The evolution and function of sperm head morphology in songbirds

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Summary

The sperm cell is one of the most morphologically diverse cells in the animal body. Despite the similar function of all sperm cells – to fertilise the egg – there is extraordinary diversity in shape and length across taxa, from multiflagellate crustacean sperm to the extremely long sperm of *Drosophila*. However, this diversity is poorly understood. Why are sperm cells so variable? What are the factors that have influenced sperm cells to evolve in such different directions?

In this PhD thesis, I have attempted to contribute to our understanding of sperm evolution and function, using an animal group with high levels of sperm diversity; the songbirds. Songbirds (Passeriformes: Passerida) are a well-known study system for sperm evolution – they are promiscuous to a varying degree, which means that there is varying levels of post-copulatory sexual selection pressure; additionally, sperm length varies considerably within this group. Sperm length has been shown to correlate with sperm competition intensity as well as with sperm swimming speed, and so sperm morphology appears to be an important trait for songbird males. To date, research in this area has been focused on one-dimensional length measurements, usually using images from a relatively low-resolution light microscope; however, songbirds have a characteristic helical sperm head, which appears to vary in shape and size across species. This morphology is difficult to accurately assess using light microscopy, and therefore, the foundation of my work has been to use scanning electron microscopy (SEM) to build a collection of high-resolution images of sperm heads across songbird species. I have, in total, acquired 2289 such images from 36 species, and from each image I have obtained a number of measurements. These types of measurements are a novel addition to the literature, and open many doors for research into the evolution and function of sperm head morphology.

My work describes intra- and interspecific variation in sperm head morphology of songbirds, and considers both the functional significance of sperm traits (i.e. influences of sperm morphology on swimming speed and sperm abnormalities), and external factors that influence the evolution and function of these traits (i.e. the influence of mating behaviour, geography and diet on sperm morphology). **In the first chapter,** I describe geographic variation in sperm traits in a common songbird species, the willow warbler *Phylloscopus trochilus*, and find that although total sperm length did not vary across Norway in this species, sperm heads were longer in the southern part of the country than in the north. This demonstrates that sperm head traits can diverge in a population that is otherwise only weakly differentiated in genetic and morphological traits. **The second chapter** comprises the main body of work in my PhD, and presents the novel data from the SEM images. First, I describe the intra- and interspecific variation in sperm head traits across 36 songbird species, including sperm head length, width, volume, and shape. Second, I use comparative methods to assess the relationship of these traits to sperm swimming speed and sperm competition intensity. I

find that sperm head traits are correlated with one another and with total sperm length, so that longer sperm cells tend to have a more distinct helical shape, with a wide, corkscrew-shaped head, and I show that these sperm cells swim faster than the straighter cells. As such, there appears to be a fitness benefit of the distinctive helical sperm morphology in terms of increased swimming speed. I find no support for the hypothesis that sperm competition intensity is driving interspecific diversification in this group, although I do find that the intra-specific and intra-male variation in sperm head traits are correlated with sperm competition intensity, which suggests that post-copulatory sexual selection pressure has led to stabilising selection on sperm head traits. **In the third chapter,** I combine the data from the SEM images with data on the frequency of abnormal sperm heads, using the same 36 songbird species. I find that species with more helical sperm cells also have higher frequencies of abnormal sperm, indicating that helical sperm cells are more fragile, possibly due to a wide, thin helical membrane that may be susceptible to mechanical and/or oxidative stress. This suggests that there is a fitness cost associated with the fast, strongly helical sperm head shape, and my work thus indicates that there could be a trade-off between sperm swimming speed and sperm cell integrity. **In the fourth and final chapter,** I consider how the environment and diet of the individual can influence sperm function, by assessing the relationship between fatty acid composition in the blood of two finch species and the frequency of abnormal sperm heads. I show that the proportion of linoleic acid (18:2n-6) as well as the omega-6/omega-3 ratio in the blood is correlated with the frequency of sperm head abnormalities. Linoleic acid is an omega-6 fatty acid that is found in large quantities in sunflower seeds, which most of the birds in the study had free access to, and such omega-6 fatty acids are associated with increased levels of oxidative stress. As sperm are particularly vulnerable to oxidative stress, there is a possibility that a diet consisting mainly of sunflower seeds can negatively impact sperm quality in birds, although this needs to be experimentally tested to verify a causal effect.

In summary, I find that sperm head morphology in birds is variable within and among species; both in length and in several other dimensions including shape. My work shows that the distinctive helical morphology of songbird sperm is associated with an increase in swimming speed, but that the fitness benefit of having strongly helical sperm heads is associated with a cost of more fragile sperm. Finally, I find indications that feeding habits might influence sperm quality in urban birds. My results highlight that it is advisable to adopt a multi-dimensional approach when studying sperm form and function; one-dimensional length measurements give an incomplete picture of the sperm cell, and studies that neglect to include further traits run the risk of misinterpreting biological patterns or missing interesting patterns altogether. My thesis contributes to our understanding of both the evolution and function of sperm morphology in songbirds, and I anticipate that this improved understanding may even be relevant for other taxa with similar sperm morphology.

List of papers

I. Støstad, H. N., Rekdal, S. L., Kleven, O., Laskemoen, T., Marthinsen, G., Johnsen, A., & Lifjeld, J. T. (2016). Weak geographical structure in sperm morphology across the range of two willow warbler *Phylloscopus trochilus* subspecies in Scandinavia. *Journal of Avian Biology*, 47(5): 731-741.

II. Støstad, H. N., Johnsen, A. Lifjeld, J. T. & Rowe, M. Sperm head morphology is associated with sperm swimming speed: a comparative study of passerine bird sperm using electron microscopy. Manuscript in review following revision in *Evolution*.

III. Støstad, H. N., Johnsen, A., Rowe, M. & Lifjeld, J. T. Sperm head abnormalities are more frequent in songbirds with more helical sperm. Manuscript prepared for submission to *Biology Letters*.

IV. Støstad, H. N., Rowe, M. & Lifjeld, J. T. Sperm head abnormalities are associated with excessive omega-6 fatty acids in two finch species feeding on sunflower seeds. Manuscript.

Introduction

The sperm cell is, arguably, one of the most influential cells in the bodies of sexually reproducing animals. Without a well-functioning sperm, there can be no reproduction, and therefore, no life. Thus, sperm function is essential for much of the animal life on the planet. Considering this vital role, it is rather surprising that sperm cells are also one of the most diverse cell types in terms of morphology. The familiar, rounded sperm head of humans, with a single short tail, is certainly not representative of all sperm cells – for example, frog sperm have a strikingly elongated and straight sperm head; mice have hook-shaped sperm heads that attach together to form sperm "trains" (Breed 2004); whereas the sperm of some crustaceans do not have a flagellum at all but instead have a distinct protruding spike (Kim et al. 2003; for numerous examples of sperm diversity, see The San Diego Zoo Sperm Atlas (http://institute.sandiegozoo.org/resources/sperm-atlas)). Similarly, total sperm length is extremely variable across taxa; ranging from the tiny sperm of the parasitoid wasp *Cotesia congregata* (7 µm) to the 6 cm long sperm of the fruit fly *Drosophila bifurca* (20 times its entire body length), a difference of almost four orders of magnitude.

