Master's Thesis

The relative roles of natural and sexual selection in the evolution of tardigrade gamete morphology

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Spermatazoa are the most diverse cell type in the animal kingdom. In many animal taxa, sperm variation across and within species has been shown to be associated with the degree of postcopulatory sexual selection and fertilization conditions. For example, promiscuous species have reduced intraspecific sperm morphometric variation and internal fertilization is linked with more complex sperm designs. Tardigrades are micrometazoans best known for their cryptobiotic ability (i.e. a reversible dormant state when environmental conditions are unfavourable). Moreover, they have incredible yet unexplored variation in reproductive biology and gametes. I quantified the variation in tardigrade primary reproductive traits (e.g. relative gonad size, sperm and egg morphology) at intra- and inter-specific levels. I also investigated the relative roles of the evolutionary processes of natural (egg shell complexity) and sexual (presence of the spermatheca) selection on tardigrade sperm design. My comparative approach used data from 12 *Macrobiotus* species (order: Parachela). I found a large difference in the effect size of the relative midpiece length in the species with and without the spermatheca, indicating that the prolonged storage requires more energy that is generated in the midpiece. This corroborates similar findings across other animal taxa. The categorization of the egg type based on the morphological patterns did not seem to be associated with differences in the sperm head size; further investigation into the question of how the male and female gametes are associated in their morphology is currently hindered by a lack of data on the mode of fertilization and mating behavior in Tardigrada.

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Siittiösolut ovat eläinkunnassa esiintyvistä solutyypeistä monimuotoisin ryhmä. Siittiösoluissa esiintyvän muuntelun on useissa eläintaksoneissa havaittu olevan yhteydessä parittelun jälkeisen seksuaalivalinnan tasoon sekä hedelmöittymisolosuhteisiin. Esimerkiksi, seka-avioisilla lajeilla lajien sisäinen vaihtelu siittiöiden rakenteessa on vähäisempää, kun taas sisäinen hedelmöitys on yhteydessä siittiöiden monimutkaisempaan rakenteeseen. Karhukaiset ovat selkärangattomien eläinten ryhmä, joka on parhaiten tunnettu joidenkin lajien kyvystä vaipua ympäristöolosuhteiden muuttuessa epäsuotuisiksi kryptobioosiin, jopa vuosia kestävään horrostilaan. Karhukaisten lisääntymisbiologia ja sukusolut ovat varsin vähän tutkittuja aihepiirejä, mutta niissä tiedetään esiintyvän valtavasti muuntelua. Tutkielmassani vertailin kahdentoista Macrobiotus-suvun karhukaislajin ensisijaisten lisääntymisominaisuuksien, kuten sukurauhasen suhteellisen koon, sekä siittiöiden ja munien rakenteen, muuntelua lajien sisällä sekä lajien välillä. Lisäksi selvitin luonnonvalinnan ja seksuaalivalinnan suhteellisia vaikutuksia siittiöiden rakenteeseen käyttäen mittareina munien kuorten monimutkaisuutta (luonnonvalinta) sekä siittiösäiliön esiintyvyyttä (seksuaalivalinta). Siittiöiden keskiosan suurempi koko oli yhteydessä siittiösäiliön esiintyvyyteen, mikä viittaa pitkäaikaisen sperman säilömisen vaativan enemmän siittiöiden keskiosien tuottamaa energiaa. Muista eläintaksoneista on tehty samankaltaisia löydöksiä. Munien rakenteellisen vaihtelun ja siittiön kärkiosan koon välillä ei vaikuttanut olevan yhteyttä. Jatkotutkimuksia koiraiden ja naaraiden

sukusoluissa esiintyvän rakenteellisen muuntelun yhteydestä rajoittaa tällä hetkellä karhukaisten lisääntymismuotoihin ja parittelukäyttäytymiseen liittyvän aineiston vähäisyys.

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TERMS AND ABBREVIATIONS

TERMS

Acrosome	An organelle covering the head or protruding from the nucleus of animal sperm cells and containing enzymes that digest the egg cell coating, thus permitting the sperm to enter the egg
Anhydrobiosis	A form of cryptobiosis that allows organisms to withstand prolonged desiccation by slowing their level of metabolism to barely measurable levels
Chorion	The outer membrane or shell of the eggs of insects and other invertebrates
Parthenogenesis	A form of asexual reproduction in which an embryo can develop without the fertilization of the egg by a sperm cell
Spermatheca	A small sac or cavity in female or hermaphroditic invertebrates used to store sperm for fertilizing eggs
Sperm midpiece	The component of the spermatazoon that contains mitochondria and provides ATP for the cell's motility.
Sperm nucleus	The component of the spermatozoon that contains the genetic material in the form of densely coiled chromatin fibers

ABBREVIATIONS

HIM	Helium ion microscopy
SEM	Scanning electron microscopy

1 INTRODUCTION

Research on gamete variation within and across species as well as on intra-individual gametic variation is an important prerequisite for understanding evolutionary relationships in biology. Isogamy occurs when all gametes are morphologically the same and it is generally accepted as the ancestral state (Charlesworth 1978, Bulmer and Parker 2002a, Lehtonen et al. 2016). We do not yet fully understand how gametic dimorphism has evolved (Maynard 1982) and a lot of research in this area has relied on mathematical modelling of gamete competition and evolutionary pressures for sperm development (Parker et al. 1972, Bulmer and Parker 2002b). The importance of the female gamete – the egg cell or the ovum - in the development of a zygote might seem to be self-evident due to its relatively large size and small number produced per ovulation. However, the exact roles and relative contribution of the egg cell and spermatozoon as well as their interaction have been the focus of reproductive biology over the past few decades, especially spurred on by the increase in technological advances that allow for such methods as molecular techniques and comparative analysis (Harvey and Pagel 1991, Freckleton et al. 2002a, Primig 2012). These studies have made an immense impact on our understanding of the male gamete biology in general and its interaction with the egg cell. Research on sperm started to take off in a very productive manner in the 1960's and a large number of sperm types and species have been described since then (Jamieson 1987a, 1987b, 1991a, 1999a, 1999b, 2000a, 2000b, 2005, 2007, Jamieson et al. 1995, 1999, Rouse 2006, Scheltinga and Jamieson 2003a, 2003b, 2006). The effects of reproductive biology and ecology on spermatozoa and their association with gametic variation have been studied in a large number of organisms of different taxonomic groups with each group and individuals within them possessing their own morphological and biochemical traits (Franzén 1970, 1977a, 1977b, Jamieson 1987a, Rouse and Jamieson 1987) and, according to Alberti (1990),

spermatozoa are subject to diverse modifications that result in extremely high species-specific variation. Sperm are the most diverse cell type known and this explains why, according to Jamieson (1987b), it is possible to figure out the exact placement of an organism within the taxonomic tree based on the examination of a single spermatozoon. Figure 1 shows some examples of this great diversity (Birkhead et al. 2008).

Making sense of sperm diversification in the evolutionary context involves studying such complementary approaches as intra-male variation, inter-male variation, inter-populational variation, interspecific variation and egg-sperm interaction. As mentioned above, attempts have been made (as well as great progress) to answer these questions in many taxa (Prakash et al. 2014, Dallai et al. 2016) but until now there has been a lack of research in tardigrades.

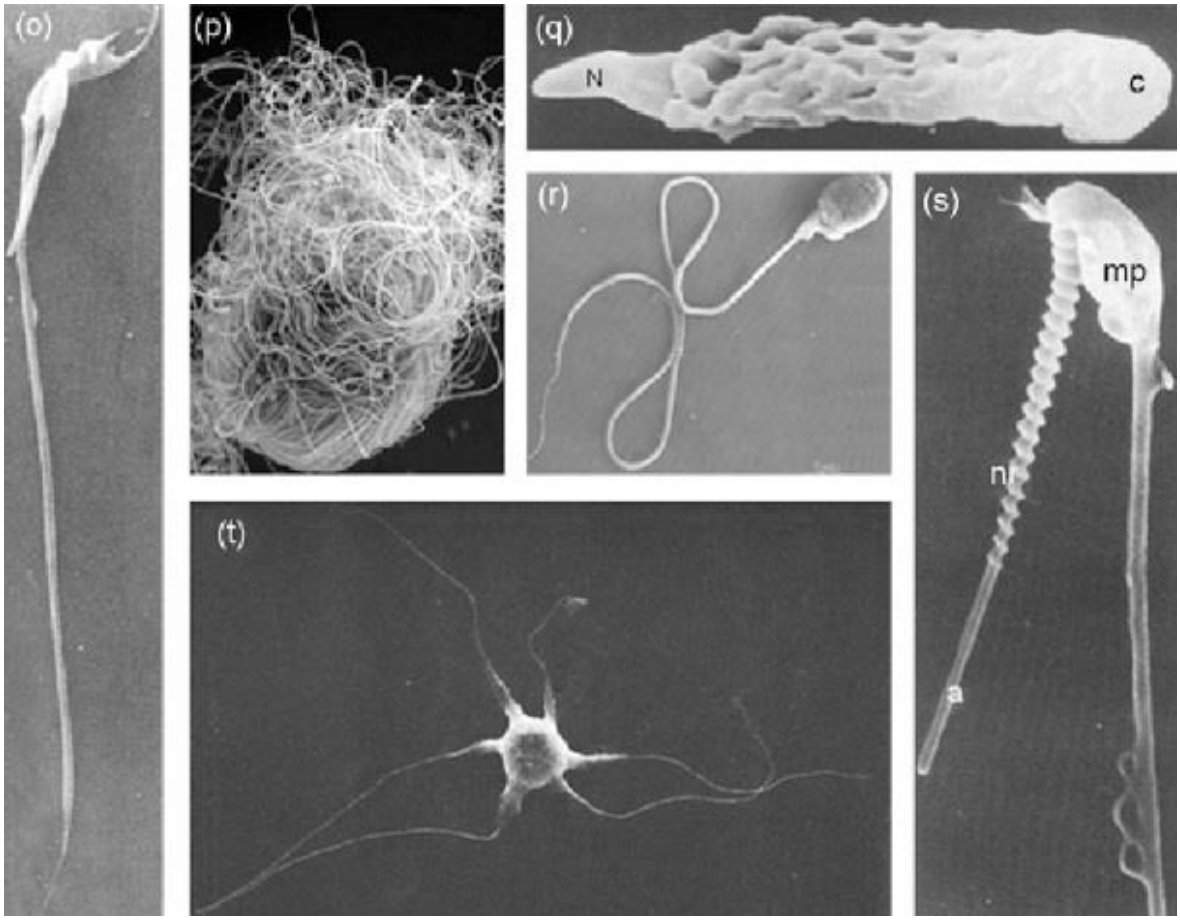

Figure 1. Spermatazoa SEMs (scanning electron microscope images) (Pitnick et al. 2009). Heterotardigrade and eutardigrade spermatozoa are shown in (o)

Pseudechiniscus facettalis (Tardigrada) (Rebecchi et al. 2000) and (s) *Macrobiouts joanne* (Tardigrada) (Rebecchi et al. 2000). Note the spiral shape of the nucleus characteristic for *Macrobiotus* sperm. (p) *Drosophila bifurca* (Bjorn and Pitnick 2006). (q) *Heligmosomoides polygyrus* (Nematoda) (Justine and Jamieson 2000). (r) *Hexagrammos agrammus* (Chordata: Osteichthyes) (Hara and Okiyama 1998). (t) *Procambrus sp.* (Crustacea: Decapoda) (Anderson and Personne 1975). Abbreviations: a, acrosome; C, anterior cap; mp, midpiece; N, posterior cap; nr, nuclear region.

1.1 Study organism

Tardigrada is a phylum of charismatic microscopic animals that have recently come to the spotlight in the media and popular science due to their peculiar appearance and interesting cryptobiotic abilities. These animals have a great diversity of sperm designs that also substantially differ from other phyla but there has been a clear lack of comprehensive studies in this regard. Despite the fact that tardigrades have been generally studied for over two centuries and over a thousand species have been described (Degma et al. 2019), rigorous scientific research and formal studies focusing on these tiny animals started only some decades ago, which has resulted in very limited information on their mating behaviour and reproductive sexual traits and strategies (Bingemer et al. 2016a, Sugiura et al. 2019).

This work tested the relative roles of natural and sexual selection in the diversity of reproductive traits in Macrobiotidae eutardigrades.

