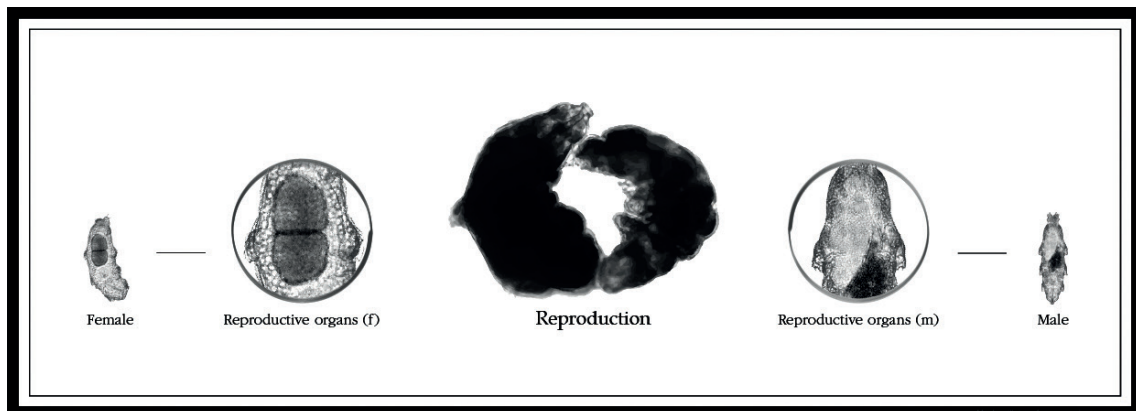


JYU DISSERTATIONS 797

Justine Chartrain

Exploring Tardigrade Reproduction

Multifaceted Insights into Sexual Selection and Evolutionary Strategies



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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Resilience is accepting your new reality, even if it's less good than the one you had before. You can fight it, you can do nothing but scream about what you've lost, or you can accept that and try to put together something that's good. – Elisabeth Edwards

*In memory of my mother,
I hope this thesis is something worthwhile despite the challenges.*

ABSTRACT

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The concept of mate choice involves complex behaviours and physiological adaptations that individuals use to select reproductive partners. In the past, research has mainly focused on mate choice in one sex, largely ignoring the involvement of the other. However, recent studies emphasize the importance of considering mate choice in both sexes to understand reproductive dynamics comprehensively. Environmental stressors play a crucial role in shaping mate choice strategies, influencing which traits are preferred by individuals and altering their reproductive behaviours. Understanding how ecological stress affects condition-dependent mate choice provides insights into the adaptive responses of organisms to changing ecological conditions. With their remarkable ability to withstand extreme environmental conditions, tardigrades offer a unique opportunity to investigate mate choice mechanisms under environmental stress. Despite their small size, tardigrades exhibit complex reproductive behaviours, including communication, courtship, and sperm storage. Moreover, due to the scarce knowledge of tardigrade behaviour and behavioural ecology, this study offers a chance to bridge crucial gaps in our understanding of these resilient organisms. My work aimed to investigate the process of mate selection throughout the reproductive process and in both sexes, providing a comprehensive understanding of the mechanisms involved in identifying and choosing a partner, engaging in sexual interactions, and investing in reproduction. Firstly, I showed that tardigrades can distinguish between sexes, determine their physiological state through chemical cues, and use those cues for mate choice. Then, I documented a consistent pattern of reproductive behaviour within a family and suggest that close-range contact pheromones could trigger courtship behaviour. Finally, I showed that although sperm storage organ presence in tardigrades does not result in greater female reproductive fitness, it is still beneficial. Overall, my thesis contributes to a better understanding of tardigrade evolutionary ecology, especially the fascinating world of mate choice and reproductive behaviour in these resilient organisms.

Keywords: Anhydrobiosis; chemical cues; mate choice; reproductive behaviour; sperm storage.

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TIIVISTELMÄ

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Diss.

Aiempi tutkimus on usein painottunut vain yhden sukupuolen parinvalintaan liittyvien mekanismien selvittämiseen jättäen toisen sukupuolen vähemmälle huomiolle. Viimeaikaisissa tutkimuksissa on kuitenkin todettu, että lisääntymisen dynamiikan kokonaisvaltainen ymmärtäminen vaatii molempien sukupuolien parinvalintaan liittyvien mekanismien huomioimista. Ympäristön stressitekijät muovaavat lisääntymiskäyttäytymisen ja parinvalinnan strategioita, esimerkiksi vaikuttaen yksilöiden suosimiin piirteisiin. Käsitys siitä, kuinka ekologinen stressi vaikuttaa parinvalintaan liittyviin mekanismeihin, auttaa ymmärtämään eliöiden adaptiivisia vasteita muutoksiin ekologisissa olosuhteissa. Karhukaistet kestävä t huomattavan hyvin äärimmäisiä muutoksia ympäristössään ja täten tarjoavat erityislaatuisen mahdollisuuden tutkia parinvalinnan mekanismeja suhteessa ympäristöstressiin. Pienestä koostaan huolimatta karhukaisten lisääntyminen on monimutkaista sisältäen viestintää, soidinmenoja ja siittiöiden säilömistä. Tämä tutkimus tarjoaa uusia työkaluja karhukaisten heikosti tunnetun käyttäytymisen ja käyttäytymisekologian ymmärtämiseen. Väitöstutkimukseni tavoitteena oli tutkia karhukaisten parinvalintaa läpi lisääntymisprosessin molempien sukupuolien näkökulmasta. Tutkimukseni antaa kattavan kuvan mekanismeista, jotka liittyvät parittelukumppanin löytämiseen ja valintaan, seksuaaliseen kanssakäymiseen sekä lisääntymispanostukseen. Osoitan, että karhukaistet käyttävät kemiallisia signaaleja erottaakseen sukupuolet toisistaan ja tekevät niiden avulla päätelmiä yksilön fysiologisesta tilasta, ja hyödyntävät näitä signaaleja parinvalinnassa. Tämän lisäksi kuvaan lisääntymiskäyttäytymiseen liittyviä malleja, jotka toistuvat yhdenmukaisina kaikilla tutkituilla lajeilla sekä esitän, että lähikontaktissa välittyvät feromonit toimivat karhukaisten soidinkäyttäytymisen laukaisevana tekijänä. Lopuksi osoitan, että karhukaistetilla esiintyvä siittiösäiliö on hyödyllinen, vaikka se ei paranna naaraiden lisääntymiskelpoisuutta. Tutkimukseni lisää ymmärrystä karhukaisten evolutioekologiasta, erityisesti niiden kiehtovasta parinvalinnasta ja lisääntymiskäyttäytymisestä.

Avainsanat: Anhydrobioosi; kemiallinen viestintä; lisääntymiskäyttäytymisen; parinvalinta; siittiöiden säilöminen.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-III.