Despite this remarkable variation, there is still considerable uncertainty as to why sperm cells are so variable. There has been some progress towards answering this question during the last few decades; the work of Geoff Parker on sperm competition in the 1970s (e.g. Parker 1970) triggered an increased interest in sperm evolution research. He showed how promiscuous mating can lead to competition among the sperm of different males, which in turn can lead to strong post-copulatory sexual selection pressure on sperm morphology. In the following years, a large body of work have documented a relationship between sperm competition intensity and sperm morphology (see Pitnick et al. 2009, Pizzari & Parker 2009 for reviews), and the importance of male-female interaction and the characteristics of the female reproductive tract have been recognised (e.g. Miller & Pitnick 2002). Nonetheless, there are still a number of unanswered questions. One such question relates to the evolution and function of sperm shape. Nearly all sperm morphology research to date uses single-dimension length measurements; yet sperm are also variable in width, volume, and form. The sperm head in particular is comprised of several parts that also appear to vary in size and shape among taxa. Are these types of variation driven by sperm competition? What are the fitness costs and benefits associated with the various sperm head shapes? In this thesis, I have attempted to answer these questions, or, at least, contribute to answering them.

The majority of the work focuses on the evolution and function of sperm head morphology, both intra- and interspecifically, whereas the final chapter takes a more ecological approach, aiming to understand how diet can influence sperm performance. By approaching the topic from several angles, I have gained a more thorough understanding of the processes that influence sperm morphology, and as such I hope to contribute to the wider body of knowledge on this important aspect of evolutionary and reproductive biology.

Study system

First, a note on terminology. Throughout this thesis and the articles therein, I have generally used the term “songbirds”, as all the study species are in the clade Passeri, also known as the songbirds or oscines, which is a suborder of the order Passeriformes (passerine birds). I have not included any representatives of the Tyranni (suboscines) which is the other clade in Passeriformes. Passeri also includes the parvorder Corvida, from which there are no species in this thesis, i.e. all relevant species in this thesis are found in the parvorder Passerida; but there is no well-known common name to describe birds in the clade Passerida, and so I have used “songbirds” throughout. However, the term “passerine birds” is used when describing literature or knowledge that refers to passerines in the broader sense, as in the paragraphs below.

Passerine birds are a widely used study system for sperm research for several reasons. Firstly, they are numerous, easily observable and relatively straightforward to catch for sampling, making them a convenient study organism in the wild. Secondly, the phylogenetic relationships are quite well established, with available DNA sequence data, facilitating comparative studies. Thirdly, they are promiscuous (i.e. a female may mate with several males in addition to her social mate), but the frequency of these extra-pair matings varies among species (Griffith et al. 2002), meaning that it is possible to compare closely related species that experience different levels of sperm competition. Finally, the sperm cells of passerine birds exhibit considerable variation in length, as well as in shape (see below); total sperm length ranges from 43 to 292 µm (Pitnick et al. 2009) – for reference, the diameter of a human hair is roughly 50-100 µm. Thus, passerine birds constitute a taxon that is both relatively easy to study, as well as being a biologically interesting system.

For the purpose of this thesis, passerine birds are particularly relevant, as the sperm bank at the Natural History Museum in Oslo (NHMO) provides access to a large number of sperm samples from passerines. These samples have been the foundation for my thesis, and it is unlikely that the work would have been possible in a reasonable timeframe without these samples. The 36 species chosen for the comparative studies (chapter 2 & 3) were selected partly on the basis of which relevant species were well represented in the NHMO sperm bank. Additionally, these species exhibit a variety of sperm lengths and sperm competition levels, and I also took into account the availability of further data such as rates of extra-pair young.

The study species in chapter 1, the willow warbler *Phylloscopus trochilus* (Fig. 1a), was of particular interest due to the existence of two subspecies within Norway which are differentiated in certain genetic markers, body morphology and migratory routes. The two study species in chapter 4, the greenfinch *Chloris chloris* (Fig. 1b) and the hawfinch *Coccothraustes coccothraustes* (Fig. 1c), were chosen partly because of their feeding habits; both species are seed eaters that are common in urban areas and tend to forage extensively on sunflower feeders. In addition, there had been reports of

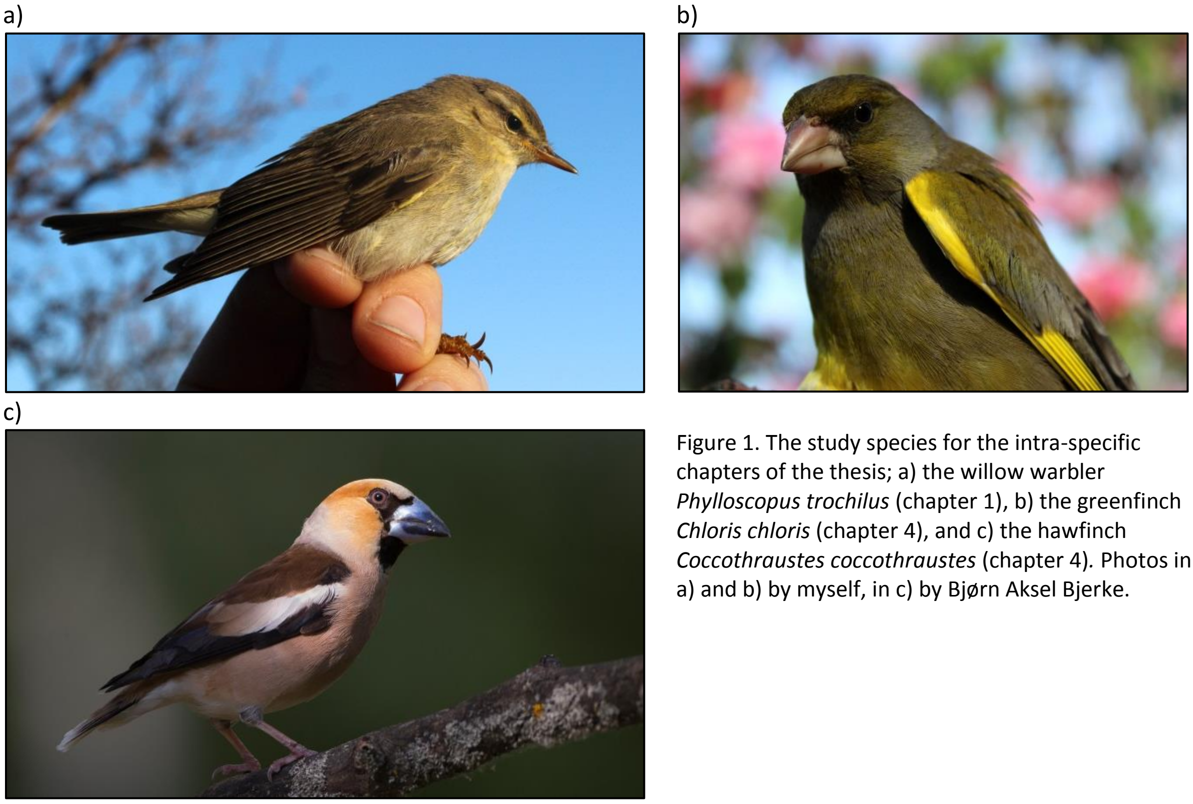

Figure 1. The study species for the intra-specific chapters of the thesis; a) the willow warbler *Phylloscopus trochilus* (chapter 1), b) the greenfinch *Chloris chloris* (chapter 4), and c) the hawfinch *Coccothraustes coccothraustes* (chapter 4). Photos in a) and b) by myself, in c) by Bjørn Aksel Bjerke.

high frequencies of damaged sperm in greenfinch sperm samples collected by the research group in previous years. The greenfinch is also a species that is declining in Norway (Falkenberg et al. 2018), further increasing the relevance of this research.