1.2 Short overview of the general and reproductive biology of Tardigrada

The first systematic description of a tardigrade emerged in the beginning of the 19th century, which was conducted by the German anatomist Carl August Sigismund Schultze. Tardigrades are more commonly known outside of the scientific community by their colloquial name “water bears”. This name was given to them when their discoverer was reminded of bears while watching their peculiar behaviour and gait on a microscope slide. Figure 2 shows a scanning electron

micrograph (later in the text referred to as SEM) image of a *Paramacrobiotus* sp. tardigrade (Bertolani et al. 2014a).

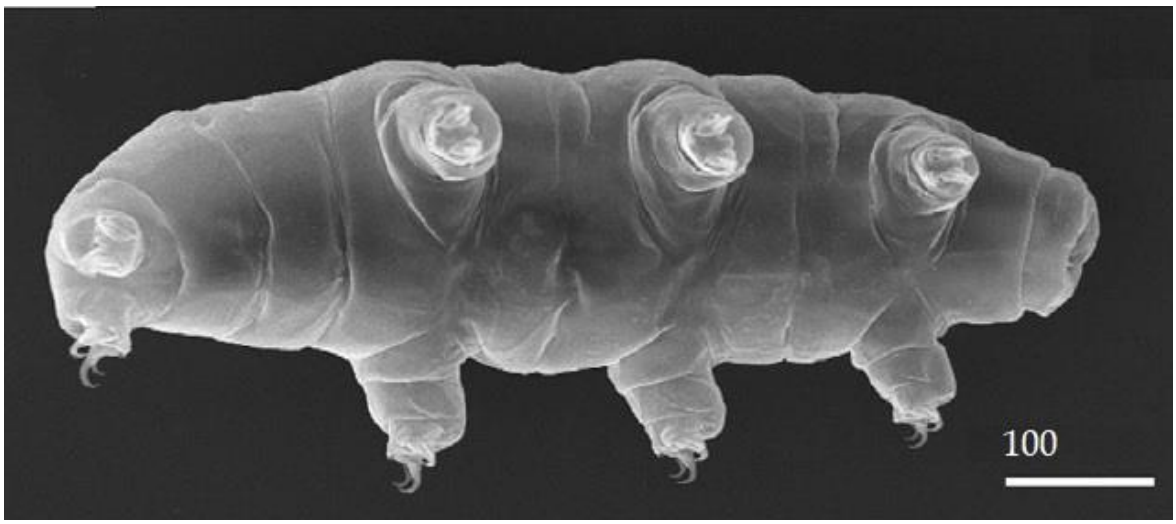

Figure 2. SEM image of a *Paramacrobiotus* sp. tardigrade (Bertolani et al. 2014a). The scale bar is in μm.

The tardigrade-related research throughout the 20th century turned out to be fruitful and these microscopic benthic invertebrates, also known as meiofauna, were established as a separate phylum. Generally, their size is less than a millimetre and they have a broad diet with many species feeding on different plant material such as algae and mosses, whereas others consume bacteria and detritus, microscopic animals such as nematodes, rotifers as well as other tardigrades (Nelson et al. 2015). The animals have been shown to be ubiquitous in their biogeography and they inhabit various types of sediments, the water film in soil and plants, moss, lichen, leaf litter etc. This broad range of ecological and biogeographic factors is predicted to be associated with primary sexual trait diversity. Tardigrades' taxonomic classification within the phylum has been undergoing large changes (Ramazzotti and Maucci 1982, Bertolani et al. 2011, Marley et al. 2011, Bertolani et al. 2014b,), especially recently, with the advances in molecular methods that have become an additional standard in taxonomic and phylogenetic descriptions of organisms. All the organisms used for this work now belong to the superfamily Macrobiotoidea,

which has been recently reduced to this status by Morek at al. (2020) from its former position of an order. Two well-established classes have been described with their own distinct morphological features and this taxonomic configuration has been stable for a while. Tardigrades' closest relatives are Athropoda and Onychophora but the exact phylogenetic position of Tradigrada relative to these two groups is still unresolved (Kinchin 1994, Nichols et al. 2006, Ramazzotti and Maucci 1983).

Eutardigrades are currently used much more extensively as model organisms for research than heterotardigrades and this work will also focus on the diverse primary sexual trait biology and morphology of the Macrobiotidae family within the Eutardigrada class. Tardigrades have a semi-transparent body, a trait which is beneficial for many observational and identification tasks. For example, it is often possible to see whether an individual has sperm or eggs in its gonad. Tardigrades have a relatively simple morphology; therefore, molecular methods are currently in wide use for accurate identification of species. Figure 3 shows light microscopy images of female and male eutardigrades *Macrobiotus polonicus* (Vecchi, personal communication).

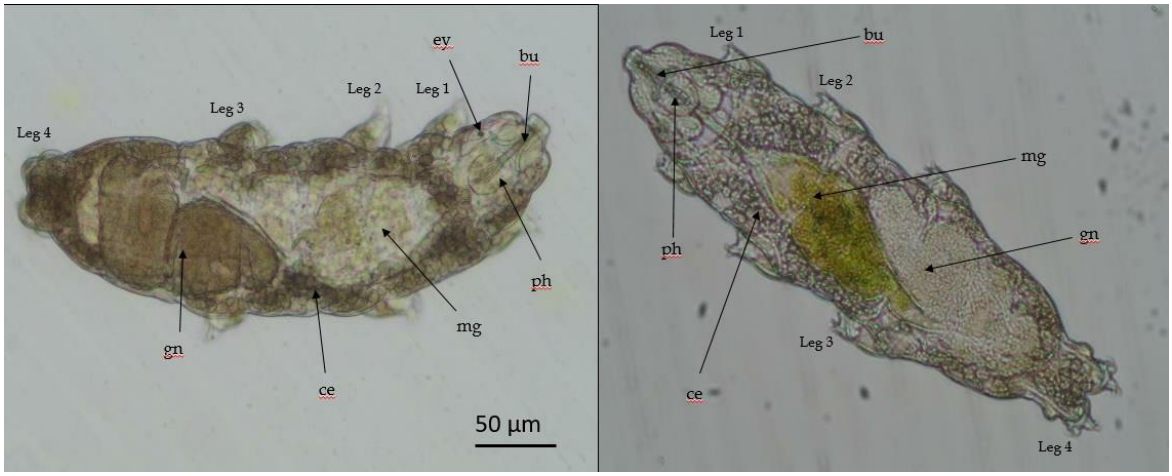

Figure 3. Left: *Macrobiotus polonicus*, female. Reight: *Macrobiotus polonicus*, male. Note the presence of eggs and sperm in the gonads. Abbreviations: bu, buccal apparatus; ce, coelomocyte; ey, eye; mg. midgut; ph, pharyngeal bulb; gn, gonad (photographs by Matteo Vecchi).

Figure 6 demonstrates another example of a female eutardigrade with some important morphological features. Note the presence of the spermatheca, a female sperm storage organ, an important feature for studies related to sperm competition and reproductive biology. It is a sac or cavity inside a female or a hermaphrodite that is used to store sperm until it is used to fertilize eggs (Bertolani 2001). The buccopharyngeal apparatus is a structure used for feeding and it is unique to Tardigrada (Schuster et al. 1980) and it is one of the focal morphological features that is used for taxonomic identification of tardigrades.

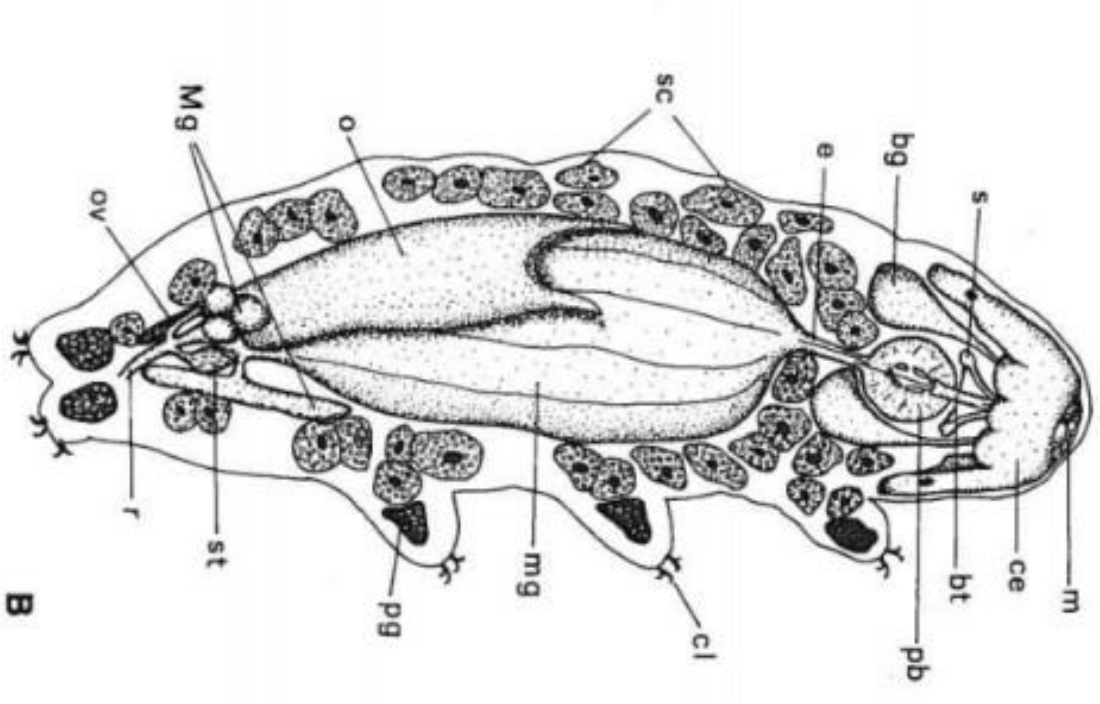

Figure 4. A generalized semiterrestrial female eutardigrade. Abbreviations: bg, buccal glands; bt, buccal tube; ce, cerebrum; cl, claws; e, esophagus; m, mouth; Mg, Malpighian glands; mg, midgut; o, ovary, ov, oviduct; pb, pharyngeal bulb; pg, pedal glands; r, rectum; s, stylets; sc, storage cells, st, spermatheca (Bertolani and Rebecchi 1999).

Two modes of reproduction have been observed in tardigrades – parthenogenesis as well as sexual reproduction. Parthenogenesis is a form of asexual reproduction in which an embryo can develop without the fertilization of the egg by a sperm cell. There are a few types of parthenogenesis in animals and tardigrades have been

observed to undergo only thelytokous parthenogenesis, which produces only females from unfertilized eggs. This has led to some female only tardigrade populations (Dastych 1984, Dastych 1987, Miller and Heathwole 1995, Claxton 1996). Sexual reproduction takes place in the form of gonochorism or hermaphroditism. Hermaphroditic tardigrades are rare and they are able to self-fertilize (Bertolani 1979, Bertolani 2001a).

In this work, I will consider only the sexual reproductive strategy, because I aim to see how the evolutionary forces affect sperm morphology. In eutardigrades, both male and females have a cloaca that is formed by reproductive tracts that lead into the anus (Kinchin 1994). A large number of heterotardigrade species is bisexual (see pp. 4 – 5 for a short description of the phylogenetic relationship within the phylum), which could be conducive to studying mating behaviour, reproductive traits and gamete variation and establish them as model organisms in this phylum on par with eutardigrades. Unfortunately, heterotardigrades are difficult to collect, rear and their abundance is generally low (Gross et al. 2015). There is only one large gonad in both classes of tardigrades and it is dorsal (Figures 3 and 4) to the midgut (Dewel et al. 1993). There has been overwhelming reliance on a small number of bisexual species of limnoterrestrial eutardigrades in this field of research, since parthenogenesis is a very common reproduction mode among many eutardigrade species (Bertolani 2001b). The abundance of parthenogenetic limnoterrestrial species as opposed to marine tardigrades has been hypothesized to evolve due to the relative instability of limnic and terrestrial environments (Nelson et al. 2010). As mentioned above, hermaphroditism occurs as well (Bertolani et al. 2009) and can be found in approximately 0.9 % of tardigrade species (Matteo Vecchi, personal communication).

