavní výběr je důležitou evoluční silou, která se může projevovat v různých fázích reprodukčního období. Výběr sociálního a extra-párového partnera jsou formy pohlavního výběru, které probíhají před kopulací. Sekundární pohlavní znaky mohou být indikátory samčí kvality, a proto by pro ně samice měly mít preference. Samci s většími ornamenty mohou získat více partnerek a zvýšit tak počet svých potomků. Samice mohou volbou svých sociálních partnerů získat přímý i nepřímý, genetický užitek. Když ale mají samice nepreferovaného sociálního partnera, mohou si za toho extra-párového vybrat samce s lepšími znaky, než jaké má ten sociální, protože extra-pároví samci samicím žádný přímý užitek neposkytují a tak se dá očekávat, že jim poskytují užitek nepřímý. Když samice kopuluje s více samci, konkurují si jejich spermie o oplodnění sady vajíček. Výsledek této kompetice spermií a tedy počet potomků by měl být ovlivněn délkou spermií. Pokud je pohlavní výběr silný, jak tomu bývá v případě vysoké míry extra-párové paternity, stabilizující selekce by měla snižovat aditivní genetickou varianci pro délku spermie, v důsledku čehož by měly spermie optimální morfologii pro vítězství v konkurenci s ostatními samci.

Tato práce se zaměřuje na pre-kopulační a post-kopulační fáze pohlavního výběru u lejska bělokrkého (*Ficedula albicollis*), migrujícího pěvce s vysokou mírou extra-párové paternity. Manipulovali jsme sekundární pohlavní znaky samců, abychom zjistili, zda si na jejich základě samice vybírají partnera. Genetické analýzy nám umožnily přiřadit k mláďatům jejich genetické otce a zjistit míru extra-párové paternity. Srovnávali jsme pak znaky samců, kteří zplodili mláďata ve stejném hnízdě. Zaznamenávali jsme také přílet samců na hnízdiště a vzdálenosti mezi jednotlivými hnízdy, což jsou faktory, které mohou ovlivňovat výběr partnera a paternitu. Spermie jsme odebírali v různých fázích hnízdní sezóny, abychom zjistili, jak se v sezóně mění jejich délka a variabilita. Na základě dlouhodobých pozorování jsme sestavili rodokmen dané populace a pomocí kvantitativně genetického modelu jsme zjistili jaká část variability v délce spermií je daná geneticky a jaká prostředím.

Samice si nevybíraly za sociální partnery jen samce s většími ornamenty. Samci s většími ornamenty byli dokonce na konci sezóny pro samice méně atraktivní a měli ve svých sociálních hnízdech více extra-párových mláďat. Extra-pároví samci neměli větší ornamenty než samci sociální, ale obě tyto skupiny měly větší ornamenty než jiní samci dostupní v blízkosti sledovaných hnízd. Časný přílet umožnil samcům lepší ochranu paternity ve vlastních hnízdech. Samci s velkými ornamenty nezplodili více extra-párových mláďat v jiných hnízdech a ani délka spermií neměla vliv na úspěch oplodnění. Délka spermií vykazovala v průběhu sezóny fenotypickou plasticitu a byla středně heritabilní.

Další výzkum by se měl zaměřit na prostředí, ve kterém probíhá kompetice spermií. Dosavadní studie, které se pokoušely zjistit, jaké znaky spermií mají vliv na jejich fertilizační úspěšnost, neuvažovaly možný vliv kryptické volby samice. Pro lepší porozumění selekčních tlaků na morfologii spermií je třeba více studií, které by zjišťovaly heritabilitu tohoto znaku v přírodních populacích a také jeho ovlivnění prostředím. Třebaže genomy mnoha druhů jsou již osekvenovány, geny zodpovědné za znaky spermií stojí dosud stranou zájmu. Jejich znalost by nám napomohla pochopit, jakou roli hrají genetické procesy v diverzitě spermií.

Introduction

Sexual selection may favor individuals that have a greater ability to reproduce. However, males and females may diverge in their interests when trying to increase their fitness. Males often display large secondary sexual traits that may indicate their quality and may be preferred by females (Andersson 1994). Those traits benefit males, increasing their reproductive success by obtaining more social mates. Females express preferences for those more ornamented males, and will increase their direct benefits and fitness by mating with them. In monogamous breeding systems, males are constrained by the number of females as they mate only with one female. A way to increase their fitness is to engage in extra-pair copulation. By copulating with other females, they will produce more offspring without paying the cost of paternal care. However, seeking extra-pair copulations could increase the risk of losing paternity in their own nest.

