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Sperm motility and postmating prezygotic isolation in two nightingale species

Motilita spermií a postkopulační prezygotická bariéra u dvou druhů slavíků

Diploma thesis

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Abstract

The motility of male gametes (sperm) is one of the important factors influencing the reproductive success of males. Because sperms are often subjected to strong postmating sexual selection and even closely related species often differ in sperm morphology, sperm motility could also differ between species, which may contribute to reproductive isolation between species. As part of my diploma thesis, I studied sperm motility in two closely related species of songbirds, the common nightingale (*Luscinia megarhynchos*) and the thrush nightingale (*Luscinia luscinia*). These two species of nightingales are an ideal model system because the areas of these two species overlap in the secondary contact zone across Central and Eastern Europe, where they occasionally hybridize and thus allow the study of speciation mechanisms in the natural environment. Both species also differ greatly in total sperm length. As part of my diploma thesis, I studied the possible influence of different sperm morphology on their motility. I further tested whether the motility of nightingale sperm differs in the fluid from the cloaca of a female of the same species and a different species, which would demonstrate the presence of postmating prezygotic reproductive isolation between species. The results of my work showed that despite the different morphology, the sperm of these two species do not differ in their motility. I also found that the sperm motility in fluid from the cloaca of a female of another species is significantly lower compared to the sperm motility in a neutral environment. In contrast, the motility of sperm in fluid from the cloaca of the same species did not differ from motility in a neutral environment. These results suggest that although the different morphology of spermatozoa in both species of nightingales does not by itself affect their motility, the presence of fluid from the cloaca of heterospecific females can significantly reduce motility. This may contribute to postmating prezygotic reproductive isolation between the two nightingale species.

Keywords: speciation, reproduction isolation, sperm, sperm motility, Nightingale (*Luscinia* sp.)

Abstrakt

Motilita samčích gamet (spermií) je jedním z důležitých faktorů ovlivňujících reprodukční úspěch samců. Protože spermie jsou často vystaveny silnému postkopulačnímu pohlavnímu výběru a i blízce příbuzné druhy se liší v morfologii spermií, dalo by se očekávat, že se spermie odlišných druhů se budou lišit také svou motilitou, což může přispívat k reprodukční izolaci mezi druhy. V rámci mé diplomové práce jsem studovala motilitu spermií u dvou blízce příbuzných druhů pěvců, slavíka obecného (*Luscinia megarhynchos*) a slavíka tmavého (*Luscinia luscinia*). Tyto dva druhy slavíků jsou ideální modelový systém, protože se areály těchto dvou druhů překrývají v sekundární kontaktní zóně probíhající napříč střední a východní Evropu, kde příležitostně hybridizují a tím umožňují zkoumat mechanismy speciace v přirozeném prostředí. Oba druhy se také velmi liší celkovou délkou spermie. V rámci mé diplomové práce jsem studovala možný vliv rozdílné morfologie spermií na jejich motilitu. Dále jsem testovala, zda se motilita spermií slavíků liší ve fluidu z kloaky samice stejného druhu a odlišného druhu, čímž by se prokázala přítomnost postkopulační prezygotické reprodukční izolace mezi druhy. Výsledky mé práce ukázaly, že navzdory rozdílné morfologii se spermie těchto dvou druhů neliší jejich motilitou. Dále jsem zjistila, že motilita spermií ve fluidu z kloaky samice jiného druhu je signifikantně nižší ve srovnání s motilitu spermií v neutrálním prostředí. Oproti tomu motilita spermií ve fluidu z kloaky samie stejného druhu se nelišila od motility v neutrálním prostředí. Tyto výsledky naznačují, že ačkoliv rozdílná morfologie spermií u obou druhů slavíků nemá sama o sobě vliv na jejich motilitu, přítomnost fluida z kloaky heterospecifických samic, může motilitu podstatně snížit. To může přispívat k postkopulační prezygotické reprodukční izolaci mezi oběma druhy slavíků.

Klíčová slova: speciace, reprodukční izolace, spermie, motilita spermií, slavík (*Luscinia* sp.)

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 4.6.2020

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Lucie Baránková

Poděkování

Chtěla bych poděkovat všem, kteří mi pomohli při sběru dat a psaní této práce. Mé veliké díky patří mé školitelce, Radce Reifové, za vedení při psaní mé práce, její cenné rady a pomoc. Děkuji take Tomášovi Albrechtovi za rady a vedení mé praxe v terénu, take za pomoc se statistickou částí mé diplomové práce. Další díky patří členům týmu věnujícího se výzkumu slavíků, Jiřímu Reifovi, Camille Sotas, Manon Poinget a Pavlovi Kverkovi za pomoc v terénu i za cenné rady.

List of abbreviations

ATP- adenosine triphosphate

CN-Common nightingale

CSP-Conspecific sperm precedence

DMEM- Dulbecco's modified Eagle's medium

PBS- Phosphate-buffered saline

SST-Sperm storage tubules

TN-Thrush nightingale

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1. Introduction

How new species arise has been one of the most important questions in biology and has been of interest of many evolutionary biologists since the end of 18th century. Famous researches of Darwin, Mayr, Haldane, Dobzhansky and many others have changed thinking about the origin of species and their evolution. They gave rise to new fields in biology focused on the origin and maintenance of biological diversity such as evolutionary biology and population genetics. In my diploma thesis, I will focus on possible mechanisms of species origin in two closely related passerine species, the Common nightingale (*Luscinia megarhynchos*) and the Thrush nightingale (*Luscinia luscinia*). Particularly, I will focus on their spermatozoa (male gametes).

Spermatozoa are under strong postmating sexual selection and from this reason their morphology often differ a lot between species. The divergence in sperm morphology can then contribute to reproductive isolation between species, particularly to postzygotic isolation (hybrid sterility) or postmating prezygotic isolation.

In the following chapters of the Introduction, I will first provide a short overview of different mechanisms of species formation and known types of reproductive isolation. Then I will describe sperm morphology, summarize the current knowledge of mechanisms affecting sperm morphology in passerines and will discuss the role of sperm in prezygotic isolation. Finally, I will describe our model system of two nightingale species, Common nightingale and Thrush nightingale.

2. Speciation

The speciation is commonly defined as “the evolution of reproductive incompatibility” (Wright, 1940). It is a natural process by which two populations from common ancestor evolve into two distinct species through the formation of reproductive barriers between populations and reducing gene flow between them (Mendelson et al., 2007). Speciation can be divided into sympatric, allopatric and parapatric speciation according to whether reproductive isolation evolves in the same geographical area, in geographical isolation or partial contact (Figure 1) (Coyne and Orr, 2004).

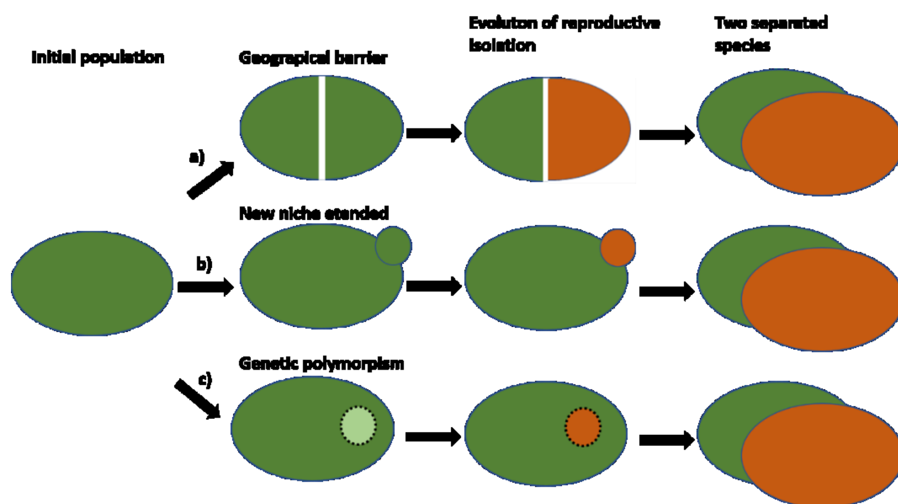


Figure 1: Types of speciation. a) allopatric speciation, b) parapatric speciation, c) sympatric speciation

Allopatric speciation is considered as the most common and includes a phase when to species evolve in geographical isolation. During this isolation, species diverge genetically and after secondary contact, they may not be able to interbreed any more. In some cases, however, species are able to interbreed to some degree after secondary contact and produce a hybrid zone (Schield et al., 2019). In this case, species can either fuse or the reproductive isolation can further evolve in sympatry and speciation is completed (Coyne and Orr, 2004). Hybrid zones are excellent natural laboratories, where we can observe and study the process of speciation under natural conditions.

Sympatric speciation occurs if reproductive isolation evolves in the same geographical area in the presence of gene flow (Coyne and Orr, 2004). The previous prevailing opinion was that sympatric speciation is rare and very unlikely because intensive gene flow can prevent species divergence (Bolnick and Fitzpatrick, 2007). Currently, it is admitted that under some conditions sympatric speciation is possible and there is a growing number of examples (Titus et al., 2019) Sutra et al., 2019). In practice, it is, however often difficult to prove the existence of sympatric speciation, as it is hard to rule out that species evolved at least for some time in allopatry.

Parapatric speciation is the case between sympatric and allopatric speciation. Populations are geographically partially separated but there is some overlap between them and gene flow can thus occur (Butlin et al., 2008). Speciation after secondary contact often evolved in parapatry.

Some authors today suggest that it is more practical to divide speciation to speciation with gene flow and speciation without gene flow. Speciation with gene flow includes both sympatric speciation and speciation after secondary contact, while speciation without gene flow includes typical allopatric speciation when species evolve in allopatry complete reproductive isolation (Smadja and Butlin, 2011).

2.1. Reproductive Barriers

To understand the mechanisms of species origin, it is important to know the reproductive barriers separating the species.

Reproductive barriers can be divided into prezygotic and postzygotic (Figure 2). Prezygotic reproductive isolation includes all mechanism of reproductive isolation until the formation of the zygote. It could be further divided into premating (so-called precopulatory) or postmating (so-called postcopulatory). Premating reproductive isolation prevents interbreeding between two species. It includes ecological isolation, chronological isolation or just simple morphological differences that prevent copulation. Postmating prezygotic reproductive isolation prevents fertilization of two different species after copulation and can occur at several points during transportation of the spermatozoa through the female reproductive tract or during the fertilization itself. This type of isolation was not studied much especially in animals with internal fertilization,

because it is harder to study. If a hybrid zygote is formed but hybrid individuals are somehow disadvantaged or die, we talk about postzygotic reproductive isolation. It is further subdivided into intrinsic (hybrid inviability or sterility), which is caused by incompatibilities among genomes of the two species, and extrinsic which depend on the environment and reflect the fact that hybrids are not well adapted to neither of the parental niches.

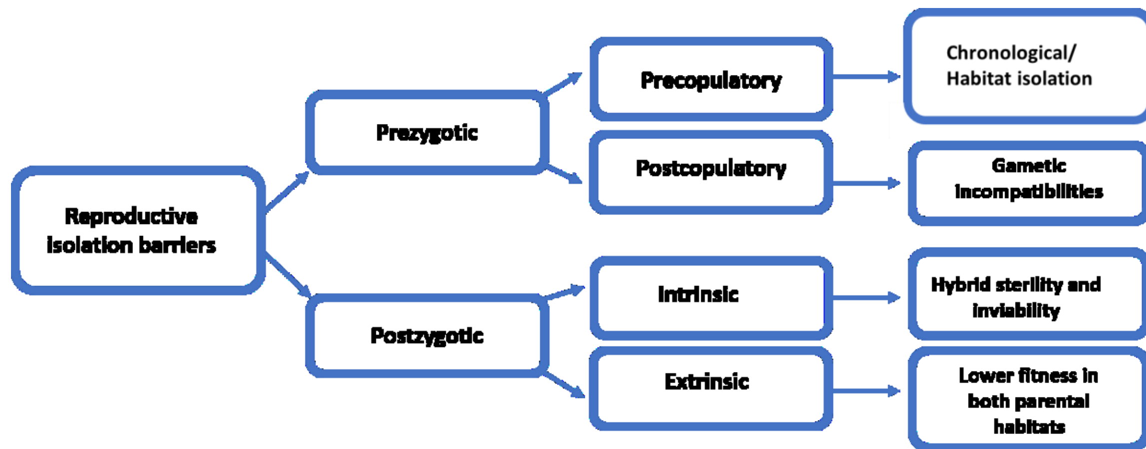


Figure 2: Detailed scheme of the division of reproductive isolation mechanisms with examples.