Generally, tardigrades reach sexual maturity within the second or third molt (Nelson 2015). Tardigrade taxa vary extensively in their reproductive strategies. My work will focus on gonochoristic iteroparous Macrobiotidae. Female eutardigrades

lay their eggs through the cloaca that is connected by the oviduct to the single ovary. The ovary changes in size depending on the reproductive stage and age of the female tardigrade (Bertolani 1983). Oocytes start maturing after the second molting but sometimes even after the first one (Rebecchi et al. 2000). Oocytes mature synchronously and the number of eggs laid can range from 2 to up to 40 and even more in eutardigrades (Altiero et al. 2006, 2015, Guidetti et al. 2019). Oocytes are formed in four stages: pre-vitellogenesis, early vitellogenesis, late vitellogenesis and mature oocyte. Then they are oviposited simultaneously with the molting process and this happens several times in female tardigrades' lives, which makes them iteroparous. This is the case in both classes – heterotardigrades as well as eutardigrades (Poprawa et al. 2015).

The exact egg laying habits of tardigrades regarding the clutch size and the inter-clutch interval, have been studied in a number of species with lab-based life history data and it is now known that female tardigrades usually deposit a number of new eggs within a couple of weeks from the previous deposition and do so several times during their adult lives (Bertolani 2001, Bingemer et al. 2016b) either freely into the environment or into an exuvium (shed integument) (Kinchin 1994). During their research on the establishment of a tardigrade rearing system, Horikawa et al. (2008) found that *Ramazzotius varieornatus* females lay their first eggs already 9 days after hatching and did so at 4 – 6 day intervals with the overall mean of 7.85 eggs per individual. Lemloh et al. (2011) found that *Paramacrobiotus tonnolii* lay their eggs after 24.4 days on average with the mean number of egg per clutch being 6.5 and 7.7 days between the clutches. They also examined the life history of *Macrobiotus sapiens* and found that these tardigrades lay their first eggs after 16.5 days with 5.1 eggs per clutch and 8.9 days between the clutches. We also used 12 *Macrobiotus* species in our investigation.

Another important consideration for this study is whether a particular tardigrade species have a spermatheca, which has been shown to affect the morphology of

sperm in many other species, due to the need for the sperm cells to stay viable in this storage organ for a prolonged period of time (Lüpold and Pitnick 2018, Zhang et al. 2015). For example, Rebecchi (1997) showed that the tail of the spermatozoa stored in spermathecae gets reduced and the cells lose their tufts. Pitnick et al. (2020) emphasize that sperm have complex and protracted live histories; therefore, it would be beneficial to carry out descriptive accounts of post-ejaculatory modifications of sperm across different taxa. Reproductive traits are very diverse in tardigrades and it is important to understand their association. For example, some arthrotardigrades (an order within Heterotardigrada, refer to pages 4 and 5 above for a short description of the phylogenetic relationship) have been found to possess external genital structures formed from extensions of the ducts of the receptacles. These structures are hypothesized to be involved in copulation and/or insemination (Hansen and Kristensen 2006). The variability of morphology and positioning of seminal receptacles is high even within a family (Hansen et al. 2012).

1.2 Tardigrade sperm and egg morphology

Tardigrade spermatozoa have been found to be quite diverse with varying sperm components. Figure 5 shows this great diversity for some tardigrade taxa. The components of a Macrobiotidae sperm cell are the acrosome, nucleus, midpiece and tail. Figure 6 shows a helium ion microscope image (later in the text referred to as HIM) of a spermatozoon of one of our studiy species *Macrobiotus polonicus*.

The tardigrade sperm have a common arrangement with the "9+2" axoneme. The axoneme is the motility motor of the spermatozoon and its microtubule arrangement can vary in different groups of organisms along with other inner structures within it (e.g. mitochondria, mitochondrial derivatives, accessory bodies, accessory tubules) (Dallai et al. 2016). The head (contains the nucleus with the haploid set of chromosomes) of the spermatozoon in eutardigrades is quite elongated and there is always a coil at the acrosome or the nucleus (Rebecchi and Guidi 1995). The acrosome is one of the main components of the spermatozoa and

is often quite distinct in various groups of organisms. It has great significance in the process of fertilization. The acrosome develops during spermiogenesis from the Golgi complex. It is located at the anterior part of the head and it has great significance in the process of fertilization (Hinrichsen – Kohane et al. 1984, Meizel 1985). Tardigrade spermatozoa very rarely lack a midpiece (often considered a part of the flagellum that contains mitochondria and the centriole with its microtubules) and the flagellum always end in a tuft, which is a bunch of fine filaments (Guidi and Rebecchi 1996). The reproductive tract and the presence as well as the design of the spermatheca in females have been shown to co-vary with the sperm length and morphometric differences in the sperm morphology in other taxa (Briskie et al. 1997, Minder et al. 2005, Anderson et al. 2006).

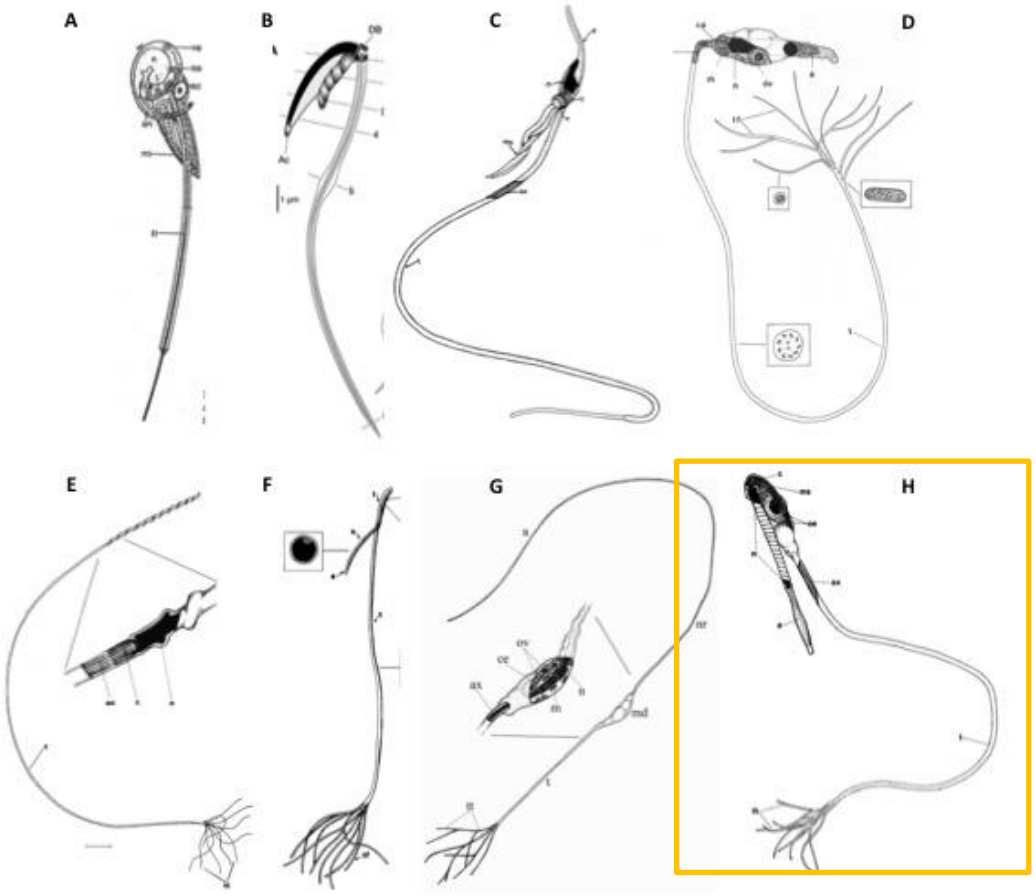

Figure 5. Sperm diversity in Tardigrada. Note the spiral nucleus and presence of a tuft in the highlighted Microbiotidae sperm in (G). Heterotardigrada: Batillipedae

(A) (Kristensen 1979), Helechiniscidae (B) (Suzuki and Kristensen 2014) and Echiniscidae (C) (Rebecchi et al. 2003); Eutardigrada: Eohypsibiidae (D) (Rebecchi and Guidi 1995), Isohypsibiidae (E&F) (Rebecchi 2001) and two different genera of Macrobiotidae (G&H). *Macrobiotus* is shown in the orange box. (Rebecchi et al. 2011, Rebecchi 1997).

Laid eggs are spherical or oval and range from the typical 50 µm to 100 µm but sometimes even up to 235 µm when the surface structures (processes) are taken into account. The eggs of tardigrades that are deposited freely tend to have complicated and unique surface patterns and these have been shown to be species-specific in many genera of Eutardigrada (Schill 2018). This phenomenon is of great importance in taxonomic considerations (Bertolani and Rebecchi 1993). Unfortunately, the function of these processes on the egg surface is still not well understood (Kinchin 1994). The eggs that are deposited into an exuvium usually have a smooth surface. Figure 7 shows the variability of egg morphology in four Macrobiotidae species.

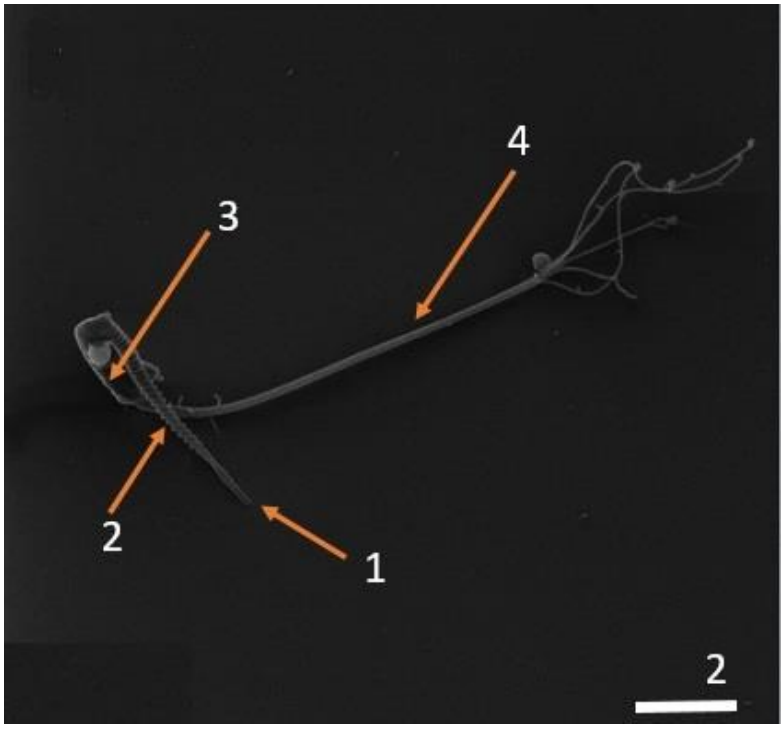

Figure 6. A HIM image of a *Macrobiotus polonicus* sperm cell (Vecchi, personal communication). 1) Acrosome 2) Nucleus 3) Midpiece 4) Tail with a terminal tuft. The scale bar is in µm.