- I Chartrain, J., Knott, K.E., Michalczyk, Ł., Calhim, S., 2023. First evidence of sex-specific responses to chemical cues in tardigrade mate searching behaviour. *J. Exp. Biol.* 226 (18): jeb245836.
- II Chartrain, J., Knott, K. E., Michalczyk, Ł., Vaherto V. & Calhim, S. 2024. The cost of being tough: Effect of anhydrobiosis on mate choice and reproduction in a macrobiotid tardigrade. Manuscript.
- III Chartrain, J., Knott, K. E., Michalczyk, Ł. & Calhim, S. 2024. Sperm storage ability is associated with oviposition but not mating behaviour: differences in macrobiotid tardigrades. Manuscript.

Table of author contributions to the original publications.

	I	II	III
Original idea	JC, SC	JC	JC, SC
Study design	JC, ŁM, SC	JC, SC	JC, SC
Data collection	JC	JC, VV	JC
Data analysis	JC	JC	JC
Writing	JC, KEK, ŁM, SC	JC, KEK, ŁM, SC	JC, KEK, ŁM, SC

JC = Justine Chartrain, SC = Sara Calhim, ŁM = Łukasz Michalczyk, KEK = K. Emily Knott, VV = Vilma Vaherto.

1 INTRODUCTION

1.1 Mate choice throughout the reproductive process

Charles Darwin initially introduced the concept of sexual selection in his seminal work, "The Descent of Man, and Selection in Relation to Sex" (Darwin 1871), presenting it as a theoretical framework to explain the evolutionary emergence of traits that do not necessarily contribute to enhanced longevity or fecundity. These traits, which may not be inherently favoured under natural selection, are subject to sexual selection operating through two primary modes: intrasexual competition, where individuals, typically males, compete for mates, and intersexual selection, where individuals, typically females, choose mates based on specific desirable traits (mate choice) (e.g. Trivers and Willard 1973, Wade 1979, Andersson 1994, Kokko *et al.* 2006, reviewed in Shuker and Kvarnemo 2021).

Despite initial controversies (Wallace 1871, Fisher 1915, Wilkinson 1993), sexual selection has been broadly defined as "any selection arising from fitness differences associated with non-random success in the competition for gamete fertilisation" (Shuker and Kvarnemo 2021). Mate choice, a crucial evolutionary process contributing to the selection of various traits, extends beyond physical characteristics to include behaviours and reproductive strategies (Andersson and Iwasa 1996). The terminology used to describe mate choice has also been examined (Edward 2015), and recent re-evaluation has defined it as an organism's inclination to engage in sexual activity with certain individuals over others based on any aspect of their phenotype (Rosenthal 2017).

Various hypotheses have been proposed to elucidate the evolution of mate choice, including direct phenotypic effects, sensory bias, Fisherian sexy sons, good genes, and handicap mechanisms (Zahavi 1975, Lande 1981, Ryan 1990, Andersson and Iwasa 1996, Møller and Jennions 2001). However, debates persist, particularly in situations where males solely contribute sperm to females and genes to offspring (Ingleby *et al.* 2010, Prum 2010, Rosenthal 2017).

Mate choice is a sequential process, encompassing distinct stages: pre-mating, peri-mating, and post-mating (Fig. 1). This progression mirrors the complexity of the reproductive process, where various behaviours and decisions come into play before, during, and after mating. These stages of mate choice intertwine with fitness components, which are broadly categorised into pre-copulatory mating success or post-copulatory fertilisation success. While mate choice involves multiple stages, fitness components are streamlined into two categories to simplify the analysis of sexual selection mechanisms, focusing on the timing of selection pressures. Thus, understanding the connection between the sequential stages of mate choice and the categorised fitness components offer valuable insights into the dynamics of sexual selection. Detecting a potential mate is a crucial initial phase in the mate selection process (Rosenthal 2017). Pre-mating choice involves detecting and evaluating courter signals, with both sexes participating in the process. For instance, many animal species employ pheromones to convey information about their sex and receptivity (reviewed in (Johansson and Jones 2007, Wyatt 2014)). Female choice based on male displays and/or courtship behaviours can lead to the evolution of complex mating rituals and elaborate courtship displays.

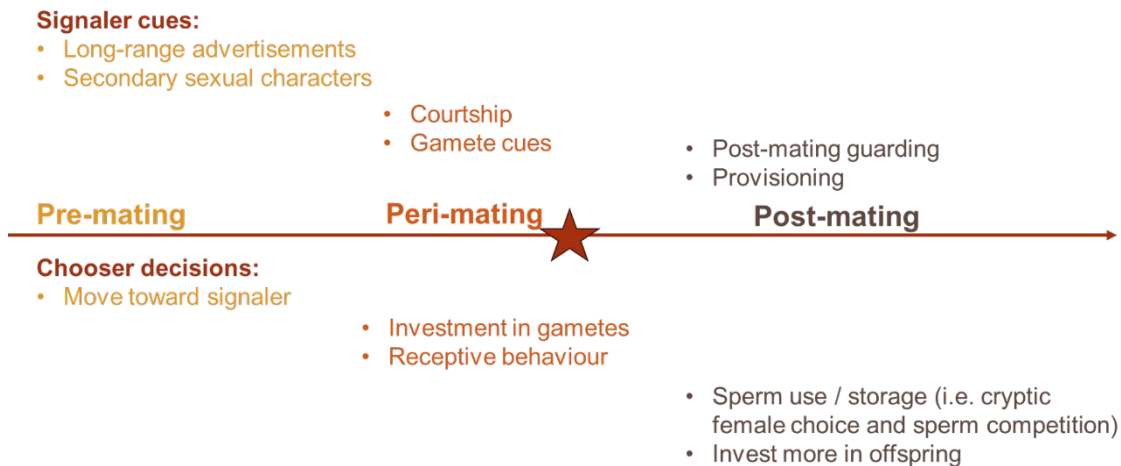


FIGURE 1 Stages of mate choice, revisited and adapted from Rosenthal (2017). At each stage, distinct signaller cues and chooser decisions are observed, irrespective of the sex assigned to the signaller or chooser roles. Star denotes the pivotal moment of fertilisation.