The order in which reproductive barriers accumulate between the species is taxon-specific. If ecological differences between species drive the speciation, premating and extrinsic postmating isolation usually evolve first. In other cases, the accumulation of genetic differences in species during geographical isolation leads first to the evolution of intrinsic postzygotic isolation (Seehausen et al., 2014). If species hybridize in the secondary contact zone and produce hybrids with lower fitness, selection can lead to the formation of prezygotic barriers to reduce the costs of hybridization. This phenomenon is called reinforcement (Butlin, 1987), and although it has been controversial for some time, there is now a growing number of examples (Nosil et al., 2003). Most of them concern premating isolation, but it has been suggested that reinforcement can occur also at the postmating prezygotic (gametic) level (Albrecht et al., 2019; Matute, 2010).

In this work, I will study the postmating prezygotic reproductive isolation in birds and from this reason, I will describe this kind of reproductive isolation with focus on avian species with more detailed in the next chapters. I will also describe sperm morphology and its role in speciation.

2.2. Sperm morphology, sperm velocity and its role in reproductive isolation

Sperms are male gametes that are one of the most variable animal cells. They are often subjected to strong postmating sexual selection, leading to their rapid interspecies and intraspecific evolution and therefore it is expected that divergence in male gametes could contribute to the formation of reproductive isolation (Pitnick et al., 2008). Sperm usually consists of three parts:

(1) head carrying a nucleus with one set of paternal chromosomes and acrosome, an organelle with enzymes that develops over the anterior half of the head
(2) midpiece that contains mitochondria that are responsible for ATP production needed for energy for sperm movement,
(3) flagellum (so-called tail) response for sperm movement

Sperm morphology varies greatly between different animal taxa (Figure 3) (Horta et al., 2018). Sperms differ in their structure as well in the number of tails. For example, we can observe spermatozoa with multiple tails as well spermatozoa with no tail. Teleostei fish, for example, differ from other vertebrates by lacking acrosome on their spermatozoa (Horta et al., 2018). Sperm vary across species also in their size. There are some extremes such as Giga spermatozoa that we can found in some drosophila species.

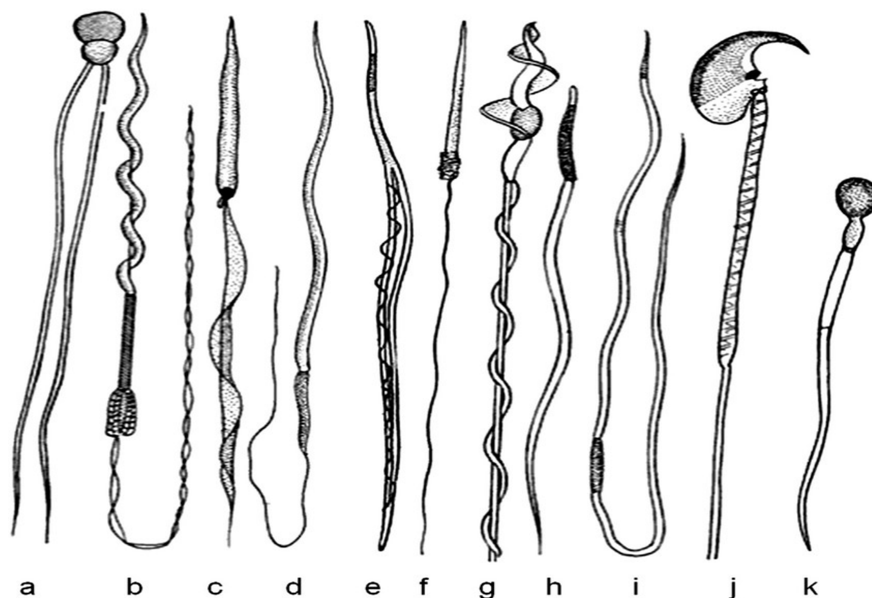


Figure 3: Variations in sperm structure across different vertebrates. (a) Toad Fish; (b) Elasmobranch (fish); (c) Toad; (d) Frog; (e) Salamander; (f) Lizard; (g) Frigilla; (h) Domestic fowl; (i) Monotreme (Echinida); (j) Mouse; (k) Man. Adapted from Horta et al. (2018)

For example, *Drosophila bifurca* is a small insect, that has sperms even 58 millimetres long(Lüpold et al., 2016).

2.2.1. Passerine sperm morphology

Although sperm morphology of non-passerine birds does not differ much from a reptile sperm, spermatozoa of passerine birds differ markedly in their phenotype (Jamieson et al, 2007; Humphreys, 1972). Passerine bird spermatozoa differ markedly in their length as well in its structure. The sperm of passerine bird has a helical conformation, including the head with the large acrosomal proportion. The midpiece is as well extremely elongated with a less noticeable transition between the tail. The midpiece, which Humphreys (1972) described as an undulating membrane, contains a single fused mitochondrion that wound helically around the sperm flagellum (Figure 4)

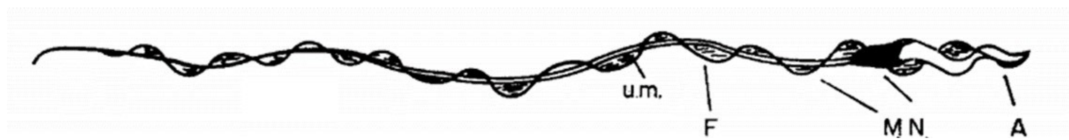


Figure 4: Representation of passerine bird spermatozoa on a canary sperm. A=acrosome; N=nucleus; M=midpiece; F=flagellum(tail); u.m.=undulating membrane. Adapted from Humphreys (1972).

Such a different structure of passerine sperm also requires a different mechanism of movement. Unlike non-passerine birds and mammals, whose spermatozoa move by the lashing of the flagellum, forward movement of passerine sperms is generated by the helical rotation of sperm, which rotates around its axis in a clockwise direction (Vernon and Woolley, 1999).

The main force driving the diversity of spermatozoa in birds is the postmating sexual selection (Immler and Birkhead, 2007) that includes sperm competition (Birkhead, 1995) and cryptic female choice (Birkhead, 1998). Sperm competition appears to be the main force in driving the diversity in sperm phenotype (Birkhead and Pizzari, 2002; Snook, 2005). In passerines, species with higher levels of copulation events have longer and faster-swimming sperm as well higher proportion of motile sperm, compared to species with lower levels of multiple mating (Kleven et al., 2009; Rowe et al., 2013).

However, the relationship between the size of sperm traits and sperm motility appear to differ across taxa (Anderson and Dixson, 2002; Balshine et al., 2001; Lüpold et al., 2009a). Theoretic assumption predicts that increased flagellum length results in increased sperm velocity. As well increased midpiece size may contain more or larger mitochondria that generate more ATP for movement. However, previous studies on passerine bird did not show any clear association between sperm length and sperm velocity (Briskie and Montgomerie, 1992; Immler and Birkhead, 2007; Lüpold et al., 2009a). I will discuss this more in chapter Discussion.

Fast divergence in sperm morphology between species may lead to intrinsic postzygotic as well to postmating prezygotic reproduction isolation. In the following subchapter, I will discuss the role of male sperm in postmating prezygotic isolation more detailed.

2.2.2. Sperm divergence and reproductive isolation

Fast divergence in sperm morphology between species caused by strong postmating sexual selection can lead to either to intrinsic postzygotic isolation (hybrid sterility), which is caused by incompatibilities between genes coming from different species (Coyne and Orr, 2004; Dobzhansky, 1937). Or it can lead to postmating prezygotic isolation. The mechanisms of postmating prezygotic reproductive isolation have been mostly studied in species with external fertilization since it is easier to observe sperm-egg interaction. In species with internal fertilization, most of the research on prezygotic postmating reproductive isolation was carried out generally on invertebrates, most intensively in Drosophila (Coyne and Orr, 1989; Manier et al., 2013). Those studies showed that during insemination, the sperm can fail to achieve sperm storage sites in the female reproductive tract, or can fail to stimulate ovulation or oviposition, also there can be a problem with the sperm-egg identification or in syngamy itself (Coyne and Orr, 2004; Patterson, 1947; Manier et al., 2013).

It has been also shown that when a female was inseminated with both conspecific (i.e. belonging to the same species) and heterospecific sperms during the same insemination cycle, conspecific sperm has been favoured over heterospecific sperm. This phenomenon is called conspecific sperm precedence (CSP) and have been described on invertebrates (Fricke and Arnqvist, 2004; Geyer and Palumbi, 2005; Price et al., 2000) as well on

vertebrates (Arkorful et al., 2018; Dean and Nachman, 2009; Ludlow and Magurran, 2006). Those studies suggest that CSP may play an important role in postmating prezygotic isolation as a part of cryptic female choice, where females can bias the outcome of sperm competition (Eberhard, 2009).

In following the chapter, I will describe more detailed what is known about postmating prezygotic isolation in birds.

2.2.3. Postmating prezygotic reproductive isolation in birds

In avian taxa, few studies have been focused on mechanisms of postmating prezygotic reproductive barrier in contrast with premating barriers or postzygotic reproductive barriers that are easier observable (Birkhead and Brillard, 2007). It is thus not known, how important this reproductive isolation is in bird speciation. Because large numbers of bird species have promiscuous females that copulate with many males, there is strong coevolution between male gametes and female reproductive tract components, such as sperm storage tubules or sperm and egg surface proteins. Relatively strong postmating sexual selection can occur also in passerine birds, which are mostly socially monogamous, but show high rates of extra-pair paternity (Birkhead, 1995; Westneat and Stewart, 2003). In birds, the female reproductive tract has several possible points where heterospecific sperm could fail in fertilization (Figure 5).

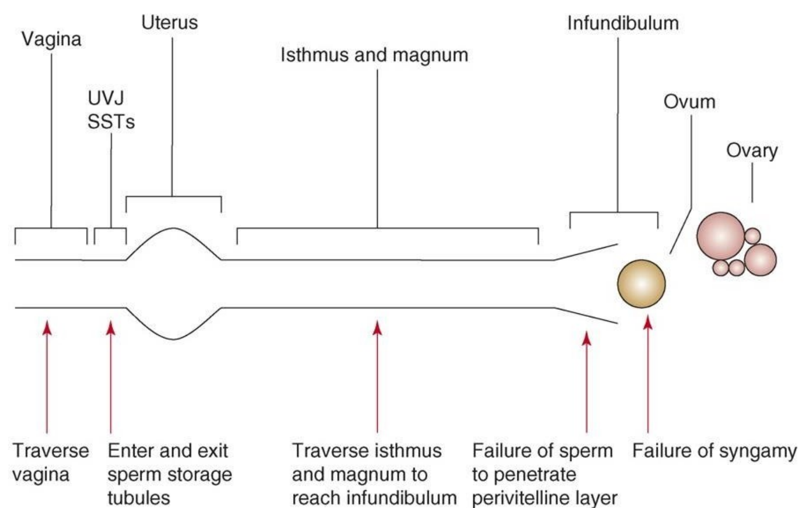


Figure 5: Female reproductive tract with possible points of failure during fertilization marked by red arrows. Adapted from fig.1 in Birkhead and Brillard (2007)**.**

Right after copulation, the sperm could fail to traverse the vagina and reach the sperm storage tubules. Studies on conspecific insemination have shown that only about 2% of sperm traverses through the vagina and gain sperm storage tubules, which implies that the traversal through the vagina is the main site of sperm selection (Bakst et al., 1994; Howarth, 1983). This was also supported by studies where sperm were placed into different parts of the female reproductive tract (vagina or magnum). The results showed that when spermatozoa were put into the vagina, females showed a lower number of fertilized eggs than when spermatozoa were placed into the magnum (Steele and Wishart, 1992). When similar experiments were performed with conspecific as well as heterospecific sperms, it was shown that fertilization success was higher with conspecific sperm than heterospecific sperms (Mcfarquhar and Lake, 1964; Sellier et al., 2005; Steele and Wishart, 1992). These studies indicate that capability of sperm to transverse the vagina could play an important role in postmating prezygotic isolation.