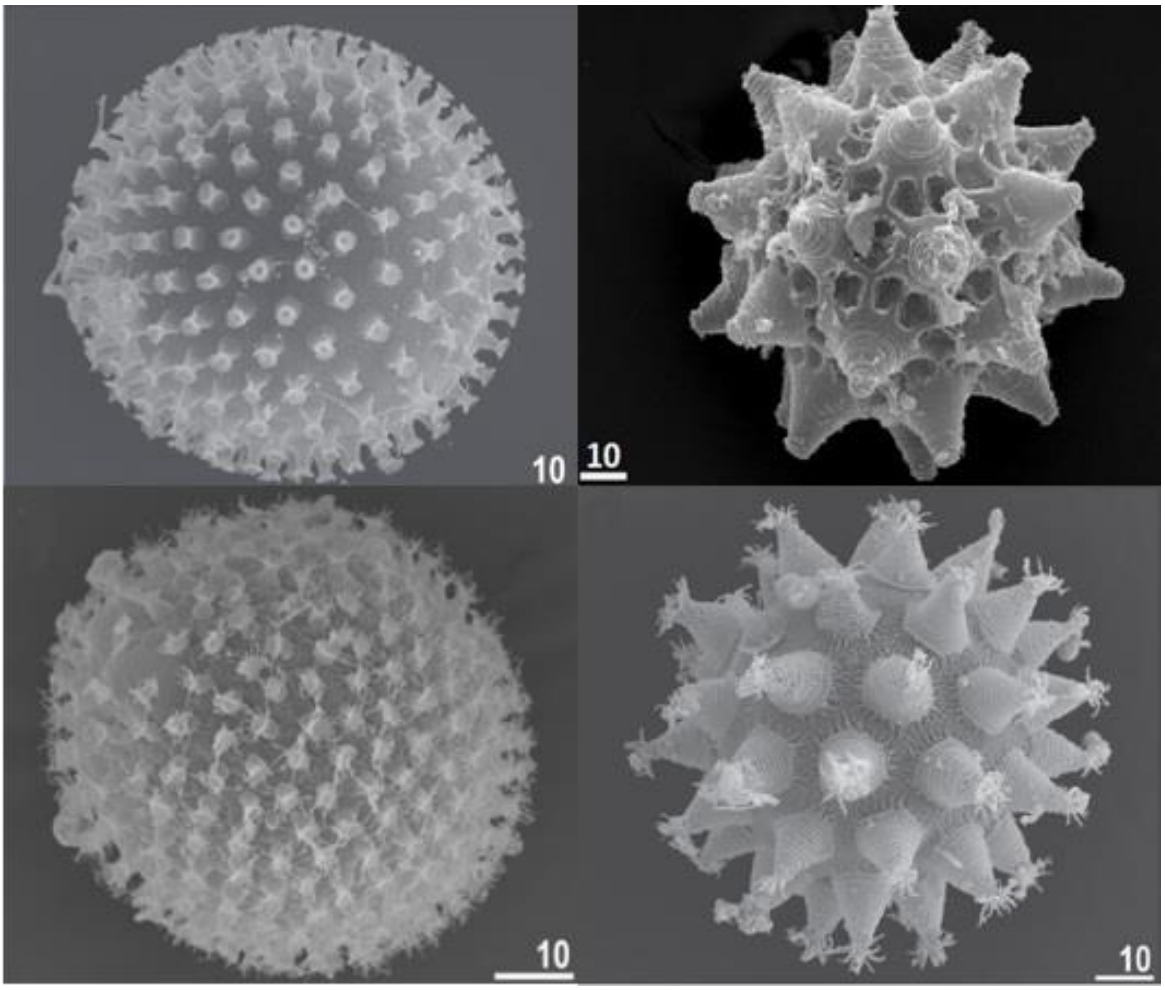

Figure 7. SEM images of variable egg morphology in the family Macrobiotidae. Top left: *Macrobiotus hannae* (from Nowak 2018); top right: *Paramacrobiotus richtersi* (Guidetti et al. 2019); bottom right: *Mesobiotus radiatus* (from Roszkowska 2018); bottom left: *Macrobiotus shonaicus* (from Stec et al. 2018). The scale bars are in µm.

The egg surface also contains the morphological features whose biological roles are still not understood – areolation and reticulation. These covetous patterns between processes are not present in every species with many possessing neither one of them. Areolation is the pattern of larger depressions, whereas reticulation is a mesh-like pattern of small and numerous cavities. Reticulation can be found within the areoles if both features are present. Figure 8 shows the examples of these features in some of the species selected for our investigation. These structures in addition to the inter-process distance can be assumed to play an important role in allowing the sperm cells to fuse with the chorion, thus resulting in an association between the

length of the sperm head and the morphology of the egg surface. This relationship has not been studied in tardigrades before in a more detailed manner and only descriptive accounts have been provided (Sugiura & Matsumoto 2020). Figure 9 shows the contact between *Paramacrobiotus* sp. sperm cells and the egg chorion. The acrosome is buried in the chorion, suggesting the fusion between the gametes and providing evidence to the hypothesis that the egg shell pattern is associated with the length of the sperm head (acrosome + nucleus).

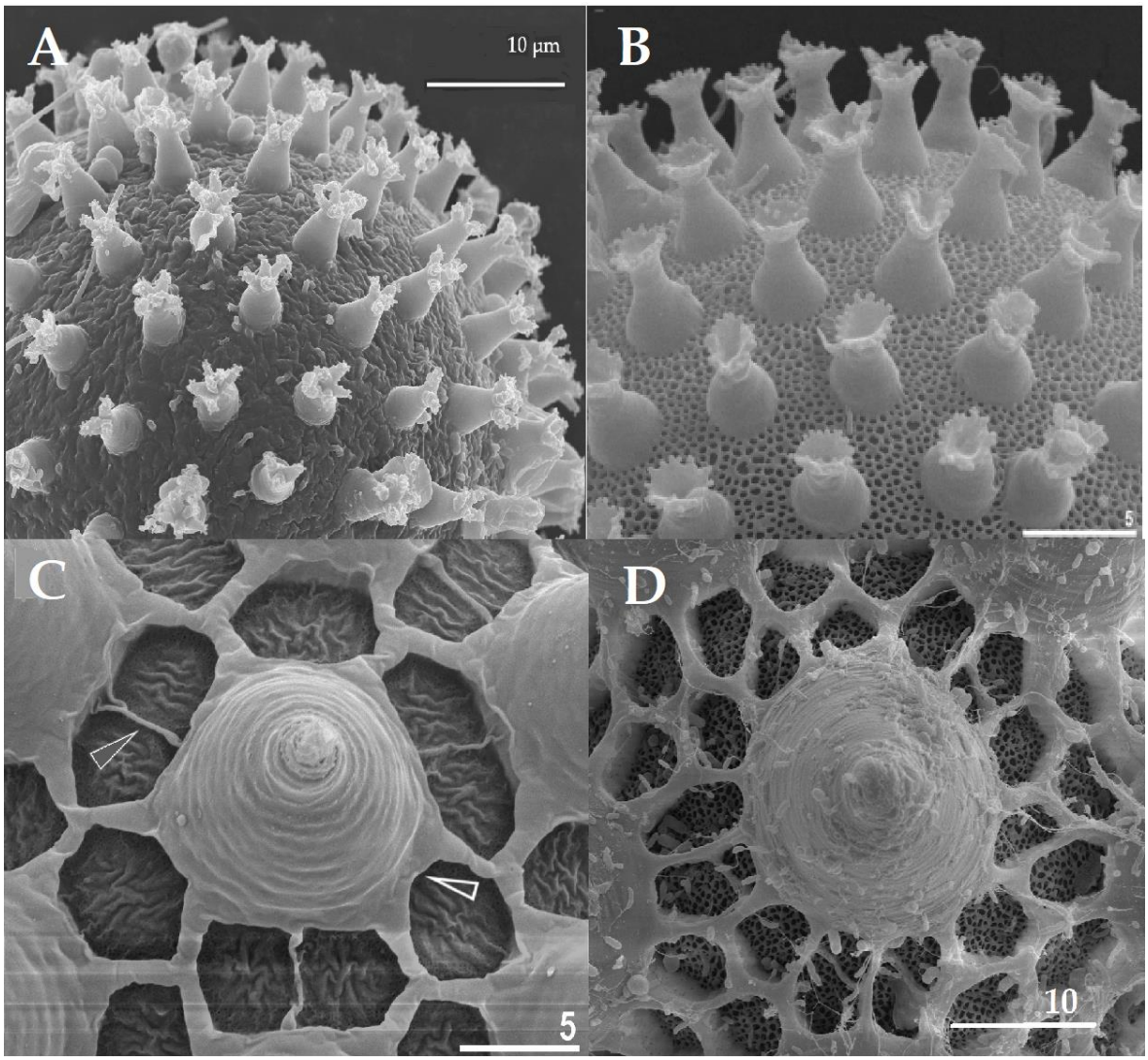

Figure 8. Diverse egg morphology in Macrobiotidae. A) Smooth surface of *M.* gr. *persimilis* "Winters". B) Reticulated surface of *M. kamilae*. C) Areolated surface of a *M.* aff. *pallarii* "Montenegro" egg. D) Areolated and reticulated surface of *M. sisubiotus* (not used in this study). The scale bars are in µm.

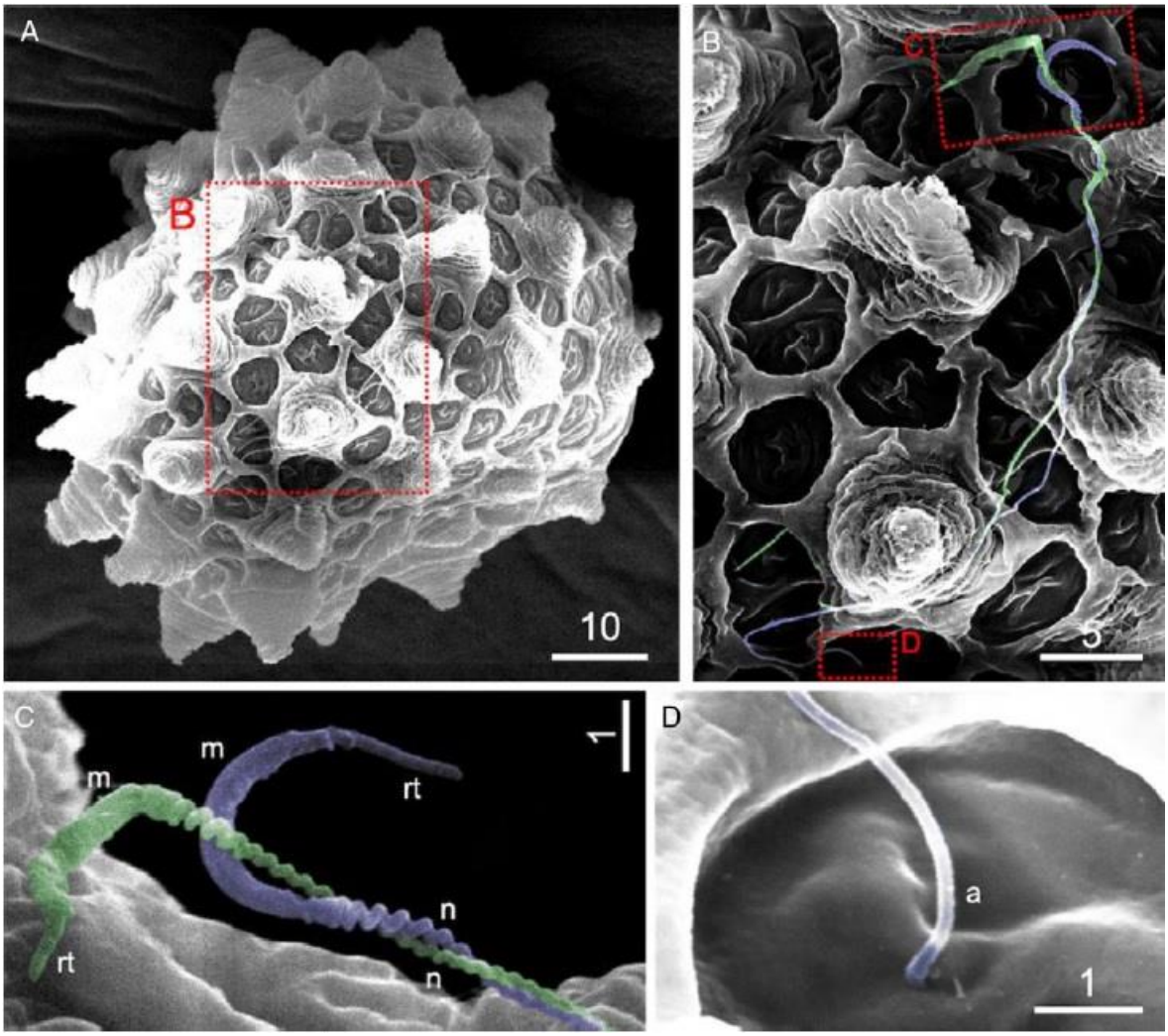

Figure 9. A SEM image of a possible fusion between the male and female gametes of *Paramacrobiotus* sp. (A) Whole image. The other images are expansions of the dashed boxes. a: acrosome, m: midpiece, n: nucleus, rt: reduced tale. The scale bars are in µm (Sugiura & Matsumoto 2020).

1.3 Important factors in the study of sperm diversity and morphology

Already in the 1950's Åke Franzén suggested that the environment within which fertilization takes place must be reflected in sperm morphology (Birkhead & Montgomery 2009). Internally fertilizing species tend to have a more complex sperm structure and have a longer sperm length as well as more evolutionary divergence. This is due to a number of processes that the spermatozoon must successfully go through in order to fertilize the ovum (Pitnick 2009), whereas in

external fertilization (e.g. water-mediated) sperm needs to only find its way to the egg surface via the constant environment, which usually involves chemotaxis (Evans and Sherman 2013).