The peri-mating choice is the time during the act of mating or fertilisation during which physical interactions occur (Mendelson and Safran 2021). It includes various opportunities for expressing choice, such as allowing, refusing, prolonging, or terminating copulation. Post-mating involves interactions after mating, encompassing sperm competition, which is the competition between ejaculates from different males for fertilisation (Parker 1970) and cryptic female choice (Thornhill 1983), where specific aspects of female physiology or behaviour bias paternity toward the sperm of a particular male. These two topics have

received a lot of attention over the years (Birkhead and Møller 1993a, Birkhead 1995, Wigby and Chapman 2004, Orr and Zuk 2012, Eberhard 2015, Orr and Brennan 2015, Firman *et al.* 2017, Parker 2020). For instance, females of various species exhibit specialised structures for the storage of sperm, generally known as sperm storage organs (SSO) (Orr and Brennan 2015, Shankar *et al.* 2022), such as spermathecae (Pascini and Martins 2017) or sperm storage tubules (Birkhead and Møller 1992), allowing them to delay fertilisation (Davey 1965,, Siva-Jothy and Hooper 1996, Neubaum and Wolfner 1998, Gobin *et al.* 2006, Orr and Zuk 2014) and choose males even after the male's death (e.g. López-Sepulcre *et al.* 2013). The propensity for pre- and post-copulatory mate selection is largely inclined towards the sex with dominion over fertilization and resource allocation (Rosenthal 2017)

The idea that males are signallers and females are choosers was reinforced by Bateman's (1948) seminal experiments with *Drosophila melanogaster*, which emphasised the predominant focus in experimental research on investigating male signals and female preferences (Andersson 1994). His experiments suggested that females could secure adequate sperm from a single mating to maximise fecundity, consequently leading to an apparent excess of males within the population (Emlen and Oring 1977). Fifty years later, Arnold and Duvall (1994) solidified this concept by utilising selection theory from quantitative genetics to establish a metric for the intensity of sexual selection. They introduced the Bateman gradient, also known as the sexual selection gradient (β_{ss}), to quantify the selective advantages stemming from intra-sexual competition for mates. A positive Bateman gradient is anticipated to stimulate mate competition, shape mating systems, and encourage the evolution of traits that enhance mating success, such as ornaments and armaments (Fromonteil *et al.* 2023).

Traditionally, it has been assumed that females invest more in reproduction; however, modern research reveals a more nuanced understanding. Across the animal kingdom, females often engage in multiple matings, and in numerous cases, having multiple male partners may enhance female reproductive success (Jennions and Petrie 2000, Taylor *et al.* 2014, Fromonteil *et al.* 2023). For instance, in certain species, such as sex-role reversed dance flies, females have abdominal sacs which inflate their body size while lekking. This makes them more attractive to potential mates (Svensson and Petersson 1988). In snapping shrimps, males and females show minor sexual dimorphisms, and females compete for access to mates using their snapping chaelae (Chak *et al.* 2015). In the Malaysian stalk-eyed fly, both sexes have laterally displaced eyes. The ornamentation is more developed in males than in females. Males lek, but they are sperm-limited and prefer to mate with more fecund females. They use eye span and other signals when choosing mates (Cotton *et al.* 2015). These few examples, show that female competition and male mate choice co-exist with male competition and female mate choice.

Recent research advocates for examination of the mechanisms of sexual selection operating in both males and females (Shuker 2010, Stockley and Bro-Jørgensen 2011, Hare and Simmons 2019, Ah-King 2022). Understanding the subtle effects of sexual selection on females, particularly in invertebrates, can

make sexual selection theory more straightforward, applicable, and more powerful (Hare and Simmons 2019). Measuring mate choice in both sexes is essential to understanding the dynamics of sexual selection, providing insights into reproductive strategies' evolutionary pressures. This approach offers a balanced perspective on mating dynamics, revealing variations in competition, preferences, and reproductive investment. Understanding mate choice in both sexes and throughout the diverse stages of the reproductive sequence is crucial for comprehending sexual selection, population dynamics, and evolutionary processes across diverse species, effectively guiding conservation and management efforts.

1.2 Signal detection

Before expressing a preference for any aspect of a potential partner's phenotype, a mate must initially detect it through the sensory process known as sensation (Lenschow *et al.* 2022). Mate choice fundamentally entails the capacity to sense, perceive, and assess signals from courtiers before arriving at a behavioural and/or physiological decision. Cues are traits assessed during mate choice and influence mating decisions (Miller and Todd 1998, Candolin 2003). Often, these traits have been modified for communication purposes, serving functions exclusively related to enhancing attractiveness to the opposite sex, even if they may be detrimental to survival, such as colourful ornaments or courtship behaviour (Ryan *et al.* 2007). Those traits can serve as honest signals of genetic or physiological conditions, indicating overall health, vigour, or genetic quality (Zahavi 1975, Penn and Számadó 2020). Choosing mates based on these signals can provide fitness benefits, as individuals gain access to superior genes or resources for their offspring. For example, in moths, males preferentially approach females with a higher reproductive potential signalled by the ratio of pheromone components (Gonzalez-Karlsson *et al.* 2021).

Mate choice unfolds through three primary sensory modalities: vision, audition, and chemoreception. Signal detection involves the nuanced evaluation of cues, manifesting as either a single cue or multiple cues assessed collectively as a cohesive signal. Females and sometimes males, rely on assessing multiple cues in their decision-making processes (Candolin 2003, Uetz *et al.* 2009). For instance, among bird species, males frequently exhibit both vibrant ornamentation and engage in intricate song performances (Candolin 2003). In peacock spiders, males employ a combination of visual and vibratory signals during courtship, with both modalities influencing mating success (Girard *et al.* 2015).

Empirical models of mate choice consistently emphasise the role of vision. Colours, in particular, play a widespread and crucial role in mate choice across diverse taxa with well-developed sight, with fish and birds being the most studied (Hill 1991, Kodric-Brown and Nicoletto 2001, Amundsen and Forsgren 2001, Endler *et al.* 2005). For instance, female guppies discriminate against males

with less-than-average orange colour (Houde 1987). Moreover, audition has been extensively studied, though predominantly limited to arthropods and jawed vertebrates. Acoustic studies of passerine birds (Searcy 1992, Byers and Kroodsma 2009, Riebel 2009), anuran amphibians (Grafe 1997, Ron 2008), and orthopteran insects (Wagner and Reiser 2000, Tregenza *et al.* 2006, Ponce-Wainer and Cueva del Castillo 2008) contribute significantly to our understanding of mate choice, where lower-frequency and higher-amplitude calls are commonly preferred (reviewed in Ryan and Keddy-Hector 1992, Ritschard *et al.* 2010).