When sperm transverse the vagina it is stored in sperm storage tubules (SST). SST are found in the oviduct as an organ for sperm storage (Sasanami et al., 2013). It has been suggested that there is a strong co-evolution between sperm morphology (especially the sperm length) and the length of female sperm storage tubules (Kleven et al., 2009). Heterospecific sperms could thus be disfavoured in storing as has been demonstrated in some studies (Briskie et al., 1997; Steele and Wishart, 1992) .

Sperm could fail during transport from sperm storage tubules to the infundibulum, where fertilization occurs. However, studies on the domestic fowl have shown that even dead sperms inseminated beyond sperm storage tubules (into the uterus) are transported by the action of the cilia in isthmus and magnum. This suggests that transport from sperm storage tubules to infundibulum is not a strong barrier for heterospecific sperm (Brillard, 1993; Wentworth and Mellen, 1964).

Heterospecific sperm could also fail during the penetration of the inner perivitelline layer of the egg because species-specific proteins are required for binding and penetration. Surprisingly, interspecific cross-reactivity between sperm and a perivitelline layer is relatively high (Bramwell and Howarth, 1992; Stewart et al., 2004). Thus, proteins required for binding and penetration of the perivitelline layer seem to be less species-specific in birds than for example in mammals (Litscher and Wassarman,

1996; Wassarman, 1995). Nevertheless, the importance of the penetration of perivitelline layer as a reproduction barrier in birds remains unclear. In the last point, sperm pronucleus can fail in syngamy due to wrong recognition or fuse with female pronucleus because of species-specific chemotaxis of sperm and egg (Perry, 1987).

Although not many studies focused on examining postmating reproductive barriers in bird species, it seems that the strongest barrier is right after copulation when sperm transfer cloaca and vagina to reach the sperm storage tubules (Bakst et al., 1994; Cramer et. al.,2014; Howarth, 1983; Moller et al., 2008). For that reason, we decided to test if the postmating prezygotic barriers are present in two passerine species by simulating insemination directly after copulation in our experiments and analysing motility of conspecific and heterospecific spermatozoa in fluid from the female cloaca.

2.3. Model System

In this diploma thesis, I focused on the measurement of sperm motility into conspecific and heterospecific female fluids in two sister species of passerines birds, the Common Nightingale *(Luscinia megarhynchos)* and the Thrush Nightingale (*Luscinia luscinia).* These are small insectivorous passerine birds belonging to *Passeriformes* order*,* which used to belong to thrush family *Turdidae*, but recent phylogenetic studies based on DNA analysis, put them to the family *Muscicapidae* (Prum et al., 2015). They have diverged from each other approximately 1,8 million years ago (Storchová et al., 2010) and during Holocene, they came into secondary contact forming a secondary contact zone running across Central and Eastern Europe (Sorjonen, 1986, Reif et al., 2018) (Figure 6a). They are still hybridizing with an approximate frequency of 4-5 % F1 hybrids (Becker, 2007; Reifova et al., 2011a). Hybrid individuals have been recognized by intermediate phenotype as well as DNA analysis.

2.3.1. Common nightingale

Common Nightingale (*Luscinia megarhynchos)* (Figure 6c) has plain brown coloured feathers with reddish undertone above and more reddish tail. It is a long-distance migrant, its breeding area cover most of Western Europe, with northern limits in south Britain (Figure 6a), and with southern limits covering also a small area in northern Africa and south-west Asia. It is wintering in sub-Saharan Africa. There is no sexual dimorphism, except that males tend to be slightly larger, with larger wingspans. Common Nightingale typically inhabits dense bushes near the ground, where the nest usually is hidden in dense vegetation (Kverek, 2007). It sometimes nests near the human dwelling and we can hear sing this species even in park bushes or in green vegetation near to the roads. The Common Nightingale can be easily recognised by its song. Though in sympatry, where its area overlaps with the Thrush Nightingale, the Thrush Nightingale can imitate a song of a Common Nightingale (Vokurková et al., 2013), which can be misleading even for experienced ornithologists.

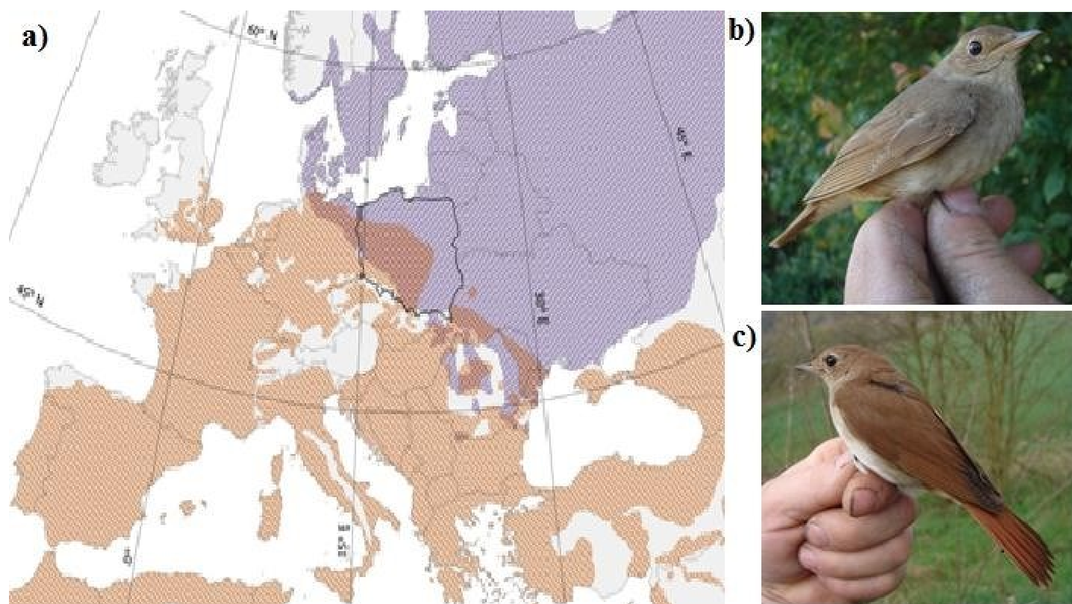


Figure 6: a) Map of distribution of Common Nightingale (red) and Thrush Nightingale (purple). The hybrid zone display is schematic. Adapted from Reif et al., (2018)**.**
b) Thrush Nightingale; c) Common Nightingale. Photographed by Pavel Kverek, Czech Society of Ornithology.

2.3.2. Thrush Nightingale

The Thrush Nightingale (*Luscinia luscinia)* (Figure 6b) has a larger body and is more robust than the Common Nightingale. Its breeding area includes Eastern Europe and the western part of temperate Asia with northern limits in southern Finland and Sweden (Figure 6a). It winters in South Africa. Similarly, as for the Common Nightingale, there is no sexual dimorphism. It occupies similar habitats as the Common Nightingale. In sympatry with the Common nightingale, however, it prefers more wet habitats, while the Common Nightingale more dry habitats. This partial habitat segregation very likely arose as a result of interspecific competition (Reif et al. 2018). The Thrush Nightingale has darker-brown feathers than Common Nightingale without reddish undertone back and has greyish-brown belly with dark spots. Besides slightly different plumage and size, the Thrush and Common Nightingales can be recognized from each other by the relative length of the first primary to the longest covert on wings. The first primary is shorter than the longest covert in the Thrush Nightingale, but longer in the Thrush Nightingale. The species also differ in the relative length of the second and the fourth primaries (Figure 7).

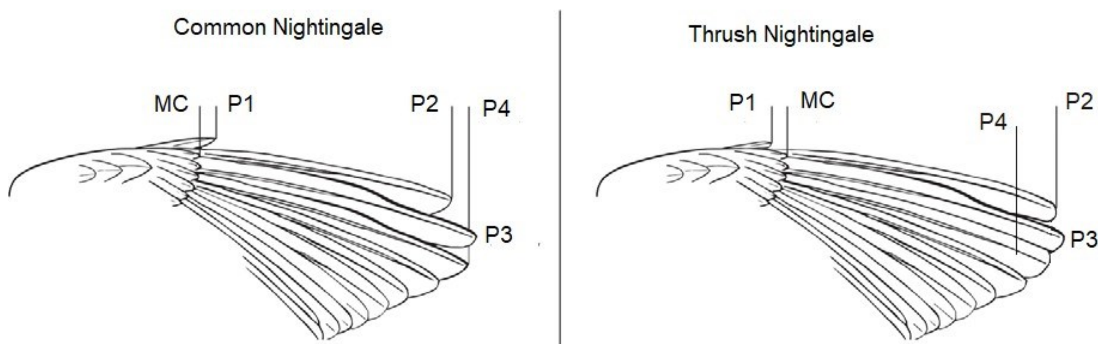


Figure 7: Comparison of the wing of Common Nightingale and Thrush Nightingale. MC = Major Coverts, P1-P4 = Primaries. Adapted and modified from Becker (2007).

2.3.3. Evolution of reproductive isolation in two nightingale species

Nightingale species pair provides an ideal model system for exploring genetic and ecological aspects of the speciation process and formation of reproductive isolation between the species. Presence of only about 4-5% of F1 hybrids in sympatric population suggests quite strong although incomplete premating reproductive isolation between the species. This can be caused by partially differentiated habitats of the two species in the sympatric area (Reif et al., 2018; Sottas et al., 2018). But slightly different plumage colouration and song can also contribute to it. However, there is a convergence of song in sympatry caused by the fact, that Thrush Nightingale sometimes imitates the song of Common Nightingale (Vokurková et al., 2013). This convergence could weaken premating isolation between the two species.

The habitat divergence between species, which also resulted in divergence in bill size in sympatry (Reifová et al. 2011, Sottas et al. 2018), very likely because species feed on a different diet in different habitats, could also contribute to extrinsic postzygotic isolation. Hybrids with intermediate morphology could be less competitive in habitats of both parental species. They can also show the intermediate migration route between the species, which might be less advantageous. Nevertheless, stronger is probably intrinsic postzygotic isolation. Although hybrids between the two Nightingales are viable, hybrid females, but not males, are sterile as has been shown by crossing experiments in captivity (Stadie (1991) as well as observations in nature (Reifova et al., 2011b). Females did not display breeding behaviour nor have brood patch. The dissection of hybrid nightingale female reproductive tract was, however, not performed yet. Thus, physical causes of female hybrid sterility remain unclear, although lack of interest in reproduction may be due to non-development of gonads and hence missing hormones affecting reproductive behaviour. On the other hand, hybrid males displayed normal sexual behaviour and are able to produce backcross progeny. They also show morphological normal spermatozoa, although they are intermediate in morphology between the species (see below, Albrecht et al. 2019).