Intra-specific sperm competition is a common process in all animal taxa, when the female copulates with more than one male during a single reproductive event (Parker 1970a, Smith 2012). Cryptic female choice cannot be observed directly because it takes place inside the female and there are many ways by which females can select for the sperm that will fertilize the ova (Wedekind 1994, Birkhead 1998). Sperm variation across and within species has been shown to be associated with the degree of post-copulatory sexual selection and fertilization conditions in many animal taxa (Parker 1970b, Minder et al. 2005, Harcourt 1991, Calhim et al. 2007, Immler et al. 2008). For example, promiscuous species have reduced intraspecific sperm morphometric variation and internal fertilization, as described earlier, is linked with more complex sperm designs (Fitzpatrick and Lüpold 2014, Alvarez 2017).

Sperm survival, transportation and, consequently, diversity and morphology are also affected by the internal female genital organs such as the reproductive tract or specialized storage organs; physicochemical and immunological factors within these organs, storage time and conditions before fertilization have an influence on the sperm design (Danchin et al. 2008, Lüpold and Pitnick 2018).

1.4 Aims and predictions

My Master's thesis had two main objectives: (1) quantification of the variation in tardigrade primary sexual traits such as the total sperm length and the length of the components of 12 *Macrobiotus* species at both intra- and inter-specific levels for the first time; collecting from the available literature, measuring and summarizing data of these species' egg shell complexity and size; (2) investigation of the relative roles

of the evolutionary processes of sexual and natural selection on tardigrade sperm design.

Based on the current available research in other taxa, sperm component length is expected to be associated with differing egg shell morphological patterns/complexity and with the presence of the spermatheca across species (controlling for phylogeny).

More specifically, I had predicted that:

- Relative midpiece size should be larger in the species with a spermatheca than those without it.
- Relative head (acrosome + nucleus) size should be associated with the egg shell pattern between processes.

2 MATERIALS AND METHODS

2.1 Overview

Any comparative study relies on an adequate and reliable sample size. The complexity of the sperm extraction process and the difficulty in rearing some of the species introduced variation in the number of obtained measures per species as well as per individual. My initial aim was to measure sperm from 10 individuals per species and 10 sperm cells per individual, however it has been shown by Calhim et al. (2011) that even low sperm numbers could provide samples with biologically meaningful mean trait values. All of the tardigrade species used for this work belong to the genus *Macrobiotus* (family: Macrobiotidae). The morphology of the studied species' eggs is quite variable, which allowed us to test for how it is associated with sperm morphology in males. I obtained the egg morphometric data by direct measurement or from literature (when available).

I used phylogenetically controlled linear mixed models using egg morphology and presence of the spermathecal as predictors and sperm head and midpiece length as response variables. Incorporating phylogeny is an important analysis tool in assessing the sperm morphology diversity and the sperm design across different species. This method provides a more reliable sample size by checking for inherent non-independence of data points due to shared evolutionary history (Freckleton et al. 2002b, Lack & Van Den Bussche 2010, Stone et al. 2011).

2.2 Materials

The 12 species used for this comparative study are listed in Table 1 along with the information on their origin and current availability at JYU.

Table 1. The list of species used in this study. Some of the cultures were obtained from our partners at Jagellonian University, whereas the rest were extracted directly from samples at JYU.

Species	Origin	Samples/cultures obtained from	In culture at JYU
Macrobiotus polonicus	Wielkopolski National Park, Poland	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Yes
Macrobiotus caelestis	Tien Shan Mountains, Kyrgyzstan	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Died during the measurement process
Macrobiotus aff. *pallarii* S14	Ponte Samoggia, Bologna, Italy	University of Jyväskylä	Yes
Macrobiotus aff. *pallarii* "Montenegro"	Crkvine, Montenegro	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Yes

Macrobiotus cf. *sapiens*	Ponte Samoggia, Bologna, Italy	University of Jyväskylä	Yes
Macrobiotus gr. *pseudohufelandi* "Rokua"	Rokua National Park, Finland	University of Jyväskylä	No
Macrobiotus gr. *persimilis* "Winters"	Jackson, Missisipi, USA	University of Jyväskylä	Yes
Macrobiotus macrocalix	Parco dei Centol Laghi Parma, Italy	University of Jyväskylä	No
Macrobiotus canaricus	Gran Canaria, Spain	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Died during the measurement process
Macrobiotus sottilei	Rewal, Poland	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Yes
Macrobiotus noongaris	King's Park, Perth, Australia	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Died during the measurement process
Macrobiotus kamilae	Mussoorie, India	Daniel Stec & Łukasz Michalczyk, Jagellonian University	Yes

For some species (*M.* gr. *pseudohufelandi* "Rokua" and *M. macrocalix*) individuals and eggs were extracted directly from samples, as these species are not amenable to laboratory rearing. Some of the species (*M.* aff. *pallarii* S12, *M.* aff. *pallarii* "Montenegro", *M.* gr. *pseudohufelandi* "Rokua", *M. gr. persimilis* "Winters") are new

records and are currently being described. However, their status as separate species has been resolved (Matteo Vecchi, personal communication).

There is available information on the egg morphology and morphometrics for the species listed above in their respective species description publications except for *M.* aff. *pallarii* S14, *M.* aff. *pallarii* "Montenegro", *M.* gr. *pseudohufelandi* "Rokua", *M. gr. persimilis* "Winters". These four species are currently being described (Matteo Vecchi, personal communication).

2.3 Methods

2.3.1 Sperm extraction, staining and measurement

I extracted the sperm by isolating possible males from each species and keeping them isolated with food for a few days before dissection. This allowed for accumulation of sperm and better gonad visibility. The process of finding and distinguishing males was very successful in specie that had a relatively larger gonad (e.g. *M. macrobiotus*, *M.* aff. *pallarii* S14). With others, the process was more or less random as it was extremely difficult to see with the stereomicroscope whether the gonad really contained sperm (e.g. *M. sottilei*, *M. noongaris*). I extracted the sperm from the isolated male tardigrades by squeezing or cutting up each individual with a dissection needle on a separate slide that had been prepared for sperm fixation by poly-lysine coating. The staining process included applying two fluorescent dyes for better differentiation of the sperm components using confocal microscopy analysis. Matteo Vecchi carried out confocal imaging of the sperm cells (Leica S18 Falcon). I used Zeiss stereomicroscopes and the Zeiss AXIO Scope A1 compound light microscope for the steps described above. The detailed protocol for sperm staining was developed by Matteo Vecchi and it is included as Appendix 1.

I measured the length of the sperm components in ImageJ v. 1.53a (Wayne Rasband, National Institutes of Health, USA). Figure 10 shows what the images and sperm

cell components looked like. The staining of the sperm cell in on the right side of the image is successful with all of the components visible. The sperm cell on the left side lacks the terminal tuft and the midpiece is not visible. I measured only the well-visible components (nucleus in this case) from these types of images. For each image, the confocal microscope produced a stack of images from different focal planes. The focal planes were summarized by using a maximum Z projection in ImageJ. The contrast of the image was adjusted to increase visibility and each measured sperm cell was tagged with a numeric identifier.

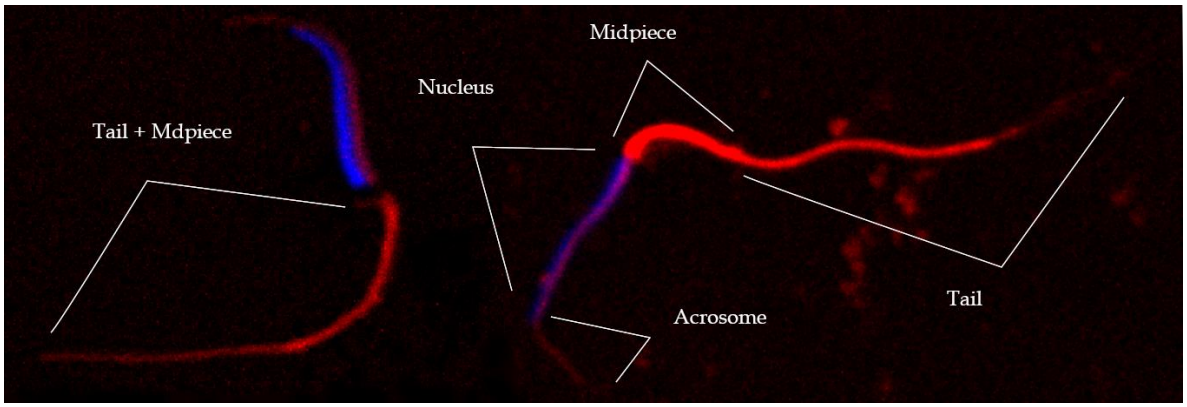

Figure 10. An example of the images obtained by confocal microscopy as measured in ImageJ. Note the variability in the tail and midpiece visibility. Only the fully visible components were included in the data set.

The original data set was tabulated for individual sperm cells as row entries and the length of the components measured in micrometers as the column entries. Additionally, I also measured the angle between the nucleus and the midpiece, which provides valuable information on the degree of "openennes" of the sperm as it changes after the release of the sperm cell from the gonad and can be used for further research to understand the morphological changes involved in the maturation process of the sperm. The original data set was condensed for statistical analysis into the mean values for each species with the standard deviations and the number of measurements presented. I also calculated the coefficient of variation of the relevant sperm components for each species.

2.3.2 Egg morphology

We collected the egg morphology data from the relevant literature in combination with our own light microscopy measurements. The literature data was available in the standardized format developed by Michalczyk & Kaczmarek (2013). We used two quantitative egg morphology variables for this study - the bare egg diameter and inter-process distance. Table 2 shows the egg morphology data that was used for our analysis and their sources.

Table 2. Egg morphology data for the variables used in our analysis. # indicates the number of measurements. Binary variables 0 and 1 indicate absence and presence, respectively.

Species	Bare diameter	# Bare diameter	Inter process distance	# Inter process distance	Egg measurements data source	Areolation	Reticulation	Spermatheca
Macrobiotus polonicus	69.3	12	3.9	36	This study	0	0	1
Macrobiotus caelestis	96.2	30	10.0	90	Coughlan et al. 2019.	0	0	1
Macrobiotus aff. *pallarii* S14	76.6	30	5.6	90	Stec et al. (in press)	1	0	1
Macrobiotus aff. *pallarii* "Montenegro"	73.8	30	4.5	90	Stec et al. (in press)	1	0	1

Macrobiotus cf. *sapiens*	91.2	6	3.0	42	This study	0	1	0
Macrobiotus gr. *Pseudohufelandi* "Rokua"	107.1	1	2.4	10	This study	0	1	1
Macrobiotus gr. *persimilis* "Winters"	65.4	10	4.1	21	This study	0	0	1
Macrobiotus macrocalix	92.2	6	3.0	30	This study	0	1	0
Macrobiotus canaricus	68.7	30	3.4	90	Stec et al. 2018.	0	1	0
Macrobiotus sottilei	78.9	30	4.9	90	Kiosya et al. 2021.	0	1	0
Macrobiotus noongaris	70.7	30	3.4	90	Coughlan and Stec 2019.	0	1	0
Macrobiotus kamilae	77.4	30	3.2	90	Coughlan and Stec 2019.	0	1	0

We carried out our own measurements for all the undescribed species as well as the species lacking standardized egg morphology data. Figure 11 shows the reticulated egg shells of *M. noongaris* and *M. kamilae* with the red lines showing how the inter-process distance was be measured.