Chemoreception, one of the oldest and most prevalent sensory modalities, encompasses smell and taste (gustation). Although the idea of chemoreception's role in sexual selection was initially proposed by Darwin in 1871, it was only in the late 1950s when the first pheromone, the sex pheromone of the silk moth *Bombyx mori*, was identified (Butenandt 1959). The most essential function of sex pheromones is to allow organisms to identify mating partners of the opposite sex (e.g. Harari and Steinitz 2013). Chemical signals advertising sex are commonly produced in a sex-specific manner, whether male-specific, female-specific or a combination of the two. Since the identification of bombykol, many of the best-characterised insect sex-pheromones are female-specific, long-range male attractants (e.g. Snell *et al.* 1995, Ando *et al.* 2004, Gaskett 2007, Lebreton *et al.* 2017). In contrast to other sensory modalities, chemoreception is likely instrumental in all stages of mate choice across various taxa, ranging from long-distance attraction to signallers (e.g. Borges *et al.* 1987, Li *et al.* 2002, Lebreton *et al.* 2017) to close-range evaluation, as seen in the use of cuticular hydrocarbons by *Drosophila* (Ferveur 2005), to peri-copulatory arousal of males by female pheromones in mice (e.g. Amstislavskaya and Popova 2004). Moreover, chemoreception is utilised in post mating discrimination among sperm (e.g. Thomas and Simmons 2007, Lane *et al.* 2015, Silva *et al.* 2021) or offspring from multiple sires (e.g. Sanchez-Andrade and Kendrick 2009, Amo *et al.* 2014). For example, female crickets discriminate among male spermatophores based on their amino acid composition (Gershman *et al.* 2012).

Our comprehension of how sexual selection and mate choice shapes chemical traits has advanced since the identification of the first pheromone (reviewed in Wyatt 2014, review in Steiger and Stökl 2014): For example, female insects can choose a mate based on the composition of cuticular hydrocarbons (e.g. Thomas and Simmons 2009, Lane *et al.* 2016). However, the exploration of mate choice concerning female chemical signals remains comparatively underexplored (Harari and Steinitz 2013).

1.3 Ecological influences and condition signalling mechanisms

The expression of assessed traits and mate choice of individuals are intricately linked to environmental conditions. The environmental dependence of costs and benefits associated with mate choice traits implies that environmental variations

can significantly impact the adaptive value of traits used to attract and select mates (e.g. Rosenthal 2017, Dougherty 2023, Dougherty *et al.* 2024).

Seasonal and daily variations in temperature, precipitation, humidity, and light levels constitute abiotic changes that often coincide with alterations in the biotic environment. Environmental change can influence the traits of individuals involved in courtship by altering external surroundings or intrinsic properties. For instance, wolf spiders adjust their communication strategies based on visibility, relying more on seismic signals in poor visibility conditions (Wilgers and Hebets 2011). Changes in intrinsic properties, such as body condition or physiological processes, can also influence individual mate attraction investments (e.g. Cotton *et al.* 2006, Hill 2015). For example, exposure to endocrine-disrupting chemicals in guppy males reduces courtship behaviour towards females (Bertram *et al.* 2015). Despite extensive knowledge on the effect of environmental changes on phenotypes, a limited understanding remains of how short-term environmental changes, occurring daily to monthly, influence reproductive behaviours like mate choice (e.g. Cronin *et al.* 2019). Recent empirical and theoretical studies, however, suggest that environmental variability is a crucial factor influencing mate choice, with examples such as alterations based on seasonality (e.g. Qvarnström *et al.* 2000, Botero and Rubenstein 2012), search costs (e.g. Milinski and Bakker 1997, Akre and Ryan 2010), hormone levels (e.g. Lynch *et al.* 2005, Husak and Moore 2008), and social context (e.g. Galef and White 2000, Bailey and Zuk 2009, Lea and Ryan 2015). Global environmental changes, driven by human activities, further compound the ecological impact on animal mate choice (e.g. Candolin 2019).

The strength of mate choice, defined as the extent to which individuals exhibit preferences for certain mating options over others (e.g. Reinhold and Schielzeth 2015), exhibits considerable variability across species, among individuals within a species, and even within an individual's own reproductive history (e.g. Jennions and Petrie 1997, Rosenthal 2017). This diversity in mate choice strength can be attributed to the inherent costs associated with the mate selection process, involving both time and energy expenditure during mate sampling (e.g. Sullivan 1994, Vitousek *et al.* 2007). Additionally, the act of making a choice necessitates rejecting potentially acceptable partners, posing a potential reduction in fecundity (e.g. Kokko and Mappes 2005, Greenway *et al.* 2015). The environmental context in which an individual engages in mate sampling plays a crucial role in shaping the costs and benefits of mate choice (e.g. Jennions and Petrie 1997, Dougherty 2021). Another pivotal factor influencing the trade-offs associated with being selective is an individual's phenotype, often called their "individual state" (e.g. Cotton *et al.* 2006, Dougherty 2023). This individual state encompasses various physical attributes such as body size, condition, and life-history factors, including age and mating history. Individuals in poor conditions can become less selective in their mate choice, accepting lower-quality mates to ensure reproductive success whereas those in good condition have more resources to invest and can afford to be more selective in their mating partner (Dougherty 2023). For instance, female zebra finches with clipped wings, representing a poor condition, spent less time associating with attractive males

than females with intact wings (Burley and Foster 2006). Additionally, the individual state can influence the perception and assessment of potential mates, leading to changes in preferences or standards depending on current needs or circumstances. For example, in brush-legged wolf spiders, food-limited females exhibit delayed development and smaller size, with varying receptivity and aggression towards males based on their body size and tuft characteristics. Short-term starvation during adulthood reduced female mass and receptivity, while long-term starvation altered female preferences, suggesting that hunger can influence mate choice and potentially strengthen selection for exaggerated male traits (Moskalik and Uetz 2011).

The condition of both the signaller and the chooser holds significance in this context. However, questions arise regarding species with limited sexual dimorphism, warranting further exploration of this phenomenon.

1.4 Entering the microscopic world: a brief introduction to tardigrades

Tardigrades, also known as water bears, are microscopic, water-dwelling organisms, sharing phylogenetic proximity with nematodes and arthropods (Fig. 2, Jørgensen et al. 2018). Initially identified in 1773 by the German zoologist Johann August Ephraim Goeze, tardigrades exhibit a diverse array, with more than 1500 formally described species (Degma and Guidetti 2023), which are divided into two classes: Heterotardigrada and Eutardigrada.

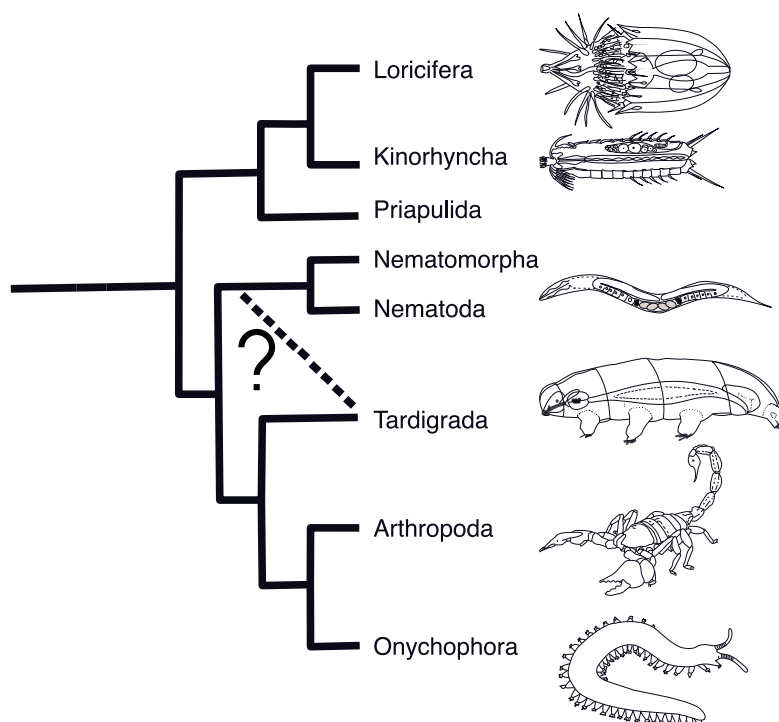


FIGURE 2 Ecdysozoan relationships adapted from Jørgensen *et al.* (2018).