Postmating prezygotic isolation has not been explored in nightingales yet. However, the previous study has shown that the two nightingale species show striking divergence in sperm morphology (Albrecht et al., 2019). The Common Nightingale has longer sperms than Thrush nightingale, which is mainly caused by longer midpiece, a part of sperm containing mitochondria (Figure 8). Interestingly, it has been also observed that nightingales show increased divergence in the sperm head length in sympatry than in allopatry, suggesting that reinforcement at the gametic level might have occurred in this species (Albrecht et al. 2019). Tail length does not differ between two species. It is possible that the divergence in sperm morphology could contribute to postmating prezygotic isolation between the two nightingale species. Indeed, the results of crossing experiments in captivity suggested that heterospecific crosses produce less laid eggs than conspecific crosses (Stadie, 1991).

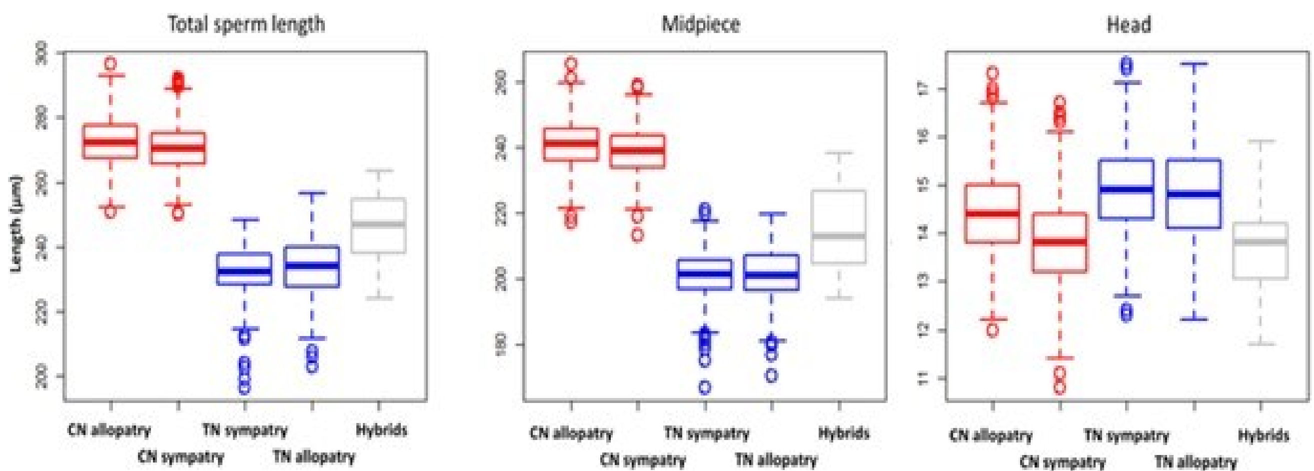


Figure 8: Size of sperm and its components. CN=Common Nightingale (Red). TN=Thrush Nightingale (Blue). There is also intermediate sperm phenotype of F1 hybrid (Grey). Adapted from Albrecht et. al., (2019)

3. Aims of the Thesis

The aim of my thesis is to test whether sperm divergence between the two nightingale species affects sperm motility and whether it could contribute to postmating prezygotic isolation. Specifically, we addressed two questions:

1) Is there a difference in sperm motility between Common Nightingale and Thrush Nightingale? We expect that Common nightingale sperm with longer midpiece will swim faster as longer midpiece could produce more ATP.

2) Is there a difference between sperm motility in conspecific and in heterospecific female fluid? If postmating prezygotic isolation is present in nightingales, we expect reduced sperm motility in the heterospecific fluid.

4. Materials and Methods

4.1. Bird Sampling

Nightingale individuals were caught between 2014 and 2019 in May, at the beginning of their breeding season. Males were caught into mist nets using playback of a conspecific male song to attract them or using food-baited ground traps. Sampling was performed in Poland, in the sympatric region of both species (Supplementary Table 1). Directly after catching, birds were ringed, weighed, measured and sex was identified as male or female. Species were identified according to the species-specific trait (see the Introduction).

From female individuals, fluid from was collected following Cramer et. al. (2014) protocol. Exterior surface of female cloaca was swabbed with a cotton tampon impregnated by 96% ethanol and allowed to dry on air. Then cloaca was gently massaged in order to expose mucus surface and small volume (5µl) of sterile phosphate- buffered saline (PBS) was pipetted in. After waiting approximately 5 sec, PBS (Phosphate-buffered saline) from cloaca was collected by pipette and dropped into cryotube. This process was repeated 3x to obtain in total 15 µl of fluid. Whole 15µl of fluid was mixed in cryotube and divided by 5µl into 3 cryotubes and directly frozen in liquid nitrogen for later use. Male individual sperm samples were obtained by gentle massage of cloaca resulting in releasing sperms. This non-invasive method was used in Albrecht et al. (2019). Ejaculate sample was taken by glass capillary preheated to 40°C and fresh sperm were directly used for the experiment. Ejaculates contaminated by faeces weren't accepted.

4.2. Comparison of sperm motility in two nightingale species

First, we evaluated motility of spermatozoa of both nightingale species in the Dulbecco's Modified Eagle´s Medium solution (Advanced DMEM, Invitrogen). It is a cell culture medium enriched with support supplements allowing cells to live longer. The collected ejaculate was diluted in the Eppendorf tube with 5µl of DMEM embedded in a heater (Eppendorf ThermoStat™ C) preheated on 40° to avoid conglutination of sperms. Then 2,8 µl of the sample was transferred by pipette on a standard 20 μm two-chamber count slide (Leja, The Netherlands) for analysis of velocity. Analysis of sperm motility

was carried out using the microscope (C40, Olympus) with the installed camera (UI-1540- C, Olympus) and preheated bottom on 40 °C to avoid premature sperm dying and reduced motility due to low temperature. Used magnification was 100x. We recorded each male sample separately for a maximum time of 15 seconds. In total, we recorded sperm motility in 19 CN males and 15 TN males (Supplementary Table1,).

4.3. Sperm motility in conspecific and heterospecific female fluids

The design of the experiment is shown in Figure 9. All samples were collected from males and females in sympatry (Supplementary Table 2). Because we found very difficult to capture Thrush nightingale females, we decided to do this experiment only with Common nightingale female fluids, which were tested in combination with conspecific Common nightingale sperm and heterospecific Thrush nightingale sperm (Figure 9). As it was difficult to obtain at the same time sperm from both species and their direct comparison would be difficult from multiple reasons (e.g. sperm motility might be affected by quality and amount of male ejaculate, sperm concentration, the time of slide preparation etc), we decided to compare the motility of sperm from both species in female fluid with motility in a neutral environment, which was PBS. Sperm motility in each male was thus measured on one microscope slide divided into two chambers, where in one chamber was sperm in fluid and in the other sperm in PBS. Preparation of samples proceeded as follows. The freshly collected male ejaculate was diluted in the Eppendorf tube with 5μl of PBS, embedded in the tube heater with set temperature 40°C. We prepared Eppendorf tubes with 5 μl of female fluid sample frozen in liquid nitrogen and other with 5 μl of PBS to have the same initial conditions for both treatments. Female fluid and PBS were thawed and 5 μl of both samples was transferred to new Eppendorf tubes. Then 2 μl of sperm sample were transferred in both fluid and PBS. The samples prepared in this way were then transferred on the Leja microscope slide and recorded. To minimalize effect of time of record we recorded both parts of microscope slide alternately, three to six times each part of slide approximately 15-20 seconds, but in total time maximum 2 mins. Exact times of switching between parts of the slide with sperm in fluid and PBS were recorded in protocols. Each male sperm sample was used only once, while female fluid was used always twice, once with conspecific and once with heterospecific sperm (Figure 9). Only one male was captured two times in different years.

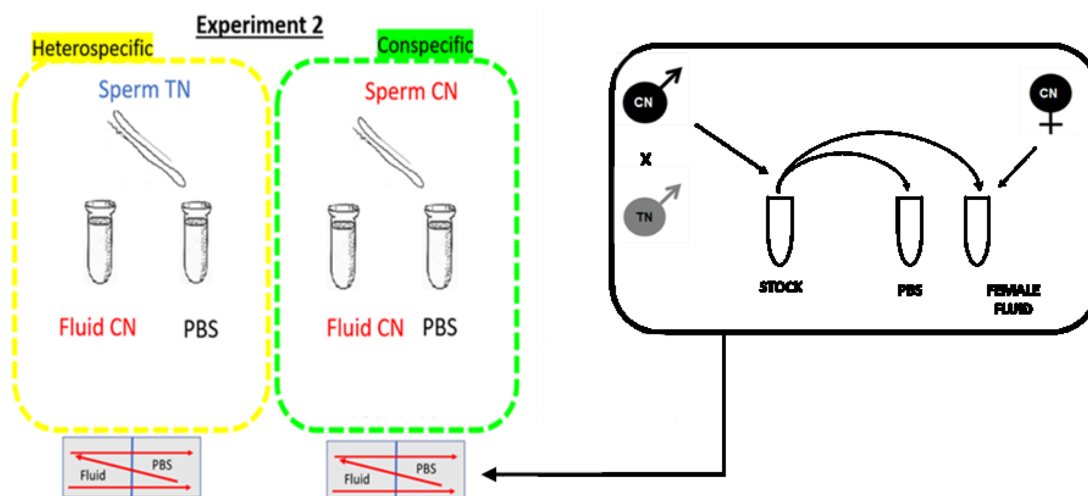


Figure 9: Design of the experiment where sperm motility was evaluated in heterospecific and conspecific fluids. Sperm motility in both heterospecific and conspecific fluids was directly compared with motility in a neutral environment (i.e., PBS, phosphate-buffers saline) on one microscope slide with two chambers.TN = Thrush nightingale, CN = Common Nightingale, PBS = (neutral control).

In total, we performed 14 experiments with conspecific sperm and 14 experiments with heterospecific sperms (Supplementary Table 3)

4.4. Analysis of sperm motility

Records of sperm motility were analysed using the CEROS computer-assisted sperm Analysis (CASA) system (Hamilton Thorne Inc., USA). From CASA software one can obtain, besides other, three main characteristics of sperm motility (Figure 10): curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP). All characteristics are measured in µm/s. (Suzuki et al., 2002). VSL is the average velocity of the sperm head through the straight line connecting the first and last position of sperm track. VAP is the average velocity of the sperm head through its average trajectory. VCL

is the average velocity of the sperm head through its real path (Hirano et al., 2003). VCL value also combines direct swimming speed with movements of sperm head, thus provide the best estimation of sperm real movement (Youn et al., 2011). CASA system sperm velocity estimations, including VCL, are also strongly correlated with fertilization success (Hirano et al., 2003). Further in the terminology of my work, I will use term sperm velocity in theAverage number meaning of measured values of VCL.

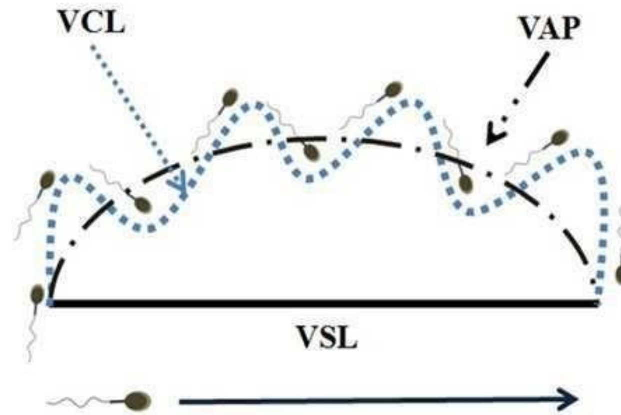


Figure 10: Three main characteristic of sperm motility. VCL=curvilinear velocity. VSL=straight line velocity. VAP=average path velocity. All three characteristics are measured in µm/s. Adapted from Suzuki et al. (2002)

To measure the VCL, set image capture was 25 frames per sec and for maximize data quality (i.e. removing poorly traced cells or contaminants), we used the following quality control. Cells with smoothed-path velocity (VAP) < 15 μm s^{-1} or straight-line velocity (VSL) < 10 μm s^{-1} were considered static and removed from the dataset. After every measurement manual control of spermatozoa track was required for removing non-sperm contaminants from the dataset. All corrections of recorded spermatozoa were done by me to maintain measurement unilaterality.