For females, mating with many males does not lead to higher fecundity so polyandry does not seem to be an advantageous behavior. Especially in birds, females obtain only sperm from their extra-pair mate. One of the expected roles of extra-pair copulation is to ensure that females obtain enough sperm, and importantly viable sperm, if their mates are infertile (Hasson and Stone 2009). But infertility in nature is highly selected against and this hypothesis receives little support. However, females should obtain indirect genetic benefits through this behavior (Akçay and Roughgarden 2007; Barbosa et al. 2012). They should increase the fitness of their offspring and there are two main theories that explain by which mechanisms they increase their fitness: the sexy-son hypothesis and the good genes hypothesis. In the first case, sons inherit their fathers' sexual traits and are more attractive than the mean of other males. In the second hypothesis, the viability of offspring is increased through higher survival ability. In both cases, females should express preferences for males expressing traits above the mean of the population. Females may also look for more compatible genes which increase the viability of offspring via a good combination of maternal and paternal genes (Neff and Pitcher 2005).

Many studies have considered female preference as static, but this view is too simplistic and mate choice may be context-dependent (Qvarnström 2001; Hale 2008; Robinson et al. 2012). Ecological and social factors could influence the opportunity for mate choice and extra-pair paternity. Protandry is widespread in migratory birds and may be explained by the opportunity to obtain better territories and/or more mate opportunities (Morbey and Ydenberg 2001). In addition to ecological context, social context like the proximity of males, especially distances between preferred males, could also influence the involvement of females in extra-pair paternity (Westneat and Mays 2005; Schlicht et al. 2015).

When females are willing, or forced, to copulate with males other than their social partner, sperm compete to fertilize the set of ova (Parker 1970; Pizzari and Wedell 2013). This sperm competition will lead to a bias in fertilization for those males which are able to provide enough sperm and/or sperm with a higher fertilizing capacity. Many factors can influence the outcome of sperm competition like number of sperm, length of sperm and viability of sperm (Immler et al. 2011; Lüpold et al. 2011; Bennison et al. 2015). The order of copulation and the number of copulations may also influence the reproductive success (Crowe et al. 2009). Among all these factors, longer sperm seems to provide an advantage for fertilization (Bennison et al. 2015). Moreover, if optimal sperm length allows fertilizing more eggs, stabilizing selection around the mean should decrease the variability of length within and also between individuals (Lifjeld et al.

2010). However, the part of variance in sperm length attributed to genetic and environmental factors is not well known as only a few studies have focused on it and none in a wild population.

Thesis Focus

Mate choice, extra-pair mate choice and the role of ornamentation have been plentifully studied. However, these studies have mainly considered mate choice as being static during the breeding season. The study of variation in mate choice according to seasonal context but also between populations of the same species in two ecological contexts is lacking. Moreover, sperm traits are also factors that influence the outcome of paternity and are rarely considered in combination with mate choice and ecological factors. The heritability of sperm length in wild populations has never previously been studied. The key aims of my thesis are to determine (1) which traits influence mate choice and extra-pair mate preference, (2) the influence of early arrival on breeding site on paternity success and (3) the heritability of sperm length in a wild population of birds with a high risk of sperm competition.

Thesis outline

Chapter 1 of this study explores all of the stages of sexual selection. A secondary sexual trait known to be preferred by females in other population was modified and all components of sexual selection were investigated: mate choice, female investment, paternity and extra-pair paternity success, the influence of sperm length on paternity and finally fledgling and recruit success. Large ornaments were not preferred by females early in the season and were less attractive late in the season as males with enlarged ornaments needed more time to pair. Females modulate their investment in reproduction according to the arrival date of their mate but not according to ornaments. More ornamented males did not obtain more offspring, chicks did not fledge or recruit more than less ornamented males, and sperm length did not influence paternity success. We discuss the variation in preference for different secondary sexual traits in two populations of collared flycatchers and the implication of the absence of an effect of sperm length on paternity.

In **chapter 2**, the factors that influence paternity within the social nest were investigated and the traits of males who share paternity were compared. As females are expected to choose extra-pair mates according to their preference, they should prospect in a close area to find males with superior traits to their social mate. The proximity of other males may favor the encounter of unfaithful birds. If females are paired to highly ornamented males, they should prospect farther to find a suitable extra-pair partner. Here, females' social, extra-pair mates and available males in a close area around the female's nest were compared. We found that more ornamented males more often share paternity in their nest. The cuckolded males were similar to the males sharing paternity in their nest, but both of them were superior to neighboring males. We discuss the strategies of both males and females in obtaining extra-pair copulations and their potential costs and benefits.