Despite their microscopic size, ranging from 0.1 to 1.2 millimetres, tardigrades exhibit sophisticated behaviours and possess a well-developed anatomy featuring a simple brain and digestive system. Asexual (parthenogenetic) reproduction is extensively observed alongside sexual reproduction in this phylum, while hermaphroditism has been reported only in a few taxa (Rebecchi 2000). Their transparent bodies facilitate easy differentiation of sexes, with gonochoristic species displaying limited sexual dimorphism, most commonly in the form of larger females (Møbjerg *et al.* 2018). Although equipped with eyes, tardigrades lack visual acuity, only perceiving changes in luminosity (Møbjerg *et al.* 2018). They exhibit a relatively short generation time, with some species reaching maturity within two weeks after hatching (Altiero *et al.* 2018, Guidetti *et al.* 2020). The moulting process, wherein tardigrades shed their exoskeletons, enables cuticle regeneration and adaptation to environmental changes.

Tardigrades inhabit diverse environments, including mosses, lichens, leaf litter, soil, ponds, lakes and marine habitats (Nelson *et al.* 2018). Renowned for their remarkable resilience, many tardigrade species can endure extreme environmental conditions. In response to sudden adverse dry conditions, they enter an anhydrobiotic state called a tun, marked by morphological adaptations such as body retraction and molecular adjustments, and promptly return to their active metabolic state upon removal of the stress factor (Møbjerg and Neves 2021), including the synthesis of protective molecules, such as trehalose and specific heat shock proteins (Arakawa 2022, Kasianchuk *et al.* 2023, p. 20). Tardigrades show remarkable resilience to environmental challenges, whether in their active or desiccated state. They regulate osmotic balance, avoid freezing through supercooling, and withstand high levels of ionizing radiation while active (Møbjerg and Neves, 2021). In a desiccated state, tardigrades from various species display extended survival when exposed to extreme conditions like immersion in nitrogen, and helium, enduring temperatures as high as 151 degrees Celsius, resisting carbon dioxide and hydrogen sulfide exposure, and withstanding high hydrostatic pressure (Rebecchi *et al.*, 2007). Moreover, desiccated tardigrades exhibit lower vulnerability to radiation compared to hydrated ones (e.g. Watanabe *et al.* 2007, reviewed in Jönsson 2019).

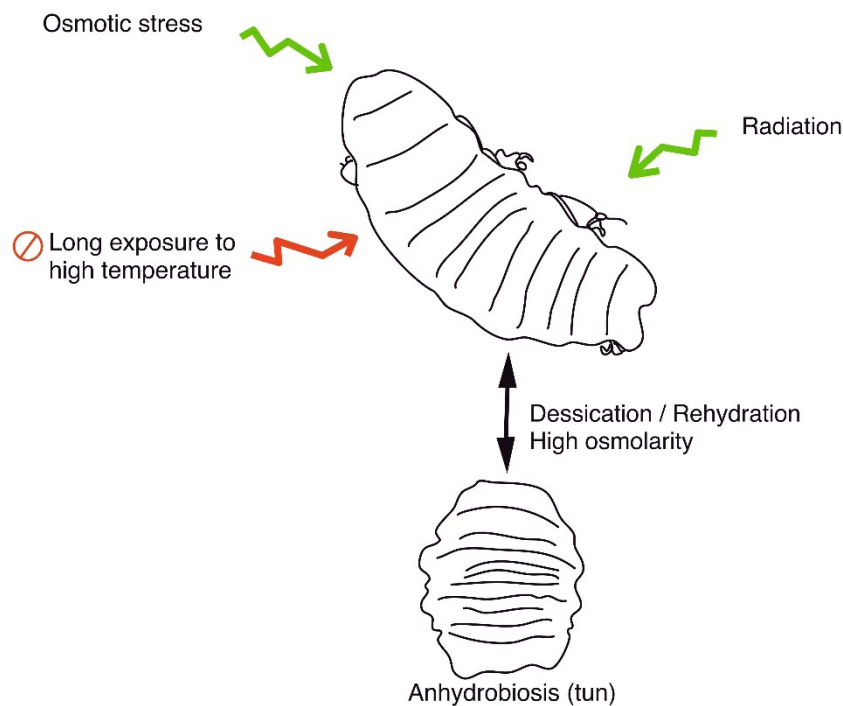


FIGURE 3 Schematic illustration of one survival strategy of tardigrades: anhydrobiosis, a sub-state of cryptobiosis, is characterised by the formation of a tun. Adapted from Møbjerg and Neves (2021). Active tardigrades demonstrate exceptional tolerance to environmental stressors. Anhydrobiotic organisms exhibit remarkable resistance to physical and chemical extremes, surpassing the tolerance thresholds of active organisms by far.

Despite extensive research on their anhydrobiosis capacity, our understanding of tardigrade behaviour, particularly in terms of communication, remains limited. Two studies have suggested the potential use of chemical cues in two distinct contexts. In a predator-prey context, individuals demonstrated the capability to discern the locations where previous prey or predators had spent time on agar (Meyer *et al.* 2020). In the context of partner finding, males exhibited the capacity to detect and find females at distances of up to 2 mm, equivalent to 2.5 times their own body length (Bartel and Hohberg 2020).

In tardigrades, females are iteroparous, laying eggs throughout their adult life, with distinct stages of ovary development and oocyte maturation (Rebecchi and Bertolani 1994). Although oviposition is often synchronised with moulting, eggs can be laid into the moulted exuvia or freely into the environment (Bertolani *et al.* 1996). The fertilisation process is poorly understood, with internal fertilisation suggested in some species based on the presence of (Rebecchi 2000, Sugiura *et al.* 2019) specialised types of sperm cells (Rebecchi 1997, 2000, Bertolani and Rebecchi 1999). Fertilisation is thought to occur externally in *Isohypsibius dastychi* because male ejaculation and female oviposition are simultaneous (Bingemer *et al.* 2016). In contrast, in *Pseudobiotus megalonyx*, *Ursulinus nodosus*, and Macrobiotidae internal fertilisation is presumed because ejaculation occurs

before oviposition, with spermatozoa entering the oviduct via the cloacal opening (Rebecchi 2000, Sugiura *et al.* 2019). Furthermore, for species with a sperm storage organ (SSO) or spermatheca that also lay eggs freely, internal fertilisation is expected because the latter is connected to the oviduct between the cloaca and ovary (Bertolani and Rebecchi 1999, Bertolani 2001). Moreover, observations of egg-laying in various species suggest that fertilisation starts only after the eggs are laid (Sugiura and Matsumoto 2020, 2021a).