Only experimental treatments with at least 3 well-tracked moving cells were included in analyses. The average number of motile spermatozoa in samples was 49, with high extremes (1-406) (Supplementary Table 3).

4.5. Statistical analysis

Statistical program R, version 3.6.1 (R Core Team, 2019) was used for statistical analyses. I used linear models for testing differences in sperm motility between Common and Thrush nightingale in DMEM. The response variable was VCL, and species (Common Nightingale/Thrush Nightingale) was used as an explanatory variable. The number of motile sperms was used as a covariate to check for the possible effect of the number of motile sperm on VCL (Gómez Montoto et al., 2011). See dataset in Supplementary Table 1.

For testing differences in sperm motility of conspecific and heterospecific sperms in fluid and PBS, we used linear mixed-effect model using the lme4 package (Bates et al., 2015). The dependent variable was VCL, the explanatory variable was treatment (fluid/PBS) (Supplementary Table 3). As covariates, we used the number of motile sperms, control order and start time of recording. The number of motile sperms was also included in the model from the reason explained above. Control order was either 1 or 2 depending on whether sperms were recorded first in PBS or in fluid. Start time was a time when recording started in PBS or in the fluid. It was included in the model because although the total time of recording was not longer than 2 mins, the effect of time could influence sperm motility, thus later records may show reduced swimming speed. Because all measurements from all treatments were used in the analysis, code of treatment measuring was used as a random effect. See supplementary table 3, which includes values of all these variables.

5. Results

5.1. Motility of Common and Thrush Nightingale's sperm in DMEM

To attain the first objective, I compared sperm motility measured as curvilinear velocity (VCL) of Thrush and Common Nightingale in DMEM. The median of VCL was 97,02 ± 2.55 µm/s in the Common Nightingale and 97,45 ± 2.23 µm/s in the Thrush Nightingale. The linear model was used for testing differences in sperm motility between the species with the number of motile sperms as a covariate. See Figure 11 and Table 1.

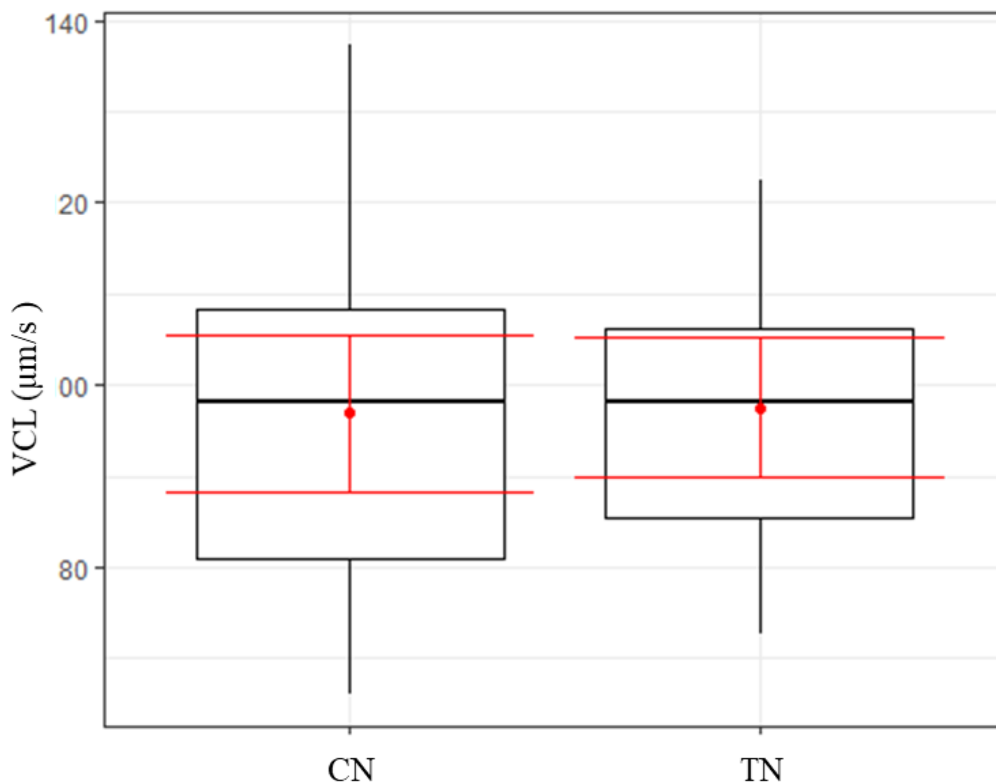


Figure 11: Comparsion of sperm motility in Thrush Nightingale (TN) and Common Nightingale (CN). Sperm motility in both species was measured as curvilinear velocity (VCL) in cell culture medium DMEM. Medians, quartiles and 1.5 interquartile range are presented. 95% confidence interval is marked by red ranges.

Table 1: Effects of species and number of motile sperms on sperm motility measured as curvilinear velocity (VCL) in DMEM. Significant P-value is indicated by asterixis. [1]SE- standard error, [2]Intercept- sperm motility of Common Nightingale. [3]Species-Thrush Nightingale

	Estimate	**SE [1]**	**T**	**P-value**
Intercept[2]	86.5502	5.1726	16.732	< 2e-16
Species[3]	3.3785	5.6158	0.602	0.55181
Number of motile sperms	**0.0497**	**0.0173**	**2.869**	**0.00734 ****

There was no significant difference in motility between two species ($p = 0.552$). But the number of motile sperms was significantly positively associated with VCL ($p = 0.007$). This fact is consistent with expectation, as there was found a strong association with sperm numbers and sperm traits that determine ejaculate quality, including sperm motility (Birkhead et al., 1999; Gómez Montoto et al., 2011).

5.2. Differences in sperm motility in conspecific/heterospecific female fluid and PBS control

5.2.1. Differences in sperm motility between conspecific fluid and PBS

I first analysed differences in sperm motility in conspecific Common Nightingale female fluid and PBS control using a linear mixed-effect model. Median VCL in PBS was 67,99 ± 2.07 µm/s, and in conspecific fluid 60,74 ± 1,53 µm/s (Figure 12). This shows that sperm velocity is slightly lower both in PBS and fluid than in cell culture medium DMEM used in the previous experiment. To test for the differences between the sperm motility in fluid and PBS linear mixed effect model was used, where the sum of motile spermatozoa, the order of control and start time of the recording were used as a covariate. Experiment (which included all records in fluid and PBS with sperms from the same male) was used as a random effect. Results of the model are shown in Table 2. There was no significant difference in VCL between conspecific treatment and PBS control (p = 0,312).

5.2.2. Differences in sperm motility between heterospecific fluid and PBS

Median VCL in PBS was 66,64 ± 1,91 µm/s, and in heterospecific fluid 57.65 ± 1,44 µm/s (Figure 13). The same linear mixed effect model as above was to test for differences in sperm motility between these two treatments (Table 3). The model has shown that there is a significant difference in sperm motility between heterospecific female fluid and neutral PBS control (p = 0,0115).

For better visualisation are Figures 12 and 13 and Tables 2 and 3 on following pages 24 and 25.

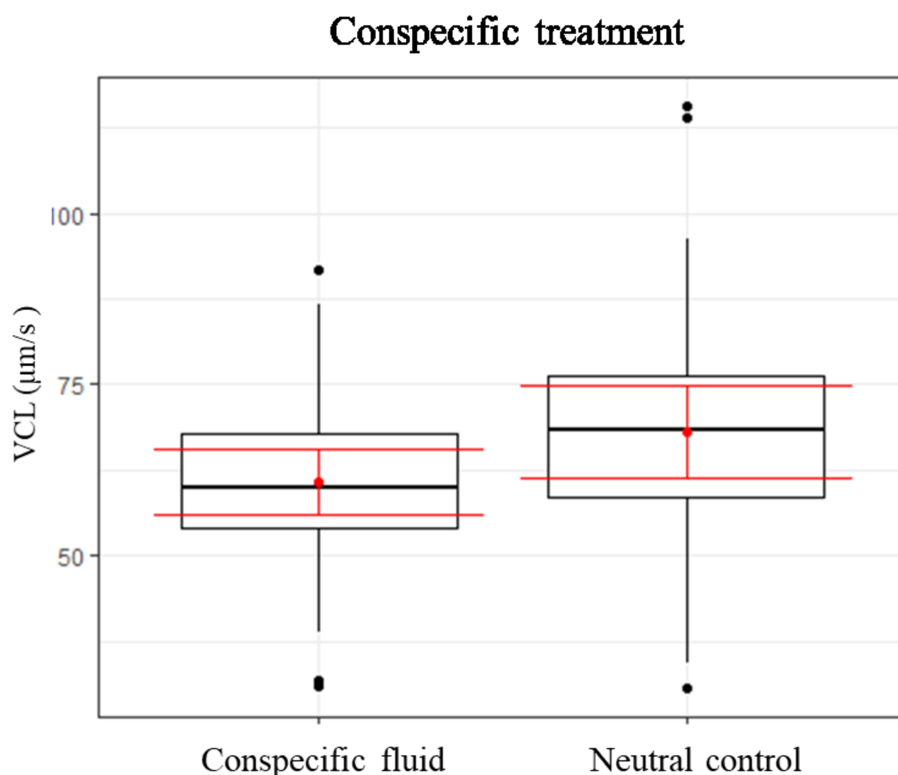
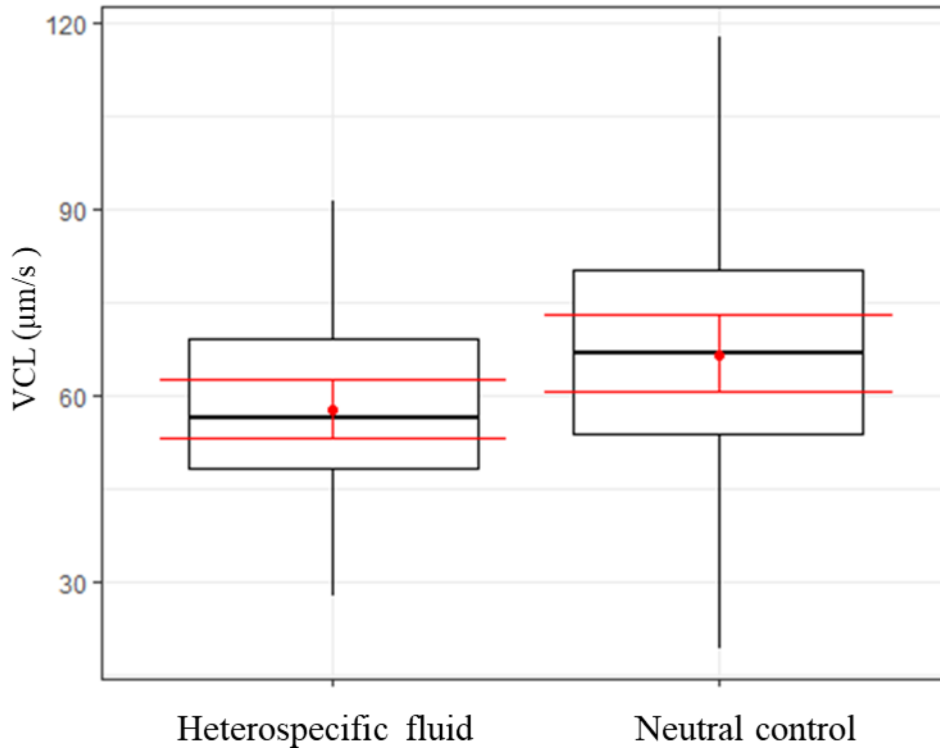


Figure 12: Sperm motility in conspecific female fluid and neutral control (PBS). Medians, quartiles, 1.5 interquartile range and outliers are presented. 95% confidence interval is marked by red ranges.