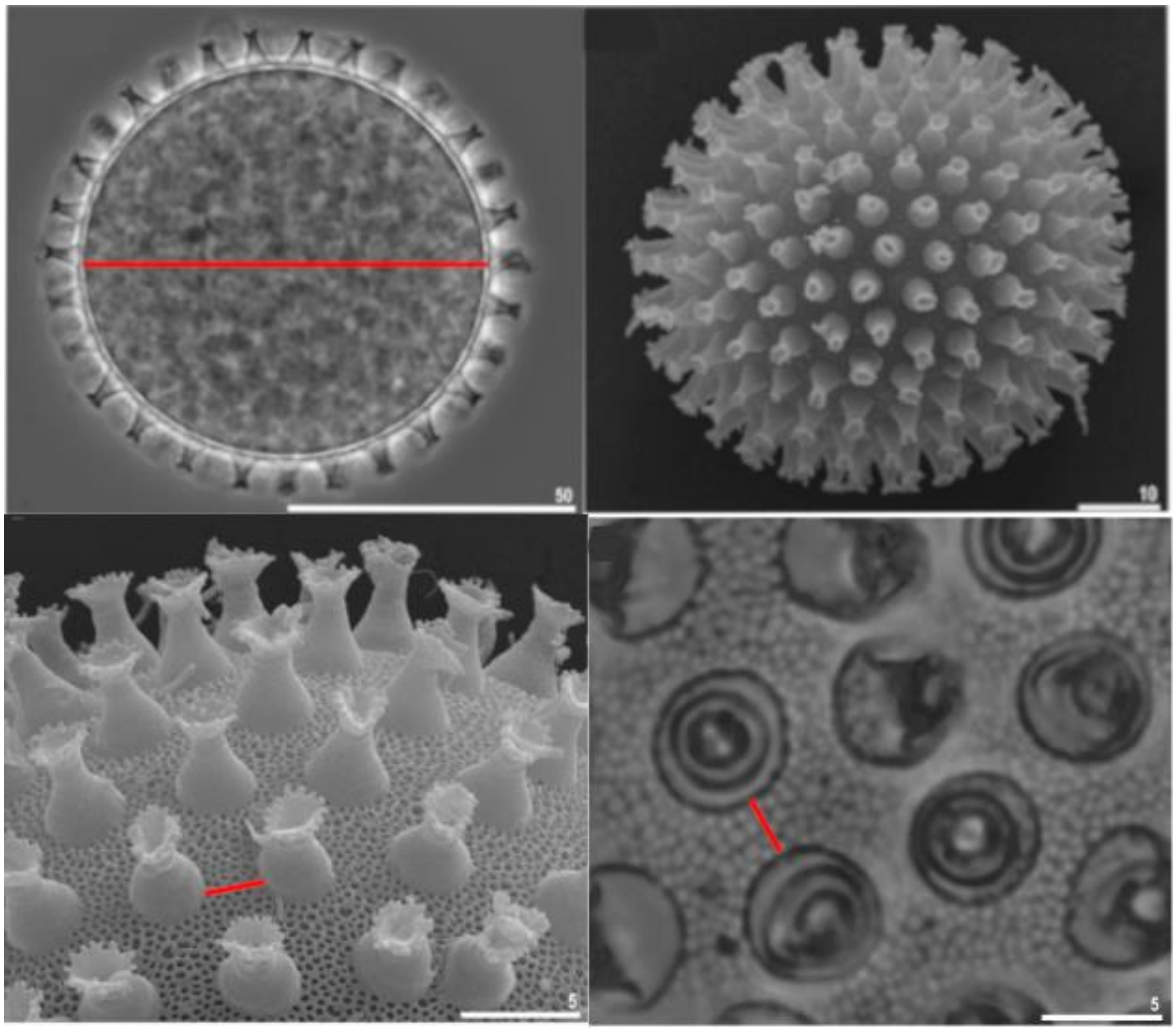

Figure 11. Top left. A light microscopy image of an *M. noongaris* egg. The red line indicates the bare egg diameter. Top right. A SEM image of an *M. noongaris* egg. Bottom left. A SEM image of an *M. kamilae* egg. Note the reticulation of the surface and the red line indicating an inter-processal distance. Bottom right. A light microscopy image of an *M. kamilae* egg (Coughlan and Stec 2019). The scale is in µm.

We coded the presence of the reticulation as well as the areolation as binary variables with 0 and 1 indicating absence and presence respectively. This allowed us to construct a new categorical variable defined as the absence of both and presence of either areolation or reticulation. No studied species had both of these morphological features present together. Table 2 above includes columns showing this information.

2.4 Statistical analysis

In order to prepare the data for the exploratory, descriptive and statistical analyses, I calculated the coefficients of variation and species means for each sperm component. I ran all of the tests in R version 4.0.4. First, I checked for any possible phylogenetic signal to see similarities in the trait values of interest due to common ancestry. This was done by calculating the K statistic with the function phylosig from the package phytools (version 0.7-70). Matteo Vecchi provided me with the phylogeny of the studied species and I rooted the tree with *Sisubiotus spectabilis*. I ran phylogenetically controlled linear mixed models to see the association between the sperm midpiece as the response variable and the presence of the spematheca as the explanatory variable across species for our sexual selection hypothesis with the sperm total length included as the covariate. I ran mixed linear natural selection models. The first one included the sperm head length (acrosome + nucleus) as the response variable and the egg shell complexity as the explanatory variable with the sperm total length as the covariate. The second model also included the inter-processal distance and the bare egg diameter (egg diameter with no processes) as the covariates. The egg shell complexity variable consisted of three levels: reticulation, areolation or neither one of these. The covariates were always centered around the mean, this resulting in the model that produced the estimate of the sperm component length for any factor of interest at the average values of the covariates. I ran these phylogenetically controlled linear mixed models by using the function phylolm from the package phytools with the bootstrap value of 1000 and the same phylogenetic tree that was used for finding the phylogenetic signal.

3 RESULTS

3.1 Quantification of sperm variation

Table 3 summarizes the quantified variation in the sperm components of the 12 studies tardigrade species. The components are acrosome, nucleus, midpiece and the total length. The table contains the number of individuals measured per species for each component, the size range of the components, their mean values and the coefficient of variation. The number of individual for each species ranged widely due to the difficulty of the sperm extraction and staining process. I successfully extracted and stained the sperm of only one *M. canaricus* individual, therefore I was not able to obtain the sperm components' range and its respective coefficient of variation for this species.

Table 3. Quantified sperm variation in the studied species. CV = coefficient of variation. The ranges and mean values are given in μm.

Acrosome						
Species	**Number of individuals**	**Mean**	**Range**	**Relative size mean**	**Relative size range**	**CV**
M. caelestis	4	2.68	2.50 - 2.82	0.09	0.08 - 0.09	0.05
M. canaricus	1	2.80	-	0.1	-	-
M. cf. *sapiens*	13	2.88	2.65 - 3.15	0.08	0.08 - 0.09	0.05
M. kamilae	3	3.26	3.02 - 3.48	0.11	0.10 - 0.12	0.07
M. macrocalix	7	2.59	2.35 - 2.99	0.07	0.06 - 0.08	0.09
M. noongaris	2	2.70	2.62 - 2.78	0.08	0.08 - 0.08	0.04
M. polonicus	12	1.85	1.71 - 2.47	0.07	0.06 - 0.09	0.11
M. aff. *pallarii* "Montenegro"	4	2.96	2.83 - 3.11	0.09	0.09 - 0.10	0.05
M. aff. *pallarii* S14	9	3.61	2.87 - 3.87	0.11	0.09 - 0.11	0.08

M. gr. *pseudohufelandi* "Rokua"	11	1.86	1.67 - 2.27	0.07	0.06 - 0.09	0.10
M. sottilei	3	3.14	2.97 - 3.28	0.1	0.09 - 0.10	0.05
M. gr. *persimilis* "Winters"	10	2.10	1.83 - 2.32	0.07	0.06 - 0.08	0.07
Nucleus						
Species	**Number of individuals**	**Mean**	**Range**	**Relative size mean**	**Relative size range**	**CV**
M. caelestis	4	6.24	6.16 - 6.34	0.2	0.2 - 0.21	0.13
M. canaricus	1	5.63	-	0.19	-	-
M. cf. *sapiens*	13	8.23	7.48 - 9.22	0.24	0.22 - 0.27	0.06
M. kamilae	3	7.52	7.20 - 7.90	0.26	0.25 - 0.27	0.05
M. macrocalix	7	8.93	8.47 - 9.26	0.24	0.23 - 0.25	0.03
M. noongaris	2	7.18	6.93 - 7.43	0.22	0.21 - 0.23	0.05
M. polonicus	12	5.42	4.80 - 5.81	0.19	0.17 - 0.21	0.05
M. aff. *pallarii* "Montenegro"	4	5.76	5.55 - 6.11	0.18	0.17 - 0.19	0.05
M. aff. *pallarii* S14	9	7.08	6.07 - 7.94	0.21	0.18 - 0.24	0.10
M. gr. *pseudohufelandi* "Rokua"	11	4.84	4.38 - 5.27	0.19	0.17 - 0.20	0.05
M. sottilei	3	8.02	7.84 - 8.32	0.24	0.24 - 0.25	0.03
M. gr. *persimilis* "Winters"	10	5.75	5.22 - 6.13	0.2	0.18 - 0.21	0.05
Midpiece						
Species	**Number of individuals**	**Mean**	**Range**	**Relative size mean**	**Relative size range**	**CV**
M. caelestis	4	4.68	4.45 - 5.04	0.15	0.14 – 0.15	0.06

M. canaricus	1	3.59	-	0.12	-	-
M. cf. *sapiens*	13	3.96	3.63 - 4.48	0.12	0.11 - 0.13	0.07
M. kamilae	2	3.80	3.72 - 3.88	0.13	0.13 - 0.13	0.03
M. macrocalix	5	4.07	3.70 - 4.33	0.11	0.1 - 0.12	0.07
M. noongaris	2	3.31	3.24 - 3.38	0.1	0.1 - 0.1	0.03
M. polonicus	10	4.71	4.43 - 4.96	0.17	0.16 - 0.18	0.04
M. aff. *pallarii* "Montenegro"	4	4.78	4.57 - 4.99	0.15	0.14 - 0.16	0.04
M. aff. *pallarii* S14	9	5.67	4.66 - 6.85	0.17	0.14 - 0.2	0.13
M. gr. *pseudohufelandi* "Rokua"	11	3.96	3.31 - 4.32	0.15	0.13 - 0.17	0.09
M. sottilei	3	4.16	4.10 - 4.19	0.13	0.13 - 0.13	0.01
M. gr. *persimilis* "Winters"	10	4.80	3.89 - 5.22	0.17	0.14 - 0.18	0.08

Total length

Species	Number of individuals	Mean	Range	Relative size range	Relative size mean	CV
M. caelestis	4	31.40	30.48 - 32.18	-	-	0.02
M. canaricus	1	29.17	-	-	-	-
M. cf. *sapiens*	12	34.03	32.81 - 35.53	-	-	0.03
M. kamilae	2	29.24	28.29 - 30.20	-	-	0.05
M. macrocalix	3	37.47	36.70 - 38.77	-	-	0.03
M. noongaris	2	32.99	31.14 - 34.85	-	-	0.08
M. polonicus	11	28.17	25.10 - 33.12	-	-	0.08

M. aff. *pallarii* "Montenegro"	4	32.07	30.60- 33.35	-	-	0.04
M. aff. *pallarii* S14	8	33.75	24.71 - 39.00	-	-	0.13
M. gr. *pseudohufelandi* "Rokua"	7	26.11	23.91 - 27.51	-	-	0.05
M. sottilei	3	32.79	30.18 - 35.63	-	-	0.08
M. gr. *persimilis* "Winters"	10	28.69	23.76 - 30.70	-	-	0.04

The egg morphology data relevant to our aims is shown in Table 2 above. This table contains the bare egg diameter (the diameter of the egg excluding the processes), the inter-process distance, their standard deviations, and egg morphology information. It was not possible to report the range and the coefficients of variation because the data obtained through the publications contained the summary values for the species only.

3.1.1 Phylogenetic signal

The phylogenetic relationship of the studied species and the lengths of each sperm component for all of them are shown in Figure 12. The only sperm trait that seemed to show a phylogenetic signal was the midpiece with a Bloomberg's K statistic close to 1 and a p-value lower than 0.05 (Table 4).