Moreover, *Pseudobiotus megalonyx* males are semelparous, as they produce sperm continuously throughout their adult life, with the entire testis dedicated to sperm production (Rebecchi 2000) and die after copulation. This raises the intriguing question of whether females of this species engage in multiple mating events during their adult life or utilise the sperm from a singular male throughout their reproductive lifespan. Conversely, males of *Macrobotus*, *Ramazzottius*, and *Diphascion* adopt a different reproductive strategy. These males demonstrate continuously maturing male germ cells and spermatozoa, with all developmental stages present in the testis. This unique physiological characteristic suggests that these males are iteroparous (i.e. are capable of engaging in multiple copulatory events throughout their adult lives), distinguishing them from the semelparous strategy observed in *Pseudobiotus megalonyx* (Bertolani 2001). Although females can take up multiple ejaculates from the same mating interaction (Sugiura and Matsumoto 2021a), multiple mating by females has not been described yet and it is unknown whether females can bias the paternity within a single batch of eggs.

Despite detailed observations of mating behaviour in some species (reviewed in Sugiura and Matsumoto 2021b), comprehensive mate choice studies, reproductive success analyses, and investigations into the effects of sperm storage in tardigrades reproductive success are extremely limited. The duration of sperm storage remains unclear, although recent findings by Vecchi *et al.* (2022) showed that sperm can be stored up to five weeks in *Macrobotus polonicus*. Furthermore, unlike parthenogenetic species (Ingemar Jönsson *et al.* 2005, Horikawa 2012), investigations into potential correlations between stress resilience and reproductive behaviour or success have yet to be explored in gonochoristic taxa. Notably, it has been shown that the parthenogenetic *Acutuncus antarcticus* is capable of reproducing after recovering from prolonged periods of cryptobiosis (Tsujimoto *et al.* 2016). These considerable gaps in our knowledge highlight avenues for future research to enhance our understanding of the intricate biology and reproductive strategies of tardigrades.

1.5 Aim of the thesis

This thesis aims to comprehensively explore mate choice in tardigrades, across all stages and in both sexes, providing an integrated understanding of the intricate dynamics involved in partner detection, selection, sexual interactions, and reproductive investment. The central research objective is to investigate the trade-off between stress and reproduction, examining how environmental factors and physiological stressors may influence the decision-making processes related to mate choice. Importantly, this study seeks to address the existing imbalance in the literature by placing equal emphasis on both sexes, recognising the nuanced roles and contributions of both males and females in the mate selection process. By examining the complexities of mate choice from initial attraction to post-mating processes, this research contributes valuable insights into the evolutionary, ecological, and behavioural aspects of reproductive strategies in this unique and resilient animal phylum.

In **Study I**, I investigate the role of waterborne diffusing signals and deposited cue trails in intersexual communication. While prior research has demonstrated the capacity of tardigrades to utilise semiochemical cues in water to locate mates (Bartel and Hohberg 2020) and detect cues deposited on agar within a predator-prey interspecific context (Meyer *et al.* 2020), potential use of such cues for the discrimination between sexes remains unexplored. Hence, using a specially designed choice-chamber arena, I test the preferences of both males and females to associate with the opposite sex in an aquatic medium and whether males and females could detect and follow an opposite-sex cue trail deposited onto agar in a water film environment.

In **Study II**, I examine the ability of tardigrades to differentiate between individuals of the opposite sex recovering from anhydrobiosis and those in a continuous active state, specifically within the context of mate selection. Given the impact of anhydrobiosis on gametes, my hypothesis posits that both male and female tardigrades are more likely to prefer counterparts of the opposite sex who have more viable gametes (i.e. control individuals), thereby enhancing their reproductive success. I also test whether the condition of the focal individual impacted this choice. I use the same mate-choice chamber arena from Study I for this aim. I assess four treatment combinations involving focal and signaller individuals under varying recovery and control conditions for both sexes. Moreover, I evaluate the effect of recovery from anhydrobiosis on individual fitness by contrasting sperm-depleted and sperm-filled females and also by examining short-term in-testis sperm motility recovery.

Successful copulation with sexually receptive mates, driven by pre-copulatory sexual selection moulding elaborate phenotypic traits, is pivotal for achieving individual reproductive success. However, the ultimate determinant lies in egg fertilisation. Tardigrades exhibit variability in sperm storage organ (SSO) presence, even among closely related species within the same family or genus. This variation prompts investigation into mating behaviours to elucidate

employed reproductive strategies. Thus, **Study III** aims to compare reproductive behaviour and success in macrobiotid species with (n=3) or without (n=2) a SSO. By quantitatively assessing mating behaviour, ovipositing behaviour, and reproductive success, I aim to understand the impact of sperm storage on tardigrade reproductive strategies. I predict that species with a SSO will exhibit more frequent and prolonged mating behaviour, increased oviposition rates, and enhanced reproductive success. This prediction stems from the advantageous ability of both sexes to engage in repeated and extended mating interactions, optimizing the transfer and storage of sperm within the SSO. Moreover, the presence of an SSO enables females to oviposit multiple times, as they can utilize stored sperm for fertilizing eggs long after the mating. This study highlights the importance of sperm storage organs in shaping tardigrade reproductive outcomes.

2 METHODS

2.1 Study species and laboratory cultures

I used strains of laboratory cultures of five moss-living eutardigrades: *Macrobiotus dolosus* Bertolani *et al.*, 2022 (formerly misidentified as *M. polonicus* Pilato *et al.* 2003), *Macrobiotus annewintersae* Vecchi and Stec 2021, *Macrobiotus ripperi* Stec *et al.*, 2021, *Macrobiotus shonaicus* Stec *et al.*, 2018, and *Paramacrobiotus richtersi* (Murray, 1911). These species are closely related (Fig. 4) and are part of the same family - Macrobiotidae. Females of all five species lay eggs freely into the environment, and the first three have an sperm storage organ, SSO (Vecchi *et al.* 2022). Mature males in all studied species have motile sperm inside the testis, that are produced continuously (e.g. Rebecchi and Bertolani 1994). Due to the transparent cuticle, the sex and reproductive status of individuals could be discerned non-invasively using light stereomicroscopy.