Table 2: The effect of treatment (conspecific fluid/PBS), the sum of motile spermatozoa, the order of control and start time of recording on the sperm motility measured as VCL. Used formula: VCL~ treatment (fluid/PBS control) + sum of motile spermatozoa +order of control +start time of recording. [1]SE- standard error, [2]Df- degrees of freedom, [3]Intercept- VCL in a conspecific female fluid.

	Estimate	**SE[1]**	**Df[2]**	**T-value**	**P-value**
Intercept[3]	65.1517	7.64474	62.99819	9.763	3.16e-14
Treatment	7.2302	4.6539	55.4638	45.836	0.127
Sum of motile sperm	0.0165	0.0275	16.2494	0.602	0.405
Control order	-2.1739	4.76271	46.59869	-0.456	0.650
Start time	-0.0403	0.0484	52.0287	-0.834	0.408



. ***Figure 13: Sperm motility in heterospecific female fluid and PBS control***. Medians, quartiles, 1.5 interquartile range and outliers are presented**.** 95% confidence interval is marked by red ranges.

Table 3: The effect of treatment (heterospecific fluid/PBS), the sum of motile spermatozoa, the order of control and start time of recording on the sperm motility measured as VCL. Used formula: VCL~ treatment (fluid/PBS control) + sum of motile spermatozoa +order of control +start time of recording. Significant P-value is indicated by asterixis. 1SE- standard error, 2Df- degrees of freedom, 3Intercept- VCL in a heterospecific female fluid**.**

	Estimate	**SE[1]**	**Df[2]**	**T-value**	**P-value**
Intercept[3]	56.7529	7.0745	63.9287	8.022	2.97e-11
Treatment	**8.5540**	**3.7399**	**55.5208**	**2.287**	**0.026 ***
Sum of motile sperm	0.0821	0.0520	48.0008	1.580	0.121
Control order	-1.3153	3.8513	57.5147	-0.342	0.734
Start time	-0.0088	0.0456	62.516	-0.192	0.848

6. Discussion

6.1. Is there a difference in sperm motility between the Common Nightingale and the Thrush nightingale?

The Common and Thrush Nightingales differ markedly in sperm length which is mainly caused by different length of midpiece between the species. The Common Nightingale has a longer midpiece resulting in longer sperm in total (Albrech et al. 2019). Effect of sperm morphology on sperm swimming speed, in this work described by sperm velocity, have been tested across passerine birds, however, there is no clear pattern in association between sperm morphology traits and sperm swimming speed (Lüpold et al., 2009a; Rowe et al., 2015). Sperm swimming speed is assumed to be important in passerines because its correlation with fertilization success in a wide range of animals (Simmons and Fitzpatrick, 2012). Theory predicts that longer sperm swim faster (Fitzpatrick et al., 2009; Lüpold et al., 2009). Sperm velocity may be also increased by enlarged midpiece (energetic component) or flagellum length (kinetic component), or by ratios between sperm components, such as between flagellum length and head size.

When the effect of total sperm length was tested, positive association between total sperm length and sperm velocity have been found in the zebra finch (*Taeniopygia guttata*) (Bennison et al., 2015) as well in the comparative study of 40 passerine bird species (Lüpold et al., 2009a). However, in another comparative study of 42 passerine species, Kleven et al. (2009) found that sperm swimming speed was not related to sperm length, although sperm length also was related to extrapair paternity and reproduction success (Kleven et al., 2009). The similar result has been found in the comparative study of 38 species of one family of passerine birds, *Icteridae* (Lüpold et al., 2009b). This study found a correlation between increased sperm size and increased postcopulatory sexual selection but found no relationship between sperm length and motility. As well in studies on sparrows (*Passer domesticus* and *P. hispaniolensis*) performed by Cramer et al., (2015). A negative correlation between total sperm length and sperm velocity has been also found in sand martins (*Riparia raparia*) (Helfenstein et al., 2008). However, there have been found negative

correlation between sperm length and initial sperm velocity, but a positive correlation between sperm length and sperm longevity. A negative correlation between sperm length and sperm swimming speed has been found as well in Simpson et. al (2014)

Some empirical observations on other passerine birds suppose that the sperms with longer midpiece have increased sperm swimming speed (Cramer et al., 2014; Rowe et al., 2015). This may be caused by the fact that longer midpiece, a part of sperm containing the mitochondria, produce more ATP providing energy for sperm movement, as argued in Cardullo and Baltz (1991). Association between generated ATP and sperm velocity was shown in several studies on mammals, fishes as well as birds species (Cardullo and Baltz, 1991; Froman and Feltmann, 1998; Johnson and Briskie, 1999; Rowe et al., 2013; Vladić et al., 2002). However, in total, a little direct empirical evidence for a positive association between midpiece size and sperm velocity was given (Lüpold et al., 2009a) and some studies even argued against this hypothesis by founding a negative correlation of argued traits (Malo et al., 2006; Simpson et al., 2014).

The theoretical hypothesis could also argue that sperm velocity could be influenced by relative flagellum length through the propulsive forces increased by longer flagellum (Katz et al., 1989). A positive correlation between relative flagellum length have been found in some passerine species (Immler et al., 2010; Lüpold et al., 2009a; Mossman et al., 2009), however, this hypothesis has been refused for example in Humphries et al. (2008) where revealed that flagellum length is unlikely to be driven by selection for increased swimming speed (Humphries et al., 2008). Any association have been found in other studies on passerines as well (Immler et al., 2010; Lüpold et al., 2009b; Rowe et al., 2013). The exact relationship between sperm components morphology and function therefore appears to vary across species in passerines, and no clear pattern is yet known (Cramer et al., 2015).

Older studies also bring evidence for the assumption that proper movement of longer flagellum requires more energy gained from ATP, thus higher amount of mitochondria contained in longer midpiece is required to compensate longer flagellum energy requirements for movement (Cardullo and Baltz, 1991). Length of sperm flagellum is indeed positively related to the length of midpiece in some birds and mammals (Birkhead and Immler, 2007). Because a positive correlation between

length of flagellum and midpiece, sperm flagellum length is considered to be relative rather to other sperm components and unlikely to be driven by selection to affect sperm velocity (Humphries et al., 2008). Moreover, even if Lüpold et al. (2009) in their study on the family of passerine birds, the New World blackbirds (*Icteridae*), found evidence for an association between sperm midpiece-flagellum ratio and sperm velocity, they consider this result more as a side effect of sexual selection rather than direct forcing of postmating sexual selection on sperm morphology to increase sperm velocity (Lüpold et al., 2009a). Overall, these results demonstrate that no general pattern between the sperm morphology and sperm swimming speed has been found in birds.

We expected that longer sperms in the Common nightingale will have higher motility. However, in contrast to this expectation, we found no significant difference in sperm motility between Common and Thrush Nightingale in cell culture medium DMEM and thus no significant effect of sperm morphology on sperm motility. No associations have been found even in close related bluethroat (*Luscinia svecica svecica*) (Sætre et al., 2018). My results support the suggestion that the relationship between the sperm components morphology and sperm motility is more complicated as has been described also in other studies on passerine birds (Immler et al., 2010; Kleven et al., 2009; Lüpold et al., 2009b). Together with previous studies, my results may suggest that other effect, such as sperm longevity (Helfenstein et al., 2008) or female reproductive tract environment (Kleven et al., 2009; Sasanami et al., 2013), could influence sperm motility and fertilisation success rather than divergent sperm morphology itself, as single components are more related to each other than are forced to directly influence sperm velocity (Humphries et al., 2008; Kleven et al., 2009).

6.2. Is there a difference between sperm motility in conspecific and in heterospecific female fluid?

The second part of my thesis shows a possible effect of reproductive isolation on heterospecific sperm. The hypothesis was that if there are some signs of prezygotic reproductive isolation acting against heterospecific sperm in female vagina, which is considered as a the strongest barrier in the female reproductive tract (Sellier et al., 2005; Steele and Wishart, 1992), sperms of Thrush Nightingale should show lower motility in Common Nightingale female reproductive tract fluid than sperm of Common Nightingale. Because we had to design the second experiment as a comparison of conspecific treatment (CN fluid +CN sperm) with sperm motility in neutral culture cell medium PBS, the hypothesis supposes, that sperms in conspecific female fluid should have same or higher motility than sperms of the same male in neutral control PBS. Indeed, our results showed that there is no significant difference in sperm motility of Common nightingale sperm in conspecific fluid and in PBS. Under the same assumption, heterospecific sperm should have lower motility in the female fluid than in PBS, if there are prezygotic reproductive isolation mechanisms acting against heterospecific sperm (Moller et al., 2008; Satake et al., 2006). My results supported this hypothesis. I found that Sperms of Thrush nightingale in heterospecific female fluid swam significantly slower than sperms of the same male in neutral control PBS. This result indeed indicates that mechanisms of prezygotic postmating reproductive isolation could act against heterospecific sperm and thus play an important role in the speciation of those two species.

Sperm performance has been examined on several taxa of passerine birds, but only a few studies tested the effect of conspecific and heterospecific female reproductive tract fluid on sperm swimming speed and motility. All experiments were performed by Cramer et al. (2014, 2016a, 2016b).

The study performed on two closely related species, house sparrow (*Passer domesticus*) and Spanish sparrow (*Passer hispaniolensis*) showed no significant difference between sperm motility in conspecific and heterospecific female fluids (Cramer et al., 2014). Species also had similar sperm morphology and sperm swimming

performance. Neither the proportion of motile sperm differed across conspecific, heterospecific, or control treatments (Cramer et al., 2014).

Similarly, in another study where three reciprocal crosses represented three taxonomic families, no evidence of females discriminating against heterospecific sperm was found (Cramer et al., 2016). This study tested sperm motility and proportion of motile cells on 3 passerine species pairs- Barn swallows (*Hirundo rustica)* versus sand martins (*Riparia riparia)*, two subspecies of bluethroats, *Luscinia svecica svecica* versus *L. s. namnetum*, and great tits (*Parus major)* versus blue tits (*Cyanistes caeruleus)*. Those taxon pairs were tested for the hypothesis that postmating prezygotic barriers arise due to divergent selection within allopatric populations or species. Chosen species were particularly likely to show such barriers, because they have divergent sperm morphology and moderate- to- high sperm competition. These species pairs also do not hybridize in the wild (Cramer et al., 2016), except swallows where only one hybrid has been documented (Heneberg, 1997). Because of this fact, detected postmating prezygotic barrier could have been attributed as a by-product of divergence in phenotypes during isolation, rather than ongoing reinforcement acting on sperm phenotypes after secondary contact (e.g., Lorch and Servedio 2007; Matute 2010). However, sperm swam equally well in fluid from conspecific and heterospecific females as well in neutral controls. That suggests that postmating prezygotic barriers do not act at the stage between copulation and fertilization in these taxon pairs.

Opposite results bring the study of Cramer et al (2016b) where collared and pied flycatchers (*Ficedula albicollis* and *F. hypoleuca*) were tested for the presence of prezygotic postmating reproductive barriers. These species commonly hybridize in nature and females face the risk of hybridization and producing unfit hybrids (Qvarnstrom et al., 2010), therefore, there is an assumption for reproductive isolation on the prezygotic level. Indeed, results showed that females are able to inhibit heterospecific male sperm motility. Furthermore, it has been shown that the negative effect on heterospecific sperm performance was strongest in pied flycatcher females that were most likely to hybridize collared flycatcher sperm (Cramer et al, 2016b).