Characteristics of passerine sperm

In passerine birds, sperm competition intensity has been shown to be correlated with total sperm length (Briskie et al. 1997; Kleven et al. 2009) as well as with intra-specific variation in sperm traits (Calhim et al. 2007; Kleven et al. 2007; Immler et al. 2008; Lifjeld et al. 2010). In turn, sperm length is associated with increased swimming speed at both inter- and intra-specific level (Lüpold et al. 2009; Bennison et al. 2016) as well as with fertilisation success in the zebra finch *Taeniopygia guttata* (Bennison et al. 2015). Hence, it is relatively clear that sperm morphology in passerines is influenced by post-copulatory sexual selection pressure, and that sperm morphology is important for male fitness in this group. Specifically, head length has been shown to vary among males and populations within a species (Schmoll & Kleven 2011; Hogner et al. 2013), although it appears to vary less among species than does total length (Lüpold et al. 2009) and might be evolutionarily constrained (Rowe et al. 2015). Additionally, Lüpold et al. (2009) found that head length was not related to sperm competition (as measured by relative testes size). However, in similarity to other taxa as described above, studies on passerine sperm are mostly one-dimensional, focusing either on total sperm length only or on the length of the various components (head, midpiece, and tail/flagellum). Thus, we have

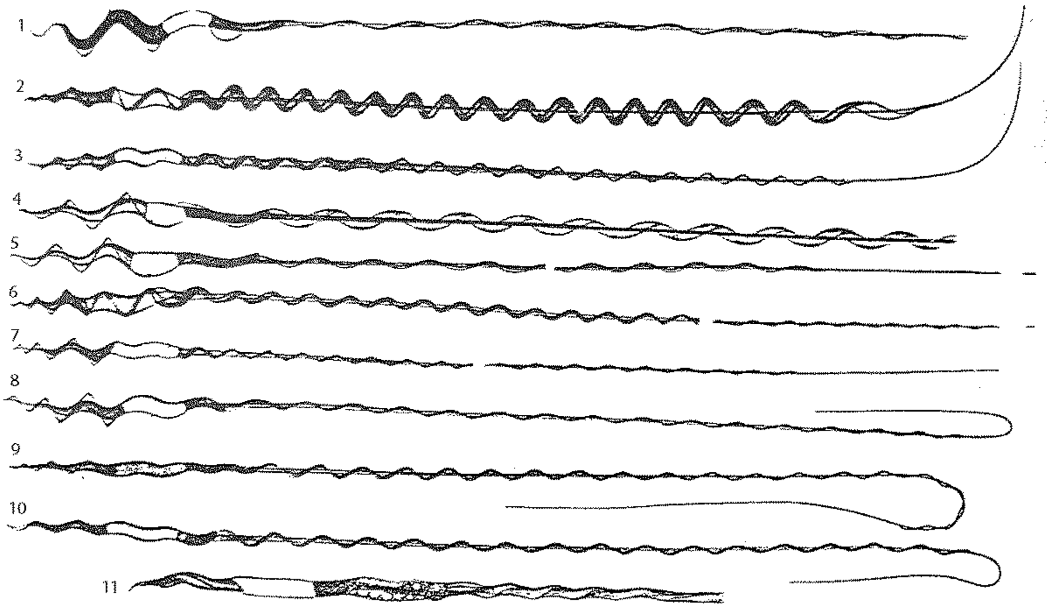

Figure 2. Line drawings of sperm cells by light microscopy, from Retzius (1909). From the top: nightingale *Luscinia luscinia* (1), starling *Sturnus vulgaris* (2,3), yellowhammer *Emberiza citronella* (4,5), pied flycatcher *Ficedula hypoleuca* (6,7), rock pipit *Anthus spinoletta* (8), wood warbler *Phylloscopus sibilatrix* (9,10), skylark *Aluada arvensis* (11). Reproduced from Jamieson (2007) with permission from the publisher.

little knowledge of the evolutionary patterns of other sperm traits such as width and shape, and how they might influence sperm function.

Passerine sperm heads have a characteristic helical shape, which has been acknowledged for more than a century. Indeed, already in the early 1900s, Retzius (1909) published line drawings based on light microscope observations (Fig. 2), clearly showing helical or spiral sperm heads with a distinct helical membrane (also known as a helical keel). However, since then, only descriptive and/or qualitative work has been published on this characteristic morphology (e.g. Schilthuizen et al. 2017). From this, it is possible to ascertain that the head shape does vary among bird species, but this variation has not been quantified. Similarly, in other taxa with spiral or helical sperm morphology (e.g. octopuses, sea slugs and monotremes), studies tend to be descriptive, focusing on the physiological process of spermatogenesis (e.g. Kubo & Ishikawa 1981), and refrain from commenting on the functional or evolutionary aspects. As Carrick and Hughes (1982) wrote in their work on monotreme sperm structure; “However tempting this might seem, it is unwise to embark on further speculation [of the evolution and function of sperm shape] in the absence of a thorough investigation of form, function and development of monotreme spermatozoa.” Considering the fact that such helical sperm morphology appears to have evolved in several different taxa, probably independently, there is presumably some associated fitness benefit, but such a benefit has never been established; neither is there any knowledge on the potential fitness costs.

The passerine sperm head consists of different parts that play different roles in the fertilisation process, and therefore may have different evolutionary trajectories. The acrosome is the anterior part of the sperm head, and contains enzymes that interact with the ovum at fertilisation (Adham et al. 1997; Nishio & Matsuda 2017); whereas the nucleus comprises the rest of the sperm head and is between the acrosome and the midpiece, containing the compacted DNA. During fertilisation, the acrosomal enzymes are released and break down the inner perivitelline layer of the ovum, a process known as the acrosome reaction, enabling the nucleus to pass through (Jamieson 2007; Nishio & Matsuda 2017). Descriptive work indicates that the relative and absolute lengths of the acrosome and the nucleus vary considerably in passerine birds (Jamieson 2007), although there have been no large-scale comparative studies to quantify this variation or investigate it from an evolutionary perspective.

The acrosome is of particular importance in this thesis. It constitutes the greater part of the sperm head (in most species – see chapter 2), and includes the helical membrane which to a large extent gives the sperm head its helical appearance. Additionally, the acrosome seems to be more susceptible to damage than the nucleus, as described in chapter 3 and 4. The long, helical avian acrosome is rather different to the human acrosome, which consists of a small cap covering the tip of the larger nucleus, and so the function of the avian acrosome is less studied and not very well understood. For example, is the helical membrane associated with sperm motion or with ovum penetration?

To improve our understanding of the evolution and function of the songbird sperm head, it is essential to move beyond simple measures of sperm length, and incorporate measurements of other aspects of sperm morphology, e.g. the width of the helical membrane and volume of the head components. Moreover, it is necessary to obtain accurate data on acrosome and nucleus lengths. For these purposes, light microscopy does not provide sufficient resolution, and therefore I have used scanning electron microscopy to obtain high-resolution images of songbird sperm.

Questions and predictions

The purpose of this thesis is to improve our understanding of sperm head evolution and function in songbirds. More specifically, I have attempted to answer a number of more concrete questions, which can be organised into four main themes. Most of these questions have been mentioned in the introduction above, but here I will summarise them. The first theme is relevant throughout the thesis, and is addressed through **all four chapters**. Both the second and third themes are dealt with in **chapters 2 and 3**, whereas the final theme is investigated in **chapter 4**.

First, how does sperm head morphology vary inter- and intraspecifically in songbirds? For example, do some species have larger acrosomes or more helical shapes than others? My prediction was that there was some variation among species, but less variation within species. This prediction was based on the patterns reported for total sperm length, but also on the early descriptive work which indicates at least some interspecific variation in head morphology. However, although several studies show intraspecific variation in head length (see above), reports of among-species variation are more scarce, such that a hypothesis of more intraspecific than interspecific variation (or simply limited variation overall) could also be reasonable.

Second, why do songbird sperm have such a distinctive helical shape? What is the function of this morphology? One possibility could be that the screw-like shape is useful for penetration of the ovum, another that it is related to sperm swimming mechanisms. Furthermore, if there is variation in this shape across species, why do some species (and taxa) have straight or round sperm instead of helical sperm? Evolutionary theory suggests that there should be some kind of cost involved, but it has been unknown what this cost might be.