Tardigrade populations were reared in the laboratory. They were kept in a climate chamber at a 14-16-18-16 °C in 6-hour shifts, 2:22 L:D cycle in 5 cm-diameter plastic Petri dishes with a scraped bottom (to aid motility) filled with mineral water and fed *ad libitum* with algae (*Chlorococcum hypnosporum* and *Chlorella* sp.; Sciento UK) and rotifers (*Lecane* sp.) or nematodes (*Panagrellus pycnus*). Half of the medium was changed weekly.

Two strains of *M. dolosus* (AT.002 in Stec *et al.* 2021 and S218) were employed in investigating sex-discrimination (I) due to an unexpected decline in the viability of strain AT.002, which was initially intended to be the exclusive strain under examination. To obtain virgin individuals, eggs from those strains were isolated and kept in groups until hatching. Hatchlings were kept individually in 3 cm diameter Petri dishes under the same conditions as that described for the main cultures. For the other two experiments (II, III), mature individuals were taken from their laboratory populations and isolated for at least two weeks, to ensure that sexually mature females do not store sperm anymore (Vecchi *et al.* 2022), in 3 cm diameter Petri dishes by sex, either individually (for females in II) or in groups (males in II, both sexes in III).

Isolated individuals were maintained under the same condition as the laboratory populations, with mature females being subjected to weekly monitoring of oviposition. If eggs were present, this was recorded, and the eggs were removed. Females that did not lay eggs during their isolation period were assumed to not have stored sperm in their sperm storage organ (II).

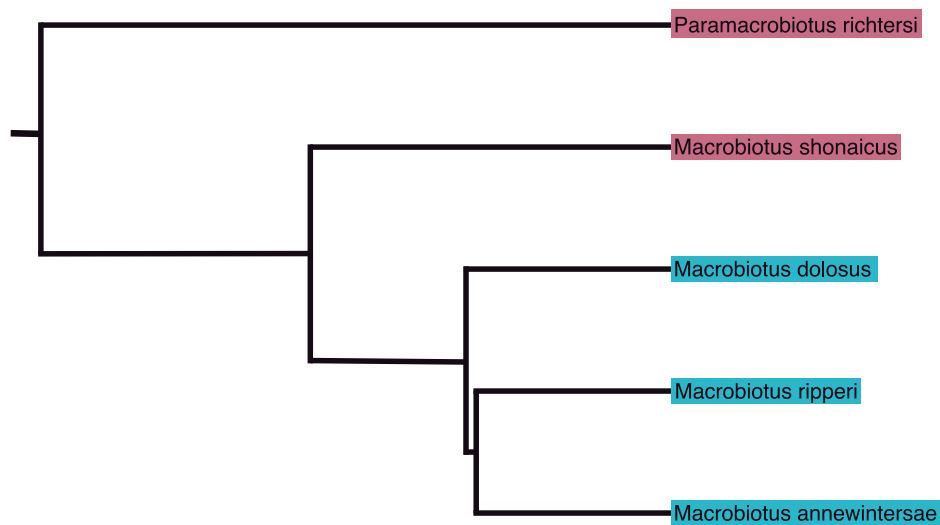


FIGURE 4 Phylogenetic relationship of the tardigrade species used in this thesis. In blue are species with a sperm storage organ (SSO) whereas red corresponds to species without an SSO. The phylogeny was constructed by concatenating 18S rRNA, 28S rRNA, ITS-2 and COI macrobiotid sequences (see Stec *et al.* 2021).

Below, I have outlined the main aspects of the methods I used to collect data on their behaviour and life history traits. For more detailed information, please refer to the respective chapters in the thesis.

2.2 Pre-mating choice (I, II)

2.2.1 Mate choice arena

I conducted experiments to investigate chemical signalling mechanisms of tardigrades in two contexts: ability to discern between sexes (I), and condition-dependent mate choice (II). These investigations were conducted using a specially designed choice chamber arena comprising a 4-well slide (Ibidi, Grafelfing, Germany) and two fishing lure rings (4 mm inner diameter). To ensure good signal detectability, the distance between the centre of each lure ring and the centre of the arena was 4 mm, based on a previous study on the distance threshold of another eutardigrade (50% detection at 2 mm, Bartel and Hohberg 2020). The arena surface was coated with 250 μ l of 1.5% agar (BD BACTOTM Agar), and the lure rings were placed according to a predetermined scheme (Fig. 5). Consequently, the critical area, where focal individuals supposedly made their choice, was defined within a 2 mm distance from the centre of the arena. Following the solidification of the agar, 1 ml of mineral water was introduced to flood the arena, permitting signallers to move freely within the lure rings. The lure rings were submerged in the water column, allowing waterborne signals to diffuse passively.

Signallers were provided with a habituation period lasting 15 to 30 minutes inside the lure rings before introducing the focal individuals into the centre of the arena. For both experiments, focal individuals of both sexes were used. Subsequently, observations were carried out for 30 minutes under a stereomicroscope equipped with diffused bottom light, linked to a camera.

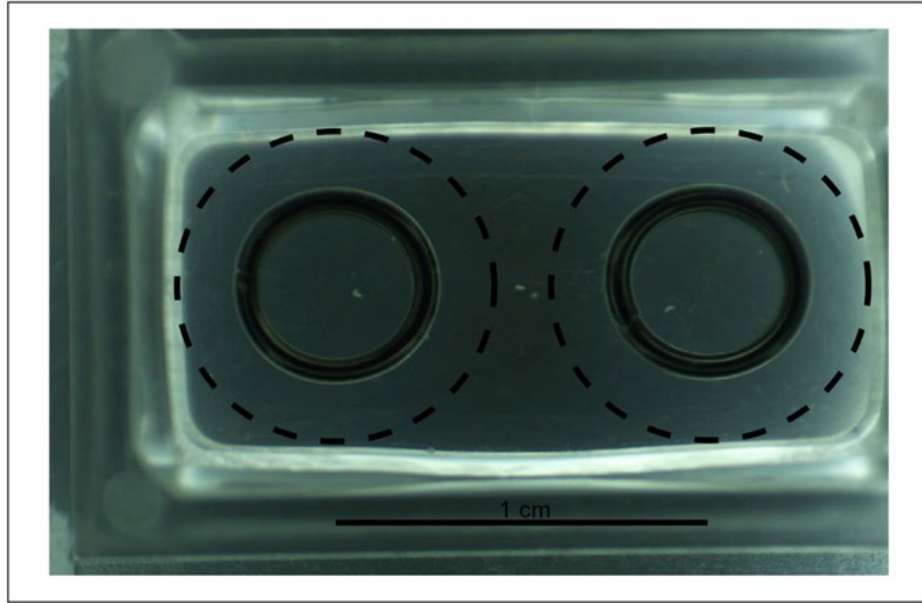


FIGURE 5 The mate choice arena used in I and II. The black dashed circles represent the critical areas around each signaller.