This work followed the same protocol as Cramer et al. (2014, 2016a, 2016b) and tested the effect of female reproductive tract fluid on sperm motility of two Nightingale species, My results could indicate the direction of further studies, as there could be

difference between sperm motility in conspecific and heterospecific female fluid. The fact, that Common and Thrush nightingale differ in sperm morphology (Albrecht et al., 2019) may indicate also divergence in other traits, such as surface proteins complement, which could be primary mechanism of cryptic female choice while sperm swim across vagina (Steele and Wishart, 1996). Thus, divergent sperm surface-associated proteins could negatively interact with female reproductive tract protein environment, resulting in selection against heterospecific sperm. Result of my thesis suggest, that there could be reproductive barriers acting after copulation and before fertilization. This assumption needs to be tested by proteomic analysis of sperm or transcriptomic analysis of testes. If is it so, my result could also contribute to assumption of possible reinforcement on gametic level, as there are signs for reinforcement acting on sperm head of Common Nightingale in sympatry (Albrecht et al., 2019). Presence of hybrid individuals in the secondary contact zone indicate that prezygotic postmating reproductive barriers are not fully formed which is one of the assumptions to prove reinforcement. However, the most majority of examples of reinforcement concern precopulatory reproductive isolation and only little examples was given for reinforcement postmating prezygotic level (Matute, 2010).

The results of my work could contribute to the explanation of the mechanisms of prezygotic reproductive isolation in two species of Nightingales. Together with the results of studies on sperm morphology (Albrecht et al., 2019) as well as results from experimental crosses in captivity (Stadie, 1991), results of my work suggesting that postmating prezygotic reproductive isolation is involved on some level and play a role in evoluton of those two species of Nightingales,.

7. Conclusion

Common and Thrush Nightingale provide useful model system for study mechanisms of speciation and reproductive isolation. Despite two species differ markedly in sperm morphology, they do not differ in sperm motility in cell culture medium DMEM. But this does not preclude assumption that there could be prezygotic reproductive isolation mechanisms acting in some level. Indeed, we showed that when sperm motility is compared in heterospecific and conspecific female fluid there is a tendency for lower motility in heterospecific than conspecific fluid. The fact, that sperms swam significantly slower in the fluid of heterospecific female than in neutral control could be sign that those barriers play role in species divergence. Contrary to the previous opinion, that in birds is important primary precopulatory prezygotic reproductive isolation, with postmating isolation evolving later, those results show, that it does not have to be true and postmating prezygotic reproductive isolation could also play an important role in the speciation of birds. However, more research is needed to better understand importance of postmating prezygotic reproductive isolation.

8. Bibliography

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9. Supplementary materials

9.1. Supplementary Table 1

List of captured individuals used in experiment 1 with capture location and GPS coordinates. Sperm motility was measured as VCL (µm/s). Number of motile spermatozoa is included as well.

Individual ID	Species	GPS_N	GPS_E	Locality	VCL	Number of motile sperms
LL30	Thrush Nightingale	53,17072	22,41963	Wizna	108,464	45,000
LL34	Thrush Nightingale	53,20000	22,4026	Wizna	96,278	169,000
LL35	Thrush Nightingale	53,20159	22,40672	Witkowo	90,694	103,000
LL36	Thrush Nightingale	53,23635	22,42335	Sieburczyn	118,114	289,000
LL37	Thrush Nightingale	53,30336	22,46024	Mocarze	114,683	46,000
S981	Thrush Nightingale	53,20009	22,40246	Wizna	103,572	514,000
S983ST	Thrush Nightingale	52,16187	17,69245	Pyzdry	84,195	58,000
S997ST	Thrush Nightingale	52,15332	17,67917	Pyzdry	122,501	380,000
S1005ST	Thrush Nightingale	52,17701	17,72444	Pyzdry	99,881	109,000
S1006ST	Thrush Nightingale	52,17668	17,73044	Pyzdry	85,593	41,000
S1007ST	Thrush Nightingale	52,17646	17,72980	Pyzdry	98,096	99,000
S1011ST	Thrush Nightingale	52,17889	17,90238	Zagorow	72,642	102,000
S1012ST	Thrush Nightingale	52,18097	17,90862	Zagorow	78,144	121,000
S1014ST	Thrush Nightingale	52,17889	17,71669	Pyzdry	85,105	57,000
S1015ST	Thrush Nightingale	52,18006	17,70967	Pyzdry	103,809	139,000
LM42	Common Nightingale	52,18134	17,69203	Dlusk	98,061	31,000
LM43	Common Nightingale	52,04330	17,72531	Czolnochów	103,017	427,000
LM44	Common Nightingale	52,04257	17,72573	Czolnochów	107,326	637,000
LM45	Common Nightingale	52,04197	17,72668	Robaków	69,216	64,000
LM46	Common Nightingale	52,04197	17,72668	Robaków	91,393	132,000
S982SO	Common Nightingale	52,17881	17,69665	Dlusk	75,500	118,000
S985SO	Common Nightingale	52,14920	17,67163	Tarnowa	112,531	141,000
S986SO	Common Nightingale	52,17948	17,69107	Dlusk	86,267	272,000
S991SO	Common Nightingale	52,17823	17,69411	Dlusk	78,394	67,000
S993SO	Common Nightingale	52,16075	17,68764	Pyzdry	137,349	536,000
S998SO	Common Nightingale	52,04799	17,70617	Prusinow	124,397	66,000
S999SO	Common Nightingale	52,04754	17,70407	Prusinow	105,393	240,000
S1000SO	Common Nightingale	52,17961	17,71470	Pyzdry	66,130	23,000
S1004SO	Common Nightingale	52,17728	17,72346	Pyzdry	92,590	358,000
S1008SO	Common Nightingale	52,18531	17,90304	Zagorow	83,458	60,000
S1009SO	Common Nightingale	52,18552	17,90236	Zagorow	109,168	207,000
S1010SO	Common Nightingale	52,20266	17,87542	Policko	76,004	282,000
S1013SO	Common Nightingale	52,18583	17,90217	Zagorow	120,959	322,000
S1016SO	Common Nightingale	52,11824	17,66426	Ruda Komorska	106,162	21,000

9.2. Supplementary Table 2

List of captured individuals used in experiment 2 with capture location and GPS coordinates. Other informations such as year of capture, experimental block, sex and species are included as well. Measured values are in Supplementary Table 3.

Individual ID	Sex	Species	GPS_N	GPS_E	Locality	Year	Experiment number
KB26645	Female	Common Nightingale	51,98386	17,84383	Kwileň most	2014	EX_1
KB26548	Male	Common Nightingale	51,96797	17,87222	Chocz	2014	EX_1
NA11009	Male	Thrush Nightingale	51,97447	17,86050	Chocz	2014	EX_1
KB26659	Female	Common Nightingale	52,04789	17,71331	Prusinów	2014	EX_2
NA05385	Male	Thrush Nightingale	52,04803	17,71311	Prusinow	2014	EX_2
KB26660	Male	Common Nightingale	52,04803	17,71311	Prusinow	2014	EX_2
KB26665	Female	Common Nightingale	52,04222	17,73011	Czolnockow	2014	EX_3
KB26667	Male	Common Nightingale	52,03186	17,73631	Grab	2014	EX_3
NA11017	Male	Thrush Nightingale	52,17906	17,91219	Zagorow	2014	EX_3
KB26671	Female	Common Nightingale	52,03731	17,73356	Robakow	2014	EX_4
NA11015	Male	Thrush Nightingale	52,18283	17,90569	Zagorow	2014	EX_4
KB26675	Male	Common Nightingale	52,20608	17,89567	Lad	2014	EX_4
kb26720	Female	Common Nightingale	51,97458	17,86064	Chocz	2015	EX_5
kb26717	Male	Common Nightingale	51,97511	17,85981	Chocz	2015	EX_5
na05385	Male	Thrush Nightingale	52,04789	17,70428	Prusinow	2015	EX_5
kb26722	Female	Common Nightingale	51,97750	17,86147	Chocz	2015	EX_6
na11001	Male	Thrush Nightingale	52,18258	17,90606	Zagórów	2015	EX_6
kb26721	Male	Common Nightingale	51,97614	17,85689	Chocz	2015	EX_6

Individual ID	Sex	Species	GPS_N	GPS_E	Locality	Year	Experiment number
kb26735	Female	Common Nightingale	51,96793	17,87233	Chocz	2015	EX_7
kb26743	Male	Common Nightingale	52,04178	17,72679	Czolnochov	2015	EX_7
na11024	Male	Thrush Nightingale	52,18308	17,92758	Zagórów	2015	EX_7
kb26740	Female	Common Nightingale	52,04821	17,71528	Prusinów	2015	EX_8
kb26663	Male	Common Nightingale	52,04283	17,73086	Czolnochov	2015	EX_8
na11023	Male	Thrush Nightingale	52,18355	17,90250	Zagórów	2015	EX_8
kb26741	Female	Common Nightingale	52,04841	17,70308	Prusinów	2015	EX_9
kb26751	Male	Common Nightingale	52,04835	17,70352	Prusinów	2015	EX_9
na11027	Male	Thrush Nightingale	52,18633	17,93744	Zagórów	2015	EX_9
kb26744	Female	Common Nightingale	52,04166	17,72384	Czolnochov	2015	EX_10
kb26752	Male	Common Nightingale	52,04799	17,71235	Prusinów	2015	EX_10
na11030	Male	Thrush Nightingale	52,19553	17,89522	Zagórów	2015	EX_10
S850	Female	Common Nightingale	52,15394	17,67921	Pyzdry	2017	EX_13
S863	Male	Thrush Nightingale	52,1614025	17,69297	Pyzdry	2017	EX_13
S878	Male	Common Nightingale	52,20595	17,79384	Ciazen	2017	EX_13
S849	Female	Common Nightingale	52,13648	17,67755	Pyzdry	2017	EX_15
S864	Male	Thrush Nightingale	52,16704	17,70060	Pyzdry	2017	EX_15
S862	Male	Common Nightingale	52,19314	17,72197	Rataje	2017	EX_15
S869	Female	Common Nightingale	52,17876	17,69441	Dtusk	2017	EX_17
S874	Male	Thrush Nightingale	52,17574	17,72611	Pyzdry	2017	EX_17
S875	Male	Common Nightingale	52,18111	17,71511	Rataje	2017	EX_17
S870	Female	Common Nightingale	52,17876	17,69441	Dtusk	2017	EX_18
S880	Male	Thrush Nightingale	52,20717	17,79409	Ciazen	2017	EX_18
S882	Male	Common Nightingale	52,20486	17,78081	Samarzewo	2017	EX_18

9.3. Supplementary Table 3

Measured values of individuals used in experiment 2.