Third, is the evolution of sperm head morphology shaped by sperm competition intensity? As total sperm length has been shown to be correlated with the level of sperm competition, there is reason to believe that post-copulatory selection pressure acts on sperm traits in general, but earlier work suggests this is not the case for head length. However, such selection pressure could still be acting on other head traits such as width and shape; additionally, the acrosome and the nucleus each play specific roles in the fertilisation process, and may be evolving independently from other sperm components.

Finally, how can the contemporary environment affect sperm quality and performance? Can diet influence sperm function in wild birds? Evidence from the poultry industry suggests that this could be the case. Due to the increasing popularity of bird feeding, the diet of urban birds has changed rather dramatically during the last few decades, and now tends to include a higher proportion of items rich in omega-6 such as sunflower seeds. Excessive omega-6 can increase oxidative stress, a particular concern for sperm cells due to the composition of the sperm membranes, which are rich in poly-unsaturated fatty acids. Therefore, I hypothesised that there could be an association between the feeding of sunflower seeds and the frequency of abnormal or damaged sperm heads.

Methods

Here, I will describe four of the methodological approaches that I feel require special attention – scanning electron microscopy, measurement of sperm head morphology, phylogenetic comparative analyses, and sperm abnormality scoring. Further details of the methods are described in detail in the chapters.

Scanning electron microscopy

For the purpose of quantifying the minute details of the sperm head, light microscopy does not provide sufficient resolution. Thus, it was necessary to use more powerful microscopy tools. Electron microscopes provide a considerable improvement in resolution compared to a standard light microscope, and have been used to observe particles on the scale of picometres (which is one thousandth of a nanometre), with magnifications up to 10 000 000x. The sperm head of a songbird is around 10 µm, and requires magnifications of around 8 000x, and so is well within the capabilities of a standard electron microscope. There are two types of electron microscopes; transmission electron microscopes (TEM) and scanning electron microscopes (SEM). TEM uses a thin slice of the sample to look at cross-sections of material, whereas SEM provides an external view of the sample surface. As my aim was to analyse the exterior structure and shape of the sperm head, SEM was most appropriate for my use.

SEM works by accelerating electrons in a beam from the top of the microscope down onto the sample. This beam moves across the sample until all areas have been traced by the beam; hence the name “scanning”. Some of the electrons in the beam are reflected off the sample surface as secondary electrons, which are picked up by receptors on the inside of the microscope (the electron beam also causes a number of other particles to be emitted from the sample surface, such as x-rays, but these were not relevant for this project). The receptors then interpret the composition of the secondary electrons, and translate this into an image on the computer screen. SEM provides a black-and-white image only, but it does give exceptional depth of focus, meaning that the entire cell can usually be kept in focus at the same time; which is a clear advantage for the helically shaped sperm cells with protruding membranes.

The SEM operates in a vacuum. Under such conditions, any water contained in the sample would instantly boil and evaporate. When water is suddenly removed from a cell, membranes tend to collapse, which damages the cell structure and changes the shape of the cell (for examples of collapsed helical membranes in songbird sperm, see Figure 3 in Schilthuizen et al. (2017)). This means that prior to the use of SEM, biological material must be completely dehydrated, and it is necessary to follow a careful drying procedure which retains the structural integrity of the cell. This process begins by substituting ethanol (which is miscible with liquid CO_2) for water by submerging the samples in solutions of gradually increased ethanol content. The samples are then put in a critical point dryer. This machine first exchanges liquid CO_2 for ethanol, before the pressure and temperature are both gradually increased until the sample reaches a state known as the “critical point”, where the CO_2 liquid can change to vapour without a change of density. Since there is no change of density during the transition phase, this can occur without causing structural damage to the cell. Finally, the dry sample is coated with a few nanometres of platinum, which enhances conductivity during SEM imaging and prevents the build-up of electrons in the sample (charging).

This complex process is time-consuming, but it results in remarkably detailed images from structurally intact sperm cells. For a comparison of a light microscope image (320x) and a SEM image (10 000x), see Fig. 3. It is clear that the SEM images provide considerably enhanced opportunities for precise measurement; for example, the acrosome/nucleus junction is not possible to distinguish in the light microscopy image, but is relatively easily identifiable in the SEM image (the white arrow in Fig. 3b). To my knowledge, the database of SEM images I have established is the first large collection of such SEM images from bird sperm cells, and thus provides a number of novel research opportunities. In this thesis, I have explored several of these opportunities, yet I believe there is considerable scope for taking further advantage of these images and the data obtained from them.

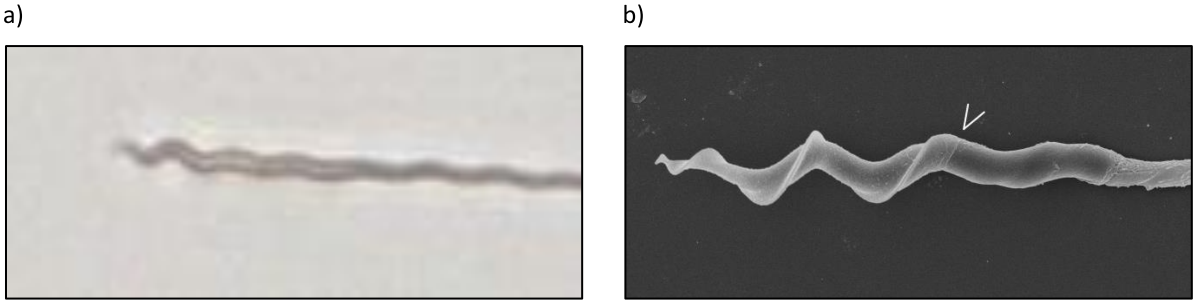

Figure 3. The sperm head of a willow warbler *Phylloscopus trochilus*, captured with a) a standard light microscope (320x) and b) a scanning electron microscope (10 000x)

Measurement of sperm head morphology

With the SEM images as the raw material, I used the software ImageJ (Schneider et al. 2012) to measure dimensions of the sperm head. At first, this only included the direct and centreline lengths of the acrosome and the nucleus (Fig. 4). However, after some preliminary analyses, it was clear that there was considerable morphological variation in the sperm heads that was not captured by such length measurements. Therefore, I measured all the images again to obtain three additional measurements; the width of the acrosome, the width of the nucleus, and the width of the helical membrane. In total, I then had seven measurements (Fig. 4), from which I could calculate a number of other traits including ratios and volumes. These measurements and calculations are described in detail in chapter 2. The ratio of the centreline head length to direct head length, termed “waveform”, is worth mentioning separately – a higher such ratio indicates a cell that has a more pronounced waveform along the core of the sperm cell, irrespective of the helical membrane. As a size-independent ratio, this can be considered to describe an aspect of cell “shape”.