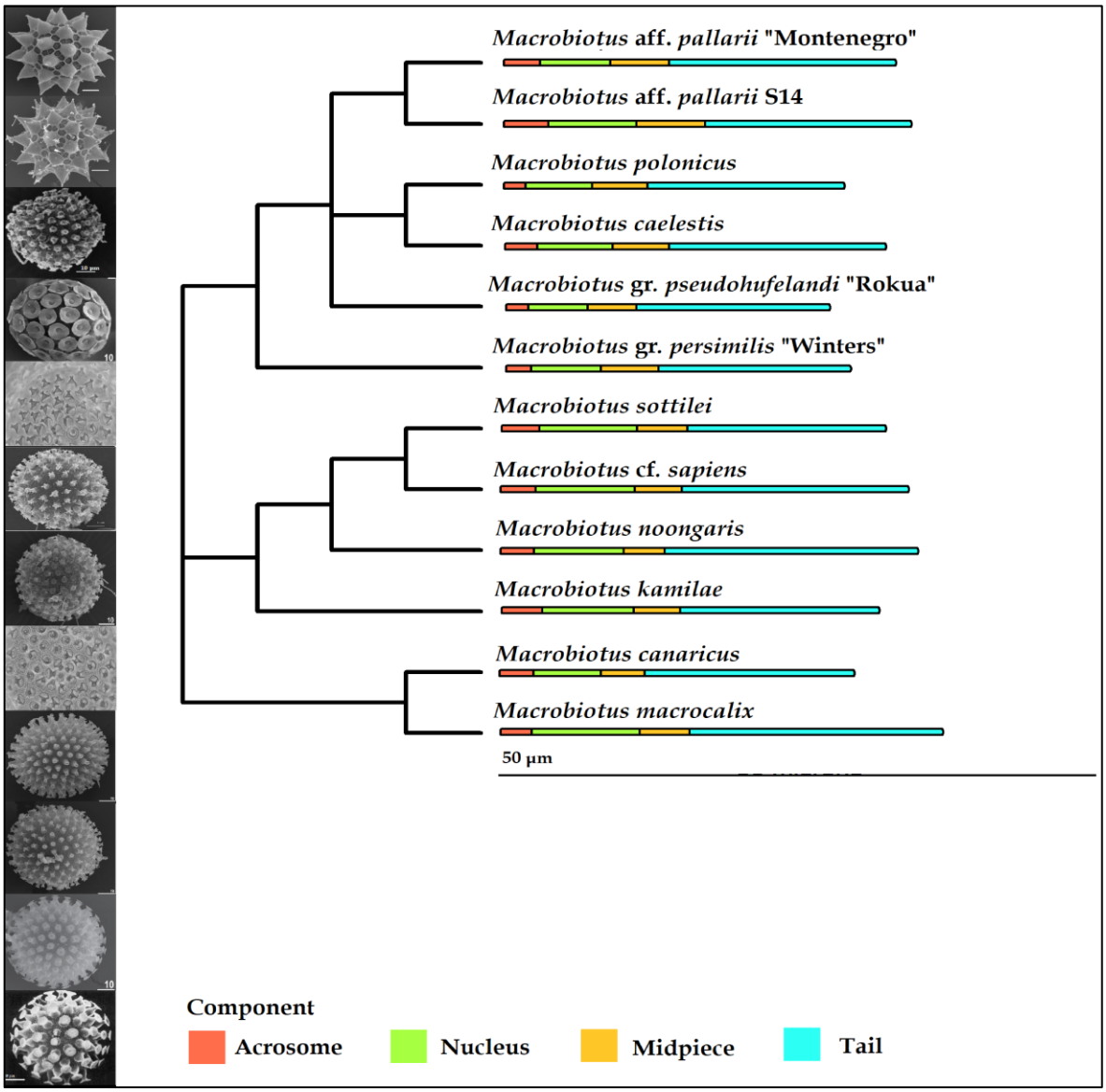

Figure 12. The phylogenetic relationship of the studied species with the quantified sperm components. The order of the egg images corresponds to the vertical arrangement of the species in the phylogenetic tree. Only light microscope images were available for *M.* cf. *sapiens* and *M.* gr. *pseudohufelandi* "Rokua". The other images are SEMs.

Table 4. Phylogenetic signal in the sperm components. The K values close or higher than 1 indicate a stronger similarity in close relatives than expected under the Brownian motion evolution.

Sperm Component	*K* Statistic	P-value
Acrosome	0.54	0.37
Nucleus	0.60	0.25

Head	0.61	0.22
Midpiece	0.89	0.03
Total	0.49	0.52

3.2 The role of sexual selection on sperm morphology

When I considered the association between the midpiece size and the presence or absence of the spermatheca, the obtained data indicates that the species with this structure tend to have relatively larger midpieces. Figure 13 shows that all of the six species with the spermatheca have larger midpieces than the species without it when this storage organ is relativized to the total length of the sperm cell.

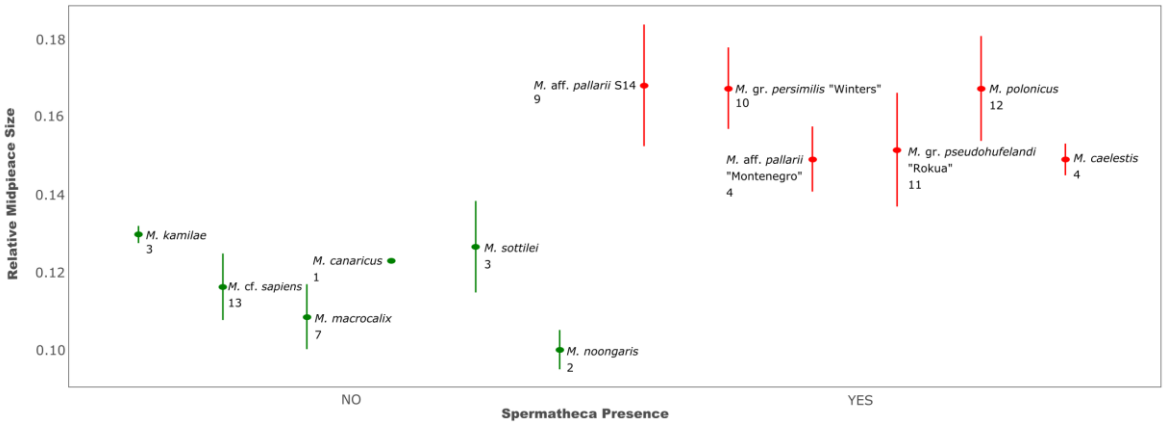

Figure 13. The difference in the relative size of the midpiece depending on the presence of the spermatheca. The vertical lines indicate the standard deviation of the relative midpiece size. The number of individuals for each data point is given below the names of the species.

The model shows that the relative midpiece size is larger in species in which the females have this sperm storage organ. Table 5 presents the output of this model.

Table 5. The output of the bootstrapped phylogenetically controlled linear sexual selection model based on 1000 replicates. The length estimates are in μm.

Sexual selection model					
	Length estimate (μm)	**t-value**	**Lower bootsrap CI**	**Upper bootstrap CI**	**p-value**
Spematheca absent	3.696	9.604	3.013	4.336	≈ 0
Spematheca present	1.200	2.123	0.271	2.086	0.063
Centered total length	0.089	2.221	0.021	0.159	0.053
Raw residuals					
Min	**1Q**	**Median**	**3Q**	**Max**	
-0.533	-0.190	0.056	0.175	0.562	
Parameter estimate using ML (σ^2)	0.080			**Mean tip height**	3.734
Bootstrap mean (on raw scale)	0.060			**Adjusted R-squared**	0.346
Bootstrap mean (back transformed from log scale)	0.053			**AIC**	20.216
Bootstrap 95% CI	(0.017, 0.128)			**Log Likelihood**	- 6.108

Note: the intercept of the model is **spermatheca absent** and the length estimate 3.696 μm indicates the length of the midpiece for the species with no spermatheca at the average total sperm length.

As can be seen from the output of the model, having a spermatheca results in a large effect size with an average increase of approximately 32.4% in the midpiece length. However, the large confidence interval (7.3% to 56.4% increase in the midpiece length) explains the p-value that is not statistically significant.

3.3 The role of natural selection on sperm morphology

There was no clear trend associated with the egg shell morphology when the sperm head was relativized to the total length. Figure 14 shows this relationship when the relative head length is plotted against the relative inter-process distance (inter-process distance/bare egg diameter).

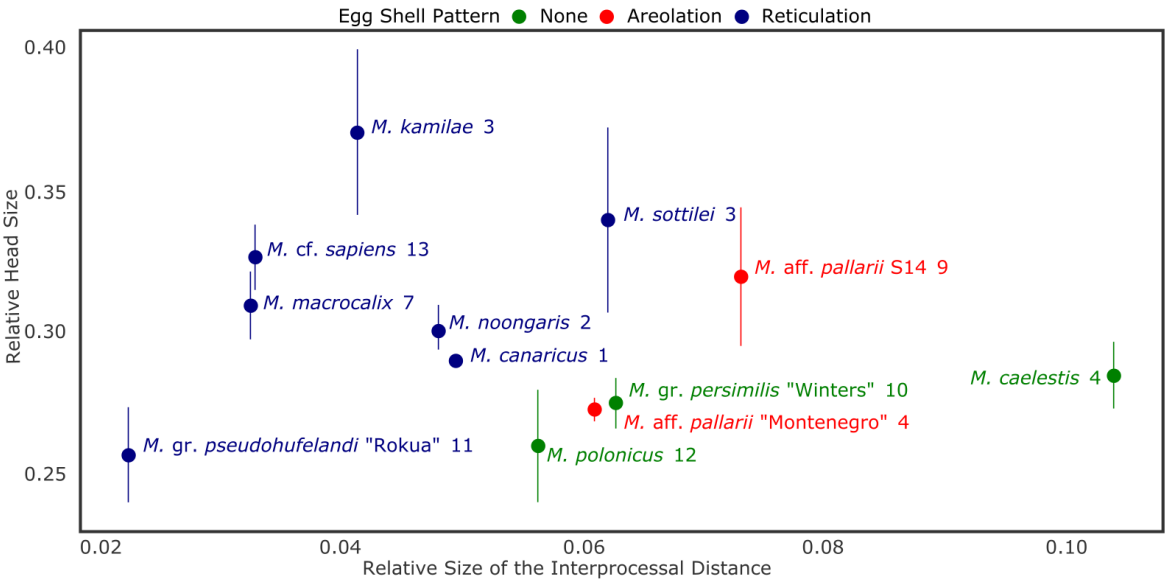

Figure 14. The relationship between the relative head size and the relative inter-processal distance. The vertical lines overlapping each data point indicate the standard deviation of the relative head length. The numbers next to the names of the species indicate the numbers of individuals for each data point.

These models did not result in any statistically significant effects, i.e. the egg shell morphology did not have an effect on the size of the sperm head. Table 6 shows the output of the two natural selection models.

Table 6. The output of the two phylogenetically controlled linear natural selection models based on 1000 replicates. The length estimates are in µm

Natural selection model with egg morphomotery and morphology					
	Length estimate (µm)	**t-value**	**Lower bootsrap CI**	**Upper bootstrap CI**	**p-value**
Smooth/None	9.085	12.199	7.893	10.259	≈ 0
Areolation	0.244	0.280	-1.169	1.611	0.786
Reticulation	0.249	0.314	-0.962	1.503	0.761
Centered total length	0.395	4.695	0.268	0.525	0.002
Raw residuals					
Min	**1Q**	**Median**	**3Q**	**Max**	
-0.904	-0.285	-0.138	0.530	2.317	
Parameter estimate using ML (σ^2)	0.279			**Mean tip height**	3.734
Bootstrap mean (on raw scale)	0.189			**Adjusted R-squared**	0.682
Bootstrap mean (back transformed from log scale)	0.166			**AIC**	37.270
Bootstrap 95% CI	(0.056, 0.398)			**Log Likelihood**	-13.630
Natural selection model with egg morphology only					
	Length estimate (µm)	**t-value**	**Lower bootsrap CI**	**Upper bootstrap CI**	**p-value**
Smooth/None	8.720	9.183	7.428	9.994	≈ 0

Areolation	0.464	0.453	-1.016	1.895	0.6666
Reticulation	0.845	0.707	-0.887	2.505	0.5059
Centered inter-processal dist.	0.129	0.922	-0.059	0.318	0.3923
Centered bare diameter	-0.008	-0.314	-0.039	0.029	0.764
Centered total length	0.379	3.483	0.221	0.522	0.013
Raw residuals					
Min	**1Q**	**Median**	**3Q**	**Max**	
-0.829	-0.334	-0.169	0.532	2.167	
Parameter estimate using ML (σ^2)	0.244			**Mean tip height**	3.734
Bootstrap mean (on raw scale)	0.123			**Adjusted R-squared**	0.629
Bootstrap mean (back transformed from log scale)	0.103			**AIC**	39.670
Bootstrap 95% CI	(0.027, 0.291)			**Log Likelihood**	-12.830

Note: the intercept of the model is **smooth/none** and its respective length estimate indicates the length of the head for the species with no spermatheca at the average total sperm length.

4 DISCUSSION AND CONCLUSIONS

This work provides useful insights into the reproductive biology of tardigrades as gamete evolution in these animals has been neglected. This is an unfortunate fact as their gamete design is very diverse (Figure 5), they are ubiquitous in their geographic distribution, and many species are amenable to rearing in lab conditions.