Chapter 3 is, to our knowledge, the first to estimate the heritability of sperm length in a wild bird population under a high level of sexual selection. In this study, sperm were sampled for five years and during two periods of the breeding season. The collared flycatcher is under strong sexual selection that should lead to stabilizing selection toward sperm morphology and consequently decrease the genetic additive variance of sperm traits. Many studies of sperm length have been conducted in farmed or laboratory species with environmental control that could influence the strength of environmental effects. High environmental variance and stabilizing selection could decrease the heritability of traits. Animal models are strong tools to tease apart the environmental and genetic parts of phenotypic variance. We discuss the role of migration and the rate of copulation on the phenotypic plasticity of sperm length. We found moderate additive genetic variance; we discuss the influence of a controlled environment and high sexual selection pressure via a high rate of extra-pair paternity. We also compared our results with the zebra finch (*Taeniopygia guttata*), a bird with a high heritability of sperm length and an inversion on the Z-chromosome which maintains genetic diversity for sperm traits through heterozygous advantage.

Conclusions and future research

The aim of this thesis was to understand how females and males increase their fitness through two steps of sexual selection. Collared flycatcher males with large ornaments are not preferred by females as mates or extra-pair mates; females prefer old males and males with longer wings. Extra-pair paternity success is not influenced by the size of secondary sexual traits, but do ornaments influence within-pair paternity. Age is the main factor influencing the outcome of extra-pair paternity; this indicates that females may obtain good genes from their extra-pair mates. Females demonstrate seasonal variation in mate preference, supporting the thesis of context-dependence of mate choice. This could reflect a population difference in collared flycatchers but also a change in costs and benefits during the breeding season. Moreover, spermatozoa length does not influence paternity success, expresses phenotypic plasticity and moderate heritability.

As female mate choice is plastic, the role of the environment (seasonal and/or social) needs to be further investigated to better understand the process of sexual selection in changing environments. If sperm traits are an important factor for fertilization, the lack of knowledge on the environment in the female genital tract where this competition takes place could lead to biased conclusions. Indeed, the studies trying to identify sperm traits that make them successful in fertilization focus on sperm traits before the bottleneck of cryptic female choice. Further study on the effect of the environment on sperm length is needed to understand the variation in heritability and whether the variation in heritability is due to an increase in environmental variance or a decrease in genetic additive variance. The genomes of many species have been sequenced but the genes coding for sperm morphology are largely unknown. More knowledge about the genes coding for these traits will help in our understanding of the genetic basis of sperm phenotypic diversity and may be the mechanism that could maintain additive variance at the genetic level like the heterozygous advantage in the zebra finch.

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Thesis results are presented as:

1. **Edme, A**., P. Zobač, P. Opatová, P. Šplíchalová, P. Munclinger, T. Albrecht, and M. Krist. 2017. Do ornaments, arrival date, and sperm size influence mating and paternity success in the collared flycatcher? Behav. Ecol. Sociobiol. 71. DOI 10.1007/s00265-016-2242-8.
2. **Edme**, **A.**, P. Munclinger, and M. Krist. 2016. Female collared flycatchers choose neighbouring and older extra-pair partners from the pool of males around their nests. J. Avian Biol. 47:552-562. DOI 10.1111/jav.00839.
3. **Edme, A**., Zobač, P., Korsten, P., Albrecht, T., Schmoll, T., Krist, M. *submitted*. Moderate heritability and pronounced seasonal plasticity of sperm morphology in a species with high risk of sperm competition, the collared flycatcher *Ficedula albicollis*.

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Edme, A., P. Zobač , Korsten, P., Albrecht, T., Schmoll, T., Krist, M. *Moderate heritability and pronounced seasonal plasticity of sperm morphology in a species with high risk of sperm competition, the collared flycatcher Ficedula albicollis*. submitted.

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Edme A., Zobač P., Korsten P., Albrecht T., Krist M., Schmoll T. 2017. *Environmental versus genetic sources of variation in avian sperm dimensions – estimates from a natural population of collared flycatchers Ficedula albicollis.* 110th Annual meeting of the German Zoological Society. 11-15 September 2017, Bielefeld, Germany (talk)

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