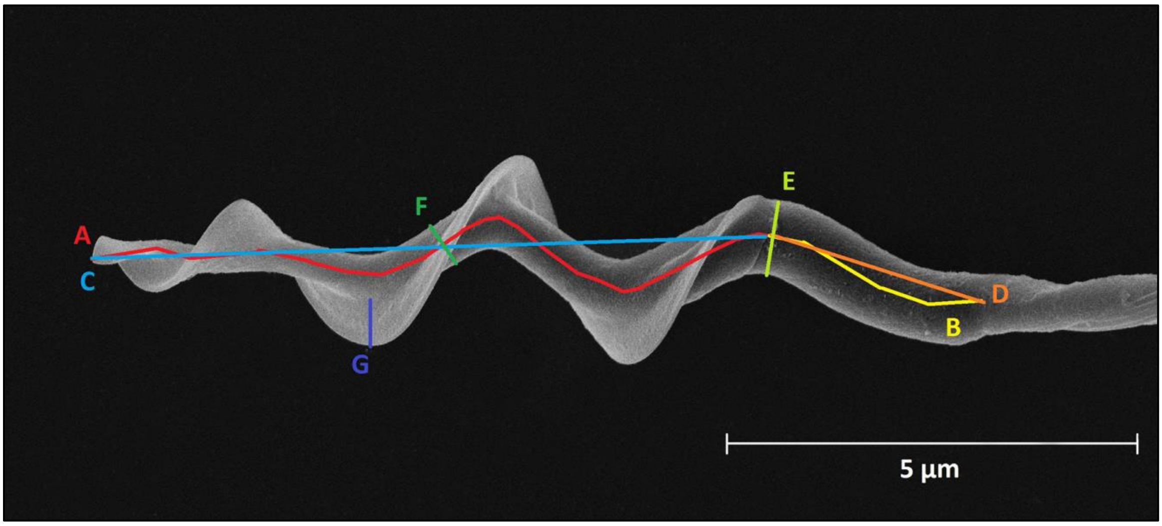

Figure 4. Sperm head morphology measurements (shown here on the sperm of the bluethroat *Luscinia svecica*). A) acrosome centreline, red line; B) nucleus centreline, yellow line; C) acrosome straight length, blue line; D) nucleus straight length, orange line; E) nucleus diameter, lime green line; F) acrosome diameter, dark green line; G) helical membrane width, purple line.

Phylogenetic comparative analyses

When analysing data from several related species, it is necessary to keep their phylogenetic relationships in mind. Due to their shared ancestry, closely related species will tend to be more similar in all heritable traits than species which have evolved independently for a long time. Thus, when considering correlative data, each species does not represent an independent data point – even if there was no causal relationship between the traits in question, there would likely be a correlation in the raw data, simply due to the shared evolutionary history of closely related species. Similarly, even if the traits in question are causally related, it is often not possible to know whether the trait has arisen independently in two closely related species or only once during their shared evolutionary history, and so the two species cannot be treated as two separate, independent data points. Therefore, considering phylogenetic effects is essential when doing comparative studies across species.

The most established method of taking phylogenetic relationships into account when analysing quantitative traits is using Phylogenetic Generalised Least Squares (PGLS) models (Pagel 1999; Freckleton et al. 2002). These models start with a phylogenetic tree (usually based on DNA sequence data), and use the distances among the species in the tree as an error term in the generalised model. Technically, PGLS models apply a maximum likelihood framework to calculate the amount of expected covariance between species based on their shared ancestry, and then apply this information as a correction to the observed variance of the traits in question. This means that in datasets with strong phylogenetic dependency, this correction is important and is applied strongly, whereas in datasets where phylogenetic correction is not strictly necessary (i.e. where the trait varies independently of the phylogeny) the correction is not so influential. Previously, there was some debate of whether PGLS models over-corrected for shared ancestry in some cases (e.g. Björklund 1997); however, due to this flexible approach where the amount of correction is based on the data in hand, this is in reality not a major concern (Freckleton et al. 2002; Blomberg et al. 2003).

Sperm abnormality scoring

In chapter 3 and 4, the primary goal was to assess sperm quality, and in particular the functional integrity of the sperm head. The standard sperm quality assessment for human sperm is based on computer-assisted sperm analysis (CASA) software, which automatically calculates metrics such as sperm swimming speed and the percent of motile sperm based on recorded video files, and these metrics are also widely used for avian sperm. However, data from CASA give little information on the cause of sperm dysfunction, for example whether the sperm is dysfunctional due to a missing tail or to head abnormalities. Since my work focuses on the morphology of the sperm head, it was necessary to supplement CASA-based data with direct assessments of sperm head abnormalities. As abnormal sperm can be visually identified at relatively low resolution, SEM images were not necessary, and thus standard light microscopy was used for this purpose. Admittedly, sperm that are only slightly damaged (for example, where the helical membrane is slightly damaged) may not be identified without using SEM, but cases where the sperm head is substantially deformed or where the acrosome is missing are relatively straightforward to determine in the light microscope.

The protocol consisted of assessing 100 sperm cells per individual, and visually determining whether or not the sperm cell was “normal” (Fig. 5a) or “abnormal” (fig. 5b, 5c). I strictly assessed the first 100 cells found in the microscope view, and if further cells were in view when reaching 100, all the remaining cells were also assessed to avoid biased selection of cells. I also used several classifications within the “abnormal” category, with abnormal heads being scored as either

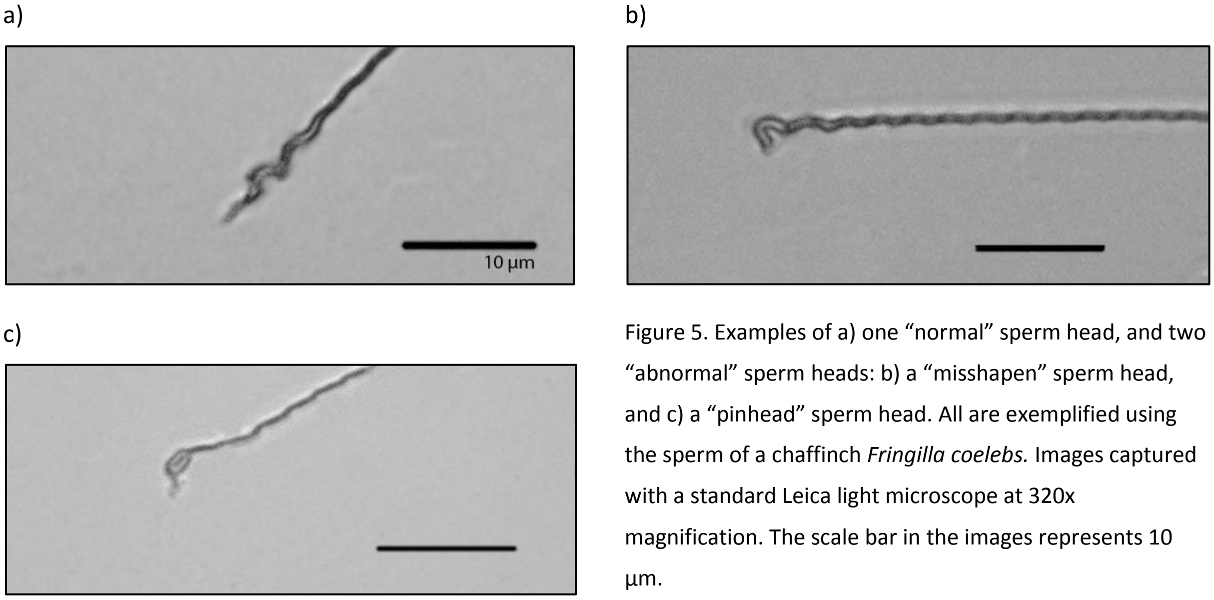

Figure 5. Examples of a) one “normal” sperm head, and two “abnormal” sperm heads: b) a “misshapen” sperm head, and c) a “pinhead” sperm head. All are exemplified using the sperm of a chaffinch *Fringilla coelebs.* Images captured with a standard Leica light microscope at 320x magnification. The scale bar in the images represents 10 µm.

“misshapen” (Fig. 5b) or “pinhead” (Fig. 5c). The latter category was colloquially named “pinhead” due to the visual appearance of these cells; they appear to have lost their acrosome entirely and therefore the nucleus is the only part left of the sperm head. It is likely that these cells have undergone a premature acrosome reaction in which the acrosome disintegrates – however, I could not prove whether this was the case; there might for example have been an error in spermatogenesis so that the acrosomes were never correctly developed in the first place. In both relevant chapters, the two groups of damaged sperm were analysed together, for two main reasons: 1) separate analyses did not provide any additional biological information to the combined analyses, and it was desirable to avoid excessive numbers of models for statistical reasons as well as for enhanced clarity; 2) it is not unlikely that the misshapen cells are in an early stage of a premature acrosome reaction, so that the two categories in effect describe the same physiological process. In any case, both types of abnormal cells are likely affected by poor membrane integrity.