The lack of available publications that focus on descriptive exploration of tardigrade sperm (Rebecchi and Guidi 1995, Rebecchi 1997, Rebecchi 2001, Rebecchi et al. 2001, Rebecchi et al. 2003, Sugiura et al. 2019) posed an obstacle to the analysis of sperm variation within and across species.

During my thesis work, I collected quantitative sperm morphometric data of the sperm morphology for 12 *Macrobiotus* species. This is the first time that this type of data is collected extensively and uniformly for a large number of tardigrade on the individual and species levels. These data allowed me to investigate the quantitative variation across and within the studies species and to test for different research hypotheses (see section 1.4).

2.1 Variation within and across species

The spermatozoa morphometric data are reported in Table 3 as absolute lengths, relative lengths and the coefficients of variation. The total length of the spermatozoa and their components falls close to or within the ranges that have been reported earlier for other *Macrobiotus* species (Rebecchi 1997, Rebecchi et al. 2011, Sugiura et al. 2019). *M. macrocalix* have the longest sperm of 37.47 µm on average and *M.* gr. *pseudohufelandi* "Rokua" (formely known as *Xerobiotus* sp., Rebecchi 1997) possess the shortest spermatozoa with 26.11 µm on average. The variability of the length of sperm components (coefficient of variation) or the total length seems to be quite large inter-specifically (Table 3). For example, the coefficient of variation, CV, in the

total length of *M.* aff. *pallarii* S14 is 0.13, whereas it is only 0.02 in *M. caelestis*. Nevertheless, the CV exceeds 0.1 in only a few cases.

It is possible that I slightly underestimated the tail and the total length of the sperm cells because of the delicate terminal tuft structures that were not always fully visible in the images, whereas the other components usually provided more accurate results. If this is truly the case, then the relative size of acrosome, nucleus, and midpiece would be slightly smaller.

The intraspecific variation in the size of sperm components has been reported to influence fertilization success across some taxa, e.g. parrots, quail and mice (e.g Carballo et al. 2019, Matsuzaki et al. 2021), play a role in the process of speciation (Landry et al. 2003, Albrecht et al. 2019), and serve as an index of sperm competition levels (Kleven et al. 2008, Immler et al. 2008, Fitzptrick and Baer 2011). Additionally, the swimming velocity is influenced by the overall shape and proportion of each component, thus linking form to function (Humphries et al. 2008, Simons and Olsen 2018, Hook and Fisher 2020). For example, Lüpold et al et al. (2009) showed that sexual selection acts concomitantly on sperm morphology and sperm velocity in passerine birds. Sperm competition in males favors sperm of larger length or larger midpieces, because these components are thought to play an important role in the velocity at which spermatozoa can reach the ovum (Anderson and Dixson 2002, Bennison et al. 2015). Sperm cell size morphometric data in tardigrades allows to test the hypothesis as to how the sperm morphology corresponds with sperm competition (Page 15). For example, it has been shown that more promiscuous species tend to have reduced intraspecific sperm morphometric variation (Calhim et al. 2009, Fitzpatrick and Lüpold 2014, Alvarez 2017).

2.2 Phylogenetic signal

I have found a statistically significant phylogenetic signal for the midpiece length, which means that it tends to correlate with the evolutionary history of the studied species (see Table 4 and Figure 12). This results in the fact that some of the

components are “less free to vary” in regards to the evolutionary process of a particular species and its deviation from the other closely related species. This question is important for our understanding of what constraints are imposed on certain structures in their evolutionary development and why this should be the case (Lüpold et al. 2008)

2.3 Sexual selection

Our sexual selection model revealed that post-copulatory sexual selection plays an important role in the sperm design, with a large increase in the relative midpiece size in the species with the spermatheca as compared to those without it (32.4 % on average) as presented in Figure 13 and Table 5. This finding makes sense in light of the energetics of sperm viability (Danchin et al. 2008, Tourmente et al. 2009, Lüpold and Pitnick 2018). Sperm cells need to stay alive during their pre-fertilization period in the storage organ, which requires a larger number of mitochondria that are contained in the midpiece (pp. 8 – 9). This fact likely played a role in leading to larger midpieces in the species with the spermatheca when compared to those without this sperm storage organ in this study.

Generally, post-ejaculatory sperm modifications will exhibit widespread phenotypic plasticity mediated by sperm–female interactions (Zhang et al. 2015, Pitnick et al. 2020). Rebecchi (1997) observed that the spermatozoa stored in the spermatheca of *Macrobiotus pseudohufelandi* undergoes a marked transformation of the midpiece. This sperm component becomes reduced and loses its ovoid (hemispherical swellings surrounding the centriole) elements, thus becoming tubular and thin. The tail is also reduced to a short stub and the spermatozoon acquires a straight profile with the head losing its bent orientation (see Figure 5 for comparison). A similar observation was made for the hermaphroditic marine tardigrade *Orzeliscus belopus* in regards to the spermatozoon straightening inside the seminal receptacle. However, the tail seems to be preserved in this case (Suzuki and Ktistensen 2014). A deeper investigation of this question includes such

considerations as whether the nucleus gets bent or distorted during the storage process in other tardigrade species (in which the spermatozoa are tightly packed), the degree of "openness" of the spermatozoon (the nucleus-midpiece angle), and how the tail tuft is being affected (for example, the tail tuft or most of the flagellum might be shortened or discarded in the species with the spermatheca) (Rebecchi 1997, Suzuki and Ktistensen 2014, Pitnick et al. 2020).

2.4 Natural selection

The collected data was not consistent with the hypothesis that the egg shell morphology in tardigrades is associated with the head size of the sperm (Figure 14 and Table 6). These negative results rely still on a limited amount of data (see Materials and Methods, Table 2). The number of species in each category was also very small with, for example, only two species with areolated eggs. An analysis with a larger sample size in each morphological category would be necessary to draw a definitive conclusion. In addition, the crude categorization of the egg morphology into three discrete types might be too simplistic and could be improved. As can be seen from Figure 14, it seems that the reticulation type occurs concomitantly with shorter inter-process distances. If more data is obtained, the interaction term relative inter-processal distance*egg shell type can be included because the trend seems to show a positive association between the relative inter-process distance and the relative head size for the reticulated egg shell (possibly also for the areolated type) but not for the egg shells that lack both of these features. Another approach to test for this hypothesis is to consider the open chorion area between the processes it would be the only part of the egg chorion that is exposed for the potential entry by the sperm (Figures 8 and 9). This eliminates the need to categorize the egg into morphological types and allows for testing the hypothesis with a continuous predictor: the morphological categorization into different types might not always reflect the size of the chorion area between the processes, therefore collapsing the inter-process distance and egg shell pattern variables into one continuous predictor

(chorion area between processes) might capture better the conditions and possible requirements for the fusion of both gametes.

2.5 Technical issues and future directions

As has been described above, the lack of descriptive and quantitative morphometric data poses an obstacle to more thorough and larger-scope research on tardigrade gamete evolution. One of the difficulties of the sperm extraction process is that no other method other than manual dissection of the gonads has been successfully implemented in tardigrades. Gonad dissection is time consuming and can lead to contamination from tardigrade cells other than sperm, thus creating background noise. More efficient and "clean" methods like electric stimulation, that is already being applied in a wide range or organisms (Abril-Sánchez et al. 2019, Beirão et al. 2019, Frediani et al. 2019), should be attempted on tardigrades in the future. The ideal sperm extraction method would also allow for the males to survive the extraction, which would be very helpful for maintaining slowly reproducing cultures or prolonging the life of species that do not breed in lab conditions. Another issue in regards to the ease of tardigrade sexing is that some species have large and well visible gonads under the stereomicroscope (for example, *M. macrobiotus* and *Macrobiotus* gr. *persimilis* "*Winters*"). This allows for much easier separation of the males to be dissected for sperm extraction; in other species (e.g. *M. noongaris*, *M. kamilae)* the gonads are not so easily discernible with a stereomicroscope. This fact played a detrimental role in obtaining a larger sample size for each species in this study, as we conducted the initial trials and tested the staining protocol on *M. polonicus*, whose contents of the male gonads are very easily observed.

Inter-specifically, the clear visual difference in the gonad size begs the question of whether there is an association between the gonadosomatic index (GSI = gonad weight/body weight) and sperm length as well as their number in tardigrades. This information would allow to test one of the central fundamental sperm competition

theory questions that states that the GSI and sperm number are positively correlated with sperm competition intensity, which leads to a decrease in the sperm size (Parker 2020).

Intra-individual variation in tardigrade sperm morphology requires more attention as we do not yet understand how spermiogenesis is reflected in the allometry of the components' length in the sperm of these animals. The following questions must be answered in order to obtain more reliable data for intra-specific studies: do all sperm cells grow more or less uniformly and do all sperm components grow equally in their proportion to the total length? A descriptive account of what happens to the spermatazoa when they are released from the gonad would also be of value for reproductive biology investigations. Another thorough examination that is pertinent to the questions of sexual selection is how the presence of the spermatheca changes the morphology of the sperm.

I would like to emphasize that my interpretation of gamete evolution in Macrobiotid tardigrades is hindered not only by a lack of well-researched information on spermiogenesis (as described above) and egg development, but also because it is still unclear where the fertilization of the egg occurs and whether there are large differences across the tardigrade taxa. For example, the role of the highly inter-specifically variable egg processes during fertilization is still not understood and requires further investigation. It is important to point out that there is limited information on the mating behavior and process of sperm transfer and uptake for only a few tardigrade species (Bingemer et al. 2016, Sugiura et al. 2019, Bartel et al. 2020). In fact, we do not yet understand how sperm enters the eggs in this group of invertebrates in general; however, the observations made by Sugiura et al. (2019) for *Paramacrobiotus* sp. and *M. shonaicus* lay the ground work for understanding how fertilization takes place in Macrbiotid tardigrades. In this work, they suggest that the tip of the acrosome penetrates the chorion.

2.6 Conclusion

I have quantified sperm morphometric data for 12 *Macrobiotus* species. This is the first time that such extensive quantitative data has been collected for Tardigrada. I have also found a large difference in the relative midpiece size in the species with and without the spermatheca, which supports our current understanding of sperm energetics and the requirements necessary for prolonged sperm viability. I did not find an association between the egg morphology in tardigrades and the relative sperm size in tardigrades based on the collected data. However, a larger sample size and a possible change in how the egg shell pattern predictor variable is constructed are needed in order to draw a definitive conclusion.

A lack of well-researched information on sperm morphology and sexual behavior of tardigrades poses an obstacle to understanding how sexual and natural selection are reflected in the design of the reproductive cells these charismatic micrometazoans. From a broader perspective, understanding possible sperm competition patterns and the exact mating processes across the phylum gives us a better overall view of gamete evolution and tardigrade reproductive biology in general.

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Appendix 1. Tardigrade sperm confocal microscopy protocol (Formaldehyde fixation):

A) Poly-lysine slides preparation:
   1) Rinse the slides with ethanol and let dry
   2) Draw a small circle in the center of the bottom of the slide (prevents washing away the ink)
   3) Place one drop of **0.1% poly-lysine solution** on the top of the slide on the drawn circle and leave for 5 minutes
   4) Remove the drop of poly-lysine and let air dry

B) Sperm staining:
   1) Place 5 µL of **dissection solution** on the top of the slide within the circle
   2) Place the animal in the drop of the **dissection solution** with a loop and open the gonad to release the sperm
   3) Leave in the humid chamber for 10 minutes
   4) Remove the solution
   5) Add µL of **fixing solution** and leave in the humid chamber for 5 minutes
   6) Add 25 µL of **Phalloidin staining solution,** place a small coverslip and keep in the humid chamber in a dark place for 1 hour at room temperature
   7) Remove the coverslip and rinse with distilled water
   8) Put 10 µL of **Mounting medium** and seal with nail polish
   9) Wait at least half an hour before imaging

Dissection solution:
0.1 X PBS

Fixing solution:
1% formaldehyde in 0.1 X PBS

Phalloidin staining solution:
1:20 phalloidin – TRITC stock (10 µM) in staining buffer (0.1 X PBD + 0.25% Triton – X100)

Mounting medium:
96% fluoromount + 4% Hoechst Working solution

Hoechst Working solution:

Hoechst stock 1:10 in Fluoromount

Phalloidin – TRITC stock:

Dissolve the 0.1 mg of Phalloidin – TRITC (P1951 Sigma) in a vial with 766 µL of DMSO