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
KB26645	KB26548	EX_1	SO	conspecific	SO	2	fluid	87,60	2	1	24
KB26645	KB26548	EX_1	SO	conspecific	SO	4	fluid	60,20	2	3	118
KB26645	KB26548	EX_1	SO	conspecific	SO	1	PBS	70,50	1	16	1
KB26645	KB26548	EX_1	SO	conspecific	SO	3	PBS	42,70	1	4	97
KB26645	NA11009	EX_1	ST	heterospecific	SO	2	fluid	91,40	2	216	8
KB26645	NA11009	EX_1	ST	heterospecific	SO	4	fluid	71,56	2	70	41
KB26645	NA11009	EX_1	ST	heterospecific	SO	1	PBS	82,54	1	95	1
KB26645	NA11009	EX_1	ST	heterospecific	SO	3	PBS	81,38	1	53	17
KB26645	NA11009	EX_1	ST	heterospecific	SO	6	fluid	68,08	2	122	112
KB26645	NA11009	EX_1	ST	heterospecific	SO	8	fluid	74,56	2	42	151
KB26645	NA11009	EX_1	ST	heterospecific	SO	5	PBS	73,62	1	77	56
KB26645	NA11009	EX_1	ST	heterospecific	SO	7	PBS	71,08	1	67	126
KB26659	NA05385	EX_2	ST	heterospecific	SO	1	fluid	69,36	1	75	1
KB26659	NA05385	EX_2	ST	heterospecific	SO	3	fluid	60,18	1	25	18
KB26659	NA05385	EX_2	ST	heterospecific	SO	2	PBS	58,81	2	30	9
KB26659	NA05385	EX_2	ST	heterospecific	SO	4	PBS	44,08	2	25	27
KB26659	NA05385	EX_2	ST	heterospecific	SO	5	fluid	51,04	1	22	38
KB26659	NA05385	EX_2	ST	heterospecific	SO	7	fluid	43,00	1	4	59
KB26659	NA05385	EX_2	ST	heterospecific	SO	6	PBS	53,53	2	25	46
KB26659	KB26660	EX_2	SO	conspecific	SO	1	fluid	72,47	1	114	1

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
KB26659	KB26660	EX_2	SO	conspecific	SO	3	fluid	54,83	1	22	23
KB26659	KB26660	EX_2	SO	conspecific	SO	2	PBS	52,67	2	74	7
KB26659	KB26660	EX_2	SO	conspecific	SO	4	PBS	76,60	2	6	41
KB26659	KB26660	EX_2	SO	conspecific	SO	5	fluid	86,70	1	10	104
KB26659	KB26660	EX_2	SO	conspecific	SO	6	PBS	58,60	2	4	127
KB26665	KB26667	EX_3	SO	conspecific	SO	1	fluid	69,02	1	13	1
KB26665	KB26667	EX_3	SO	conspecific	SO	2	PBS	96,10	2	9	9
KB26665	NA11017	EX_3	ST	heterospecific	SO	2	fluid	34,81	2	25	8
KB26665	NA11017	EX_3	ST	heterospecific	SO	4	fluid	46,80	2	11	102
KB26665	NA11017	EX_3	ST	heterospecific	SO	1	PBS	67,46	1	34	1
KB26665	NA11017	EX_3	ST	heterospecific	SO	3	PBS	48,58	1	64	27
KB26665	NA11017	EX_3	ST	heterospecific	SO	5	PBS	60,46	1	9	124
KB26671	NA11015	EX_4	ST	heterospecific	SO	1	fluid	33,00	1	13	1
KB26671	NA11015	EX_4	ST	heterospecific	SO	3	fluid	58,20	1	3	27
KB26671	NA11015	EX_4	ST	heterospecific	SO	2	PBS	43,73	2	19	12
KB26671	NA11015	EX_4	ST	heterospecific	SO	4	PBS	32,70	2	5	45
KB26671	NA11015	EX_4	ST	heterospecific	SO	5	fluid	27,63	1	11	102
KB26671	KB26675	EX_4	SO	conspecific	SO	1	fluid	67,72	1	98	1
KB26671	KB26675	EX_4	SO	conspecific	SO	3	fluid	61,15	1	23	41
KB26671	KB26675	EX_4	SO	conspecific	SO	2	PBS	87,42	2	99	13
KB26671	KB26675	EX_4	SO	conspecific	SO	4	PBS	69,90	2	8	105
KB26671	KB26675	EX_4	SO	conspecific	SO	5	fluid	55,96	1	9	126

KB26671	KB26675	EX_4	SO	conspecific	SO	6	PBS	68,40	2	8	145

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
kb26720	kb26717	EX_5	SO	conspecific	SO	1	PBS	72,80	1	15	1
kb26720	kb26717	EX_5	SO	conspecific	SO	3	PBS	114,08	1	14	39
kb26720	na05385	EX_5	ST	heterospecific	SO	2	fluid	71,57	2	49	13
kb26720	na05385	EX_5	ST	heterospecific	SO	4	fluid	51,71	2	17	105
kb26720	na05385	EX_5	ST	heterospecific	SO	1	PBS	64,03	1	15	1
kb26720	na05385	EX_5	ST	heterospecific	SO	3	PBS	101,02	1	34	39
kb26722	na11001	EX_6	ST	heterospecific	SO	1	fluid	45,66	1	26	1
kb26722	na11001	EX_6	ST	heterospecific	SO	3	fluid	48,72	1	39	37
kb26722	na11001	EX_6	ST	heterospecific	SO	2	PBS	38,85	2	50	10
kb26722	na11001	EX_6	ST	heterospecific	SO	4	PBS	51,29	2	15	55
kb26722	na11001	EX_6	ST	heterospecific	SO	5	fluid	41,20	1	40	130
kb26722	na11001	EX_6	ST	heterospecific	SO	6	PBS	53,96	2	11	144
kb26722	kb26721	EX_6	SO	conspecific	SO	1	fluid	48,00	1	5	1
kb26722	kb26721	EX_6	SO	conspecific	SO	3	fluid	31,00	1	6	30
kb26722	kb26721	EX_6	SO	conspecific	SO	2	PBS	38,40	2	5	9
kb26722	kb26721	EX_6	SO	conspecific	SO	4	PBS	80,76	2	7	54
kb26735	kb26743	EX_7	SO	conspecific	SO	2	fluid	59,78	2	30	13
kb26735	kb26743	EX_7	SO	conspecific	SO	4	fluid	47,05	2	26	109
kb26735	kb26743	EX_7	SO	conspecific	SO	1	PBS	64,89	1	19	1
kb26735	kb26743	EX_7	SO	conspecific	SO	3	PBS	49,32	1	17	41
kb26735	na11024	EX_7	ST	heterospecific	SO	2	fluid	60,77	2	211	10
kb26735	na11024	EX_7	ST	heterospecific	SO	4	fluid	54,57	2	124	58

kb26735	na11024	EX_7	ST	heterospecific	SO	1	PBS	74,34	1	115	1

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
kb26735	na11024	EX_7	ST	heterospecific	SO	3	PBS	65,76	1	112	39
kb26735	na11024	EX_7	ST	heterospecific	SO	5	PBS	80,48	1	85	127
kb26740	kb26663	EX_8	SO	conspecific	SO	3	fluid	65,97	1	62	38
kb26740	kb26663	EX_8	SO	conspecific	SO	2	PBS	61,97	2	189	13
kb26740	kb26663	EX_8	SO	conspecific	SO	4	PBS	68,62	2	72	114
kb26740	kb26663	EX_8	SO	conspecific	SO	5	fluid	47,79	2	35	141
kb26740	na11023	EX_8	ST	heterospecific	SO	1	fluid	58,72	1	69	1
kb26740	na11023	EX_8	ST	heterospecific	SO	3	fluid	49,26	1	65	39
kb26740	na11023	EX_8	ST	heterospecific	SO	2	PBS	77,61	2	245	13
kb26740	na11023	EX_8	ST	heterospecific	SO	4	PBS	67,73	2	83	101
kb26740	na11023	EX_8	SO	heterospecific	SO	5	fluid	48,06	1	27	124
kb26741	kb26751	EX_9	SO	conspecific	SO	2	fluid	83,56	2	23	12
kb26741	kb26751	EX_9	SO	conspecific	SO	4	fluid	57,24	2	5	108
kb26741	kb26751	EX_9	SO	conspecific	SO	1	PBS	66,80	1	8	1
kb26741	kb26751	EX_9	SO	conspecific	SO	3	PBS	84,70	1	1	41
kb26741	na11027	EX_9	ST	heterospecific	SO	2	fluid	63,93	2	58	16
kb26741	na11027	EX_9	ST	heterospecific	SO	4	fluid	70,58	2	22	101
kb26741	na11027	EX_9	ST	heterospecific	SO	1	PBS	80,10	1	53	1
kb26741	na11027	EX_9	ST	heterospecific	SO	3	PBS	57,08	1	26	42
kb26741	na11027	EX_9	ST	heterospecific	SO	5	PBS	117,80	1	5	118
kb26744	kb26752	EX_10	SO	conspecific	SO	1	fluid	67,38	1	206	1
kb26744	kb26752	EX_10	SO	conspecific	SO	3	fluid	59,21	1	182	40

kb26744	kb26752	EX_10	SO	conspecific	SO	2	PBS	77,60	2	406	17

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
kb26744	kb26752	EX_10	SO	conspecific	SO	4	PBS	64,44	2	243	105
kb26744	kb26752	EX_10	SO	conspecific	SO	5	fluid	66,68	1,00	116	126
kb26744	na11030	EX_10	ST	heterospecific	SO	1	fluid	66,43	1	52	1
kb26744	na11030	EX_10	ST	heterospecific	SO	3	fluid	65,79	1	30	45
kb26744	na11030	EX_10	ST	heterospecific	SO	2	PBS	43,62	2	40	15
kb26744	na11030	EX_10	ST	heterospecific	SO	4	PBS	85,14	2	7	109
S850	S863	EX_13	ST	heterospecific	SO	1	fluid	51,79	1	20	1
S850	S863	EX_13	ST	heterospecific	SO	3	fluid	51,03	1	8	49
S850	S863	EX_13	ST	heterospecific	SO	2	PBS	103,53	2	8	17
S850	S863	EX_13	ST	heterospecific	SO	4	PBS	54,98	2	5	78
S850	S863	EX_13	ST	heterospecific	SO	5	fluid	50,96	1,00	8	108
S850	S878	EX_13	SO	conspecific	SO	1	fluid	51,68	1	13	1
S850	S878	EX_13	SO	conspecific	SO	3	fluid	91,75	1	6	74
S850	S878	EX_13	SO	conspecific	SO	2	PBS	76,15	2	33	24
S850	S878	EX_13	SO	conspecific	SO	4	PBS	67,43	2	4	124
S849	S864	EX_15	ST	heterospecific	SO	2	fluid	77,48	2	26	19
S849	S864	EX_15	ST	heterospecific	SO	4	fluid	42,98	2	16	80
S849	S864	EX_15	ST	heterospecific	SO	1	PBS	86,71	1	29	1
S849	S864	EX_15	ST	heterospecific	SO	3	PBS	76,86	1	5	55
S849	S864	EX_15	ST	heterospecific	SO	5	PBS	19,24	1,00	8	111
S849	S862	EX_15	SO	conspecific	SO	2	fluid	74,65	2	15	31
S849	S862	EX_15	SO	conspecific	SO	4	fluid	53,94	2	5	123

S849	S862	EX_15	SO	conspecific	SO	1	PBS	115,67	1	3	1

Female ID	Male ID	Experiment number	Male species	Treatment	Female species	Record order	Fluid or PBS	VCL	Order of PBS control	Sum of motile sperms	Start time in seconds
S849	S862	EX_15	SO	conspecific	SO	3	PBS	32,30	1	1	93
S849	S862	EX_15	SO	conspecific	SO	6	fluid	31,77	2	9	1
S849	S862	EX_15	SO	conspecific	SO	8	fluid	59,26	2	7	93
S849	S862	EX_15	SO	conspecific	SO	10	fluid	38,72	2	11	1
S849	S862	EX_15	SO	conspecific	SO	5	PBS	64,50	1	35	149
S849	S862	EX_15	SO	conspecific	SO	7	PBS	30,66	1	7	31
S849	S862	EX_15	SO	conspecific	SO	9	PBS	34,41	1	11	123
S869	S874	EX_17	ST	heterospecific	SO	2	PBS	85,72	2	43	22
S869	S874	EX_17	ST	heterospecific	SO	4	PBS	60,60	2	3	98
S869	S875	EX_17	SO	conspecific	SO	1	fluid	78,77	1	15	1
S869	S875	EX_17	SO	conspecific	SO	2	PBS	55,80	2	18	24
S870	S880	EX_18	ST	heterospecific	SO	2	fluid	72,43	2	78	20
S870	S880	EX_18	ST	heterospecific	SO	4	fluid	86,98	2	16	82
S870	S880	EX_18	ST	heterospecific	SO	1	PBS	66,43	1	45	1
S870	S880	EX_18	ST	heterospecific	SO	3	PBS	68,35	1	28	54
S870	S882	EX_18	SO	conspecific	SO	2	fluid	62,29	2	279	17
S870	S882	EX_18	SO	conspecific	SO	4	fluid	57,04	2	62	78
S870	S882	EX_18	SO	conspecific	SO	1	PBS	72,44	1	234	1
S870	S882	EX_18	SO	conspecific	SO	3	PBS	72,20	1	269	50