The main challenge with this protocol is that it is to a certain extent subjective, and is based on assessments made by a potentially informed observer. In chapter 4, I was not aware of the fatty acid status of individuals when performing the abnormality scoring, and so the assessment can be considered to be blinded. However, for chapter 3, it was impossible to assess abnormal cells without observing the morphology of the normal cells, and it was relatively obvious which samples exhibited strongly helical sperm cells, and which had straighter cells. As the hypothesis of the study involved a correlation between sperm morphology and sperm abnormality, this was a challenge, and one that would be difficult to avoid. Importantly, however, in almost all cases it is straightforward to identify abnormal sperm heads, as the helical shape of normal sperm cells is distinctive (even the cells with straighter morphology do have a characteristic shape), and thus deviations from the normal shape are easily identifiable, minimising the opportunity for biased decisions. In a small number of cases where cell status was particularly difficult to decide, I would disregard that particular cell rather than risk making a biased decision. In sum, I am confident that the data represent real, biological patterns.

Main findings and discussion

Quantifying variation in sperm head traits

One of the original tasks of my thesis was to quantify the variation in sperm head traits. Although the thesis now encompasses several further aspects, this core challenge remained the foundation for the entire work. Firstly, this task includes obtaining and presenting novel data on sperm head traits to the scientific community, which can be used for further research. Secondly, this variation describes biological patterns which are interesting in their own right.

In **chapter 1**, I focus on the most numerous bird species in Norway, the willow warbler, and quantify sperm traits across the range of two subspecies with a hybrid zone. The findings demonstrate that sperm head length can vary geographically within a species, even when other sperm traits are homogenous. Although the absolute differences in head length were not very large, the results support the hypothesis that sperm head morphology can evolve independently of other sperm components.

In **chapter 2**, I present the data obtained from the SEM images. This includes information on traits that have not been published previously, e.g. the width of the helical membrane, and as such these data represent an important contribution to the scientific literature. I find considerable variation in sperm head traits, primarily among species, but also to a smaller extent among and within males. I show that nearly all sperm head traits are strongly correlated with one another, as well as with total sperm length, so that a long sperm cell tends to also have a long and wide acrosome, a short and wide nucleus, a wide helical membrane and a pronounced waveform. Considering that head length and total sperm length have not shown the same responses to sperm competition in comparative studies (Lüpold et al. 2009; Immler et al. 2012; Rowe et al. 2015), it is interesting that I found such strong correlations among head traits and total sperm length. However, these correlations do not explain 100% of the variation (in most cases around 80%), and the residual variation might explain the discrepancies between the trait responses in the comparative analyses.

The mean acrosome:nucleus ratio across all species was 2.14, so that the acrosome was twice as long as the nucleus. For two species (the reed bunting *Emberiza schoeniclus* and the bluethroat *Luscinia svecica*) the A:N ratio exceeded 5, whereas for eight species it was below 1 so that the nucleus was longer than the acrosome. Lüpold et al. (2009) reported that head length varied less among species than did total sperm length, but in their study head length still ranged from 10.9 to 20.6 µm, so that the longest sperm head was nearly twice as long as the shortest. My work suggests that such variation in head length is mostly due to variation in acrosome length, as the acrosome comprises most of the sperm head in the long-headed species. Interestingly, a strong negative correlation between nucleus length and width ($r = 0.95$) meant that nucleus volume varied little across species, and was generally conserved at about 2 μm^3. As genome size varies little in birds (Tiersch & Wachtel 1991; Gregory 2018), this result suggests that chromatin compaction is relatively conserved in songbirds.

Due to the correlation between head traits, and to reduce the number of measurement variables, I used six of the head traits to construct a single indicator of sperm head morphology (using the first component of a principal component analysis, see methods in chapter 2). I found that this indicator (PC1) quite well described the variation in overall shape that could be observed across all 36 species: from the short, narrow, straight cells, to the long, wide cells with a distinct helical membrane and a pronounced corkscrew-shaped appearance (Fig. 6). By quantifying the general variation in shape in this manner, I facilitated investigation of sperm head shape (see below) and simplified the interpretation of the results from the comparative analyses.

In **chapter 3 and 4**, I show that the ability of males to produce and/or maintain morphologically normal sperm cells can vary both among species and within a species, and that this depends both on sperm head characteristics (**chapter 3**) and on physiological status (**chapter 4**). This variation is further described in the sections below.

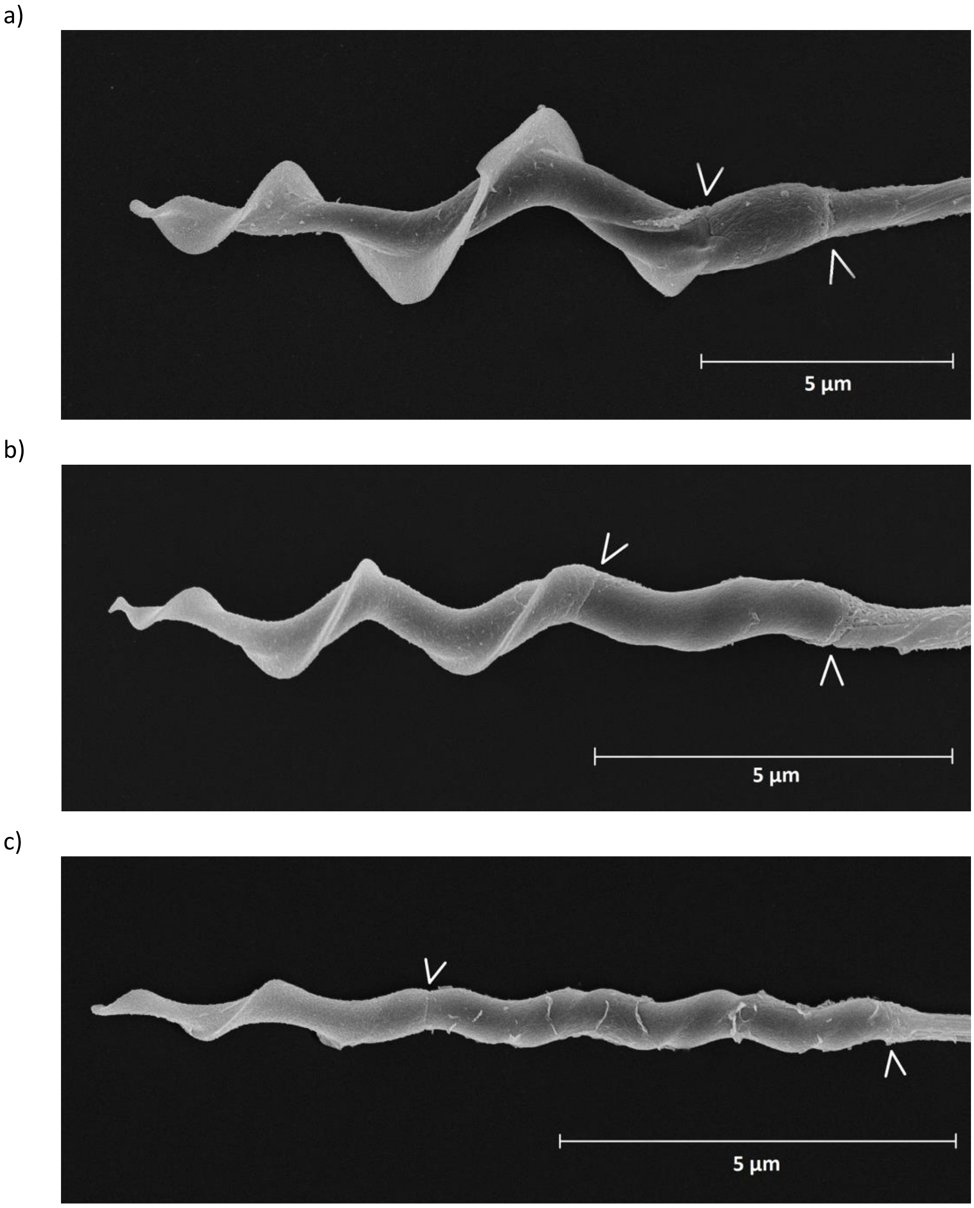

Figure 6. Examples of sperm head cells from songbird species with a) the maximum value for PC1 (reed bunting *Emberiza schoeniclus*, PC1 = 1.921), b) the closest to the median value for PC1 (willow warbler *Phylloscopus trochilus*, PC1 = 0.138), c) the minimum value for PC1 (nuthatch *Sitta europaea*, PC1 = -0.863). The top (left) white arrowhead indicates the acrosome/nucleus junction, whereas the lower (right) white arrowhead indicates the nucleus/midpiece junction.

Understanding the helical shape of passerine sperm

By using both the indicator of sperm morphology from chapter 2 (PC1) and the compound trait of “waveform” of the sperm cell, I have been able to approach the question of why songbird sperm cells have such a characteristic helical shape. PC1 to a large extent reflects the width of the helical membrane, whereas waveform reflects the amplitude of the helical “wave” of the core of the sperm cell itself (excluding the membrane), and so the two variables represent different but related (and statistically correlated) aspects of the helical, corkscrew-shaped morphology.

In **chapter 2**, I show that both PC1 and waveform are positively correlated with sperm swimming speed. Thus, the helical shape appears to provide a (presumed) fitness advantage in terms of increased swimming speed. This is an interesting finding, and for me, it was a rather unexpected finding. Larger sperm heads are predicted to increase drag due to their large surface area (Humphries et al. 2008). Thus, sperm with a large head with a wide helical membrane should experience more drag than a slimmer, straighter cell, and thus be slower. However, the biomechanics and physics of sperm motion are rather more complicated. This is described in the discussion of chapter 2, but I will attempt to use this space to explain the physical concepts in a more colloquial fashion, as they are likely to be unfamiliar to most readers.

Since sperm are so small and swim at a relatively low speed, they operate in a low Reynold’s number environment, which in simplified terms means that sperm have to struggle very hard to move forward in the much heavier material around them. (For a good in-depth description of this phenomenon and the ones described below, see Purcell (1977).) A sperm cell that swims in water would experience forces that are similar to how we can imagine swimming in thick syrup; which is exacerbated by the fluid in the female reproductive tract already being relatively viscous. In more formal terms, movement at low Reynold’s number is dominated by viscous forces, and inertia is almost irrelevant. The lack of inertial forces means that if a sperm ceases its propulsive movement, it will immediately stop (as compared to, for example, a cruise ship, which will keep moving forwards for a long distance after turning the engine off). This also means that for a sperm (and also, interestingly, for bacteria), it is inefficient to move by flapping a tail up and down – the sperm would simply move forward on the “up” stroke and backwards again on the “down” stroke (the “scallop theorem”, Purcell 1977). Thus, it needs a pattern of motion that breaks the symmetry of the movement. Avian sperm solve this problem by beating their tails in a circular fashion, twisting around their own axis (Humphreys 1972), almost like drilling into the surrounding material. In viscous or low Reynolds environments, helical shapes will always move forwards when rotated (and vice versa – which means that if you drop a helical shape such as a spring into a bucket of syrup, it will rotate whilst sinking, whereas when dropped in water, it will sink straight down). Thus, helical shapes contribute to forward movement in low Reynold’s number environments. Considering this, it should, in fact, not be surprising at all that the more strongly helical songbird sperm swim faster than the straighter ones, as the wide helical membrane and pronounced waveform will contribute to propulsion of the sperm through increased rotation. My data suggest that this effect outweighs the drag of a larger surface area. Interestingly, however, there appears to be a decline in swimming speed for the very largest sperm cells, suggesting that they might be approaching a tipping point where drag from the surface area becomes too influential.

Based on these arguments, the fitness benefit of the characteristic helical shapes seems obvious: corkscrew-shaped sperm cells swim faster. This does not exclude the possibility that such a shape is also useful for penetration of the egg; however, the acrosomal enzymes contained within the acrosome must be released early in the fertilisation process for digestion of the inner perivitelline layer (Jamieson 2007; Nishio & Matsuda 2017), presumably causing the acrosome to lose its helical shape, which supports the idea that increased swimming speed is the primary advantage of the helical morphology. In an environment of sperm competition, swimming speed is important, and has been shown to be related to fertilisation success in a range of taxa including birds (Birkhead et al. 1999; Denk et al. 2005; Pizzari & Parker 2009). Therefore, there should be strong selection for helical sperm cells in such conditions. Indeed, the question that then arises is: Why are not all sperm helically shaped? Evolutionary theory suggests that there should be a fitness cost associated with a seemingly beneficial trait that is not ubiquitous. Are such helical sperm more costly to produce, or is there another cost?

In **chapter 3**, I address this question. I find that there is a positive correlation between the indicator of sperm head morphology (PC1) and the frequency of abnormal sperm heads, as well as between sperm waveform and sperm abnormalities. Thus, the strongly helical sperm appear to be more susceptible to damage; or possibly susceptible to malformation during spermatogenesis, as I could not verify whether the abnormal cells were incorrectly developed or damaged at a later stage. A wide, thin helical membrane intuitively looks more fragile than a solid rounded structure, and could be more prone to breaking when subjected to mechanical stress; or it could have more surface area that reactive oxygen species can react with. However, it is also possible that the complex helical shape is more susceptible to developmental errors during spermatogenesis. Regardless, my data demonstrates that there is indeed a fitness cost associated with the characteristic helical shape, and suggests an evolutionary trade-off between a fast, fragile, helical sperm, and a slower, but more robust, straighter sperm.

Sperm head morphology and sperm competition intensity

Although I have found evidence of an evolutionary trade-off between sperm shapes, this does not explain why some species have evolved helical sperm and others have evolved straighter sperm. A logical answer to this question could be that males experiencing intense sperm competition are obliged to opt for a “high risk/high gain” strategy, whereas males in is less competitive conditions are able to choose a “safe option” to ensure successful fertilisation. However, my data provide little support for this hypothesis.

First, in **chapter 1**, I found that sperm head length diverged between two subspecies of the willow warbler, but I found no difference between the subspecies in the coefficient of variation of sperm traits (CVam, also known as CVbm[1]), which was the estimate of sperm competition intensity in this system. Thus, there was no indication that the observed divergence in head length was influenced by differences in post-copulatory sexual selection pressure. In **chapter 2** and **chapter 3**, I assessed the relationship between a number of sperm head traits and several indicators of sperm competition intensity, but found little support for the hypothesis that sperm competition intensity is driving interspecific variation in sperm head morphology. I did, however, find a correlation between the amount of intraspecific variation in sperm head traits and the frequency of extra-pair young, indicating that males experiencing high levels of sperm competition are selected to produce more homogenous sperm (presumably, to produce sperm that are closer to the “optimal” phenotype). This indicates that sperm competition does indeed influence sperm head morphology, but that the “optimal” sperm head differs among species.

Species-specific factors that could influence the fitness of a particular sperm shape include the viscosity of the female fluid, the architecture of the female reproductive tract, and the characteristics of the egg. There has been little research on these traits, and so it is unknown whether any of them vary sufficiently to drive the observed variation in sperm traits. Additionally, it is not clear which factors could be driving any such variation in female traits, especially if sperm competition intensity is ruled out. This is a topic that certainly warrants further research.

Importantly, the fact that I did not observe a statistically significant association between sperm head traits and my indicators of sperm competition does not necessarily mean that no such biological association exists. Sperm competition indicators are just that – indicators – and are prone to missing out on substantial amounts of biologically relevant information. For example, the rate of extra-pair young can vary considerably among years, as has been shown by my research group’s long-term studies of bluethroats in Heimdalen, Norway (Johnsen & Lifjeld 2003). Similarly, the data on extra-pair young and testes mass are not necessarily from the same population or location as the sperm data, further adding to the potential noise. Indeed, when observing the graph and trend line for the correlation between sperm head morphology (PC1) and the frequency of extra-pair young (Fig. 7), there is in fact a noticeable positive trend, but there is also considerable amounts of noise, with large residual values (note that phylogenetic correction only had a very minor influence on effect sizes in this analysis). Moreover, total sperm length has previously been shown to be correlated with

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[1] The term CVbm was previously used for “coefficient of variation between males”, but it was subsequently realised that the correct term is “among males” as there are more than two males being compared. CVam and CVbm thus represent the same variable.

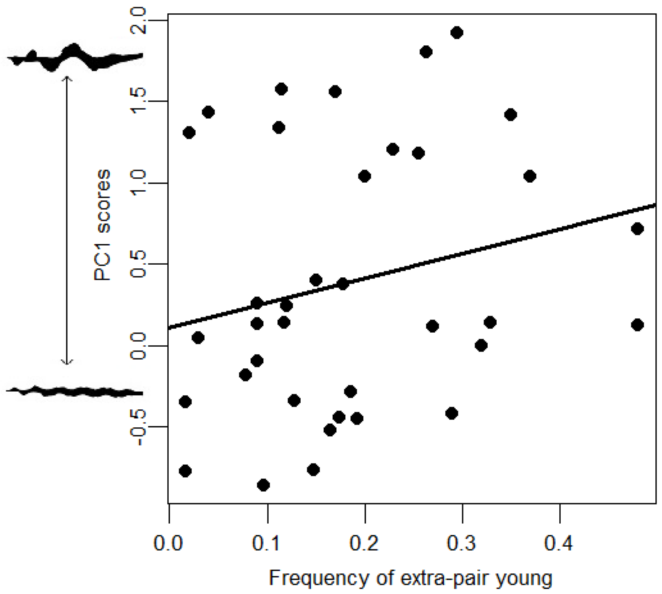

Figure 7. The relationship between the frequency of extra-pair young (proportion of young in the nest) and an indicator of sperm head morphology (PC1). The trend line is a simple linear model.

sperm competition (Briskie et al. 1997; Kleven et al. 2009), whereas I found no such correlation in my dataset. Therefore, whilst my data do not support the hypothesis that variation in sperm competition intensity drives interspecific variation in sperm head traits, there is little basis for firmly rejecting the existence of such a pattern.

The influence of environmental factors on sperm performance

Songbird males may inherit beneficial (or costly) sperm morphology traits from their parents, but they can also influence their own sperm performance within their lifetimes, by ensuring that their sperm are in good physiological condition. In **chapter 4**, I assess how individual sperm quality can be affected by the bird's contemporary environment, using two common finch species, the greenfinch and the hawfinch. Sperm are characterised by a high proportion of poly-unsaturated fatty acids (PUFAs), which the bird generally acquires through the diet. A diet deficient in the necessary PUFAs is likely to negatively affect production of normal sperm cells (Surai et al. 2000). However, dietary omega-6 PUFAs (Cherian 2007) can also be metabolised into reactive oxygen species (ROS), which readily react with the PUFAs in the sperm membranes, potentially causing oxidative damage to the sperm. Thus, the nutritional composition of the diet may influence sperm performance, which is widely recognised in the poultry industry (Cerolini et al. 2003). Despite this, many food types that are fed by households to wild birds are particularly high in omega-6, e.g. sunflower seeds, which largely consist of the omega-6 PUFA linoleic acid (18:2n-6).

My data showed a positive correlation between the proportion of linoleic acid in the blood and the frequency of abnormal sperm heads, as well as between the omega-6/omega-3 ratio and sperm abnormalities. These results support the hypothesis that excessive omega-6 PUFAs could lead to oxidative damage of sperm cells, and that omega-6 rich diets could be detrimental to males' reproductive ability; although I found little effect of fatty acid composition on sperm swimming speed. However, this study is purely correlative, and so it is necessary to be cautious when interpreting the data. I view the results as a preliminary indication that there may be an association between excessive feeding of sunflower seeds and the sperm quality of wild birds, and that this warrants further research, preferably with an experimental approach.

The feeding of omega-6-rich food items to wild birds has become a popular pastime of many households. Supplementary feeding may enhance individual survival (Robb et al. 2008; Fuller et al. 2012), but a recent report also finds that certain provided foods are linked to reduced health (Plummer et al. 2018). Although my results should be interpreted with some caution due to their correlative nature, it is still worth considering whether feeding of sunflower seeds is in fact detrimental to the birds' reproductive health. I do not think that it is necessary to stop providing food to wild birds altogether, or even avoid sunflower seeds altogether, but I do suggest that it could be advantageous to consider whether the food provided is of acceptable nutritional quality. For example, households could provide a single food type at a time rather than giving the birds a choice, and include food types that are not high in omega-6 (e.g. peanuts or coconut fat).

Conclusions and future directions

My work shows that there has been much unexplored variation in sperm head traits of songbirds. These traits vary substantially among species as well as among males within a species, and, in particular, acrosome morphology exhibits considerable interspecific variation in both size and shape.

I show that the characteristic helical sperm head of passerine birds is likely to have evolved as an adaptation for increased sperm swimming speed, but that this fitness benefit comes as a trade-off with structural integrity of the sperm cell (or, possibly, the ability of males to produce morphologically normal sperm). This insight is based on general physical and physiological principles, and as such should also be relevant to other taxa. My work demonstrates that it is essential to include multiple aspects of morphology, not only length measurements, when studying the evolution and function of sperm morphology. Additionally, my research highlights that there is currently a lack of knowledge of the mechanisms of sperm motion in birds, e.g. how the head, midpiece and flagellum interact to provide forward movement, and how this movement can be modelled mathematically. Addressing these questions would be interesting for both biologists and physicists/mathematicians, and would likely require high-resolution video imaging.

I find only limited support for the hypothesis that sperm head traits have evolved in response to sperm competition. Although there appears to be stabilising selection acting on sperm head traits, as shown by low intraspecific variation in species with high levels of sperm competition, the interspecific variation in sperm traits appears to be, at least to some extent, driven by other factors than sperm competition intensity. As such, future research should endeavour to explore potential factors that may influence sperm head evolution, with particular emphasis on the female environment. One promising approach is to investigate whether there is a relationship between the viscosity of female fluid and sperm head shape, although it may be challenging to quantify the viscosity of such small volumes of fluid. There is also considerable scope for genetic approaches, for example investigating whether there are particular genes coding for the helical shape, as well as clarifying the physiological role of the passerine acrosome.

Finally, I find indications that the frequency of sperm head abnormalities can be affected by the composition of the individual birds' diet. Thus, I demonstrate that optimal sperm performance is dependent on both inherited morphological traits as well as the contemporary environment of the bird. Based on these results, I encourage further research on the impact of supplementary feeding on the reproductive health of wild birds. Experimental studies to confirm a causal effect of diet on sperm abnormalities would be particularly useful, as well as investigating the physiological mechanisms involved, and assessing to what extent such sperm abnormalities are likely to affect the individual males' fitness in practice.

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