2.2.2 Sex-discrimination (I)

Virgin individuals from two *M. dolosus* strains were utilised, and trials were conducted within the same strain. Within the choice chambers (i.e. lure rings), individuals of both sexes were introduced. To evaluate the discriminatory abilities of each sex, we analysed their first choice, latency to cross a critical area, and duration spent within the critical area. Furthermore, we investigated tardigrade capability to discern and follow an opposite-sex deposited trail cue using the same 4-well slide, albeit without the inclusion of fishing lure rings nor the submersion of the arena with water.

2.2.3 Condition-dependent mate choice

To obtain individuals that varied in condition, we used tardigrades recuperating from an induced one-week-long anhydrobiotic period, alongside control counterparts. Individuals in an anhydrobiotic state were rehydrated for a minimum of 4 hours prior to the beginning of mate choice trials. This procedure restored their normal somatic activity while largely precluding full gametic recovery in males, specifically, sperm was immobile inside the testis. Given the inability to assess female recovery in a comparable manner, an equivalent post-rehydration recovery period was employed before initiating the trials. Signallers were exclusively of the opposite sex to the focal individuals. I subsequently analysed mate choice preference as the latency to cross a critical area and the proportion of time spent within a critical area.

2.3 Peri-mating choice: mating observation (III)

To determine whether there are differences in the reproductive behaviour of species with and without a sperm storage organ, I recorded the mating behaviours of the five species mentioned above. I placed pairs of mature individuals inside a well of an Ibidi μ slide angiogenesis, coated with 1.5% agar and flooded with water. I placed this slide under a Zeiss Cell Observer HS (2009/2009) microscope and then recorded their behaviour for a maximum of 45 minutes. I analysed the videos with the software BORIS (Friard and Gamba 2016) and categorised five behaviours (Table 1).

TABLE 1 Behaviours quantified (latency, number and/or duration) during mating interactions. Videos are available [here](#).

Contact	Contact is initiated by either individual with any part of the body of the other. Can be mutual.
Follow	Movement matching of one individual when positioned behind the other individual (with or without physical contact).
Touch	Males touch female ventral and/or posterior (cloacal) region.
Mount	In a curl position, males align their cloaca with the female cloaca and may release sperm.
Ejaculation	Observed release of sperm.

2.4 Post-mating choice: consequences of mating for males (II) and females (II, III)

To investigate the potential reproductive costs incurred during the anhydrobiosis recovery period, I provided mating opportunities to individuals. They were paired for 24 hours in one of the four possible combinations of condition: both recovering, both control, only the female is recovering, or only the male is recovering. Following the 24-hour period, sperm motility in males was assessed, while females were monitored weekly for oviposition and subsequent hatching over a span of up to two months (II).

Additionally, to ascertain whether species possessing an SSO exhibit greater reproductive success compared to those lacking such an organ, I conducted weekly monitoring of female laying and hatching over a two-month period after the recording of their mating behaviour (III).

3 RESULTS AND DISCUSSION

3.1 Pre-mating choice (I, II)

The initiation of mate choice and sexual selection entails the attraction or identification of individuals of the opposite sex. In my study, males displayed a pronounced attraction to female signallers in an aquatic setting (I, Chartrain *et al.* 2023). This finding was unsurprising as a number of well-studied insect sex-pheromones, such as bombykol (Butenandt 1959), are produced by females and serve as long-distance attractants for males. For example, in nematodes, only females produce ascr#1 which repels females but attract males (Choe *et al.* 2012).

Despite the extensive research on how sexual selection affects male responses to chemical cues (e.g. Snell *et al.* 1995, Simon and Sternberg 2002, Ando *et al.* 2004, Kiørboe *et al.* 2005, Gomez-Diaz and Benton 2013, Barrios 2014), our understanding of how sexual selection influences female responses to male olfactory cues is still limited (e.g. Bonduriansky 2001, Karlsson Green and Madjidian 2011, Hare and Simmons 2019). This knowledge gap may be due to the focus on species with sexually dimorphic sensory apparatus (e.g. Gaskett 2007, Naka 2018, Ehlers and Schulz 2023), which is not the case in tardigrades (e.g. Møbjerg *et al.* 2018). My findings shed light on this issue and show that in tardigrades, females exhibited brief yet comparable associations with individuals of either sex. It is important to note that in taxa without apparent sensory sexual dimorphism, both attraction (e.g. Passos *et al.* 2013) and insensitivity to male chemical signals were observed (e.g. Ratterman *et al.* 2009).

Moreover, my findings did not provide evidence supporting the ability of tardigrades to detect sex-specific (intraspecific) cues deposited on the surface of agar, in contrast with a previous study on interspecific cues (Meyer *et al.* 2020). This reinforces prior research indicating that diffusing waterborne chemical cues is crucial in tardigrade intraspecific communication within a reproductive context (Bingemer *et al.* 2016, Bartel and Hohberg 2020). These findings suggest that tardigrades can differentiate between sexes, with males exhibiting a preference for association with females which is in line with male-biased mate-

searching found in tardigrades previously by Bartel and Hohberg (2020) and Sugiura and Matsumoto (2021b).

When it comes to finding a mate, both males and females can choose based on their own physical condition and the condition of the potential partner (reviewed in Dougherty 2023). It has been recently highlighted that sex pheromones can vary within a population (reviewed in De Pasqual *et al.* 2021) and signal condition along with sex. My study (II) has shown that both control males and males in recovery from anhydrobiosis prefer control females (Fig. 6), indicating that the ability to discern individual physical state can result in greater reproductive success. This is also true among moths where a recent study (Gonzalez-Karlsson *et al.* 2021) underscored the significance of chemical cues as honest signals. They found that female moth sex pheromones reflect female condition and influence male mate choice. Interestingly, in my experiment, control females and those in recovery who did not lay eggs prior the experiment (assumed to have a depleted sperm storage organ) similarly preferred control males (Fig. 6). Conversely, females in recovery who had laid eggs before the experiment preferred males in recovery (Fig. 6). This suggests that physiological state can be discerned through chemical cues in tardigrades and used for mate choice.

These findings suggest a potential disruption of the detection and/or production of chemical cues due to anhydrobiosis. The latter may detrimentally impact individual condition, potentially diminishing the production and release of chemical cues or prompting strategic reallocation of resources towards chemical production. Body condition refers to the reservoir of resources available to an organism for allocation towards trait maintenance and production (e.g. Rowe *et al.* 1994, Hill 2011). Pheromone production likely utilises such energetic resources (Blomquist and Bagnères 2010), potentially relying directly on nutrition, including dietary amino acids, which serve as precursors for pheromones in mosquitoes (South and Arnqvist 2011) or act as sex pheromones themselves, as in fish (Stacey 2011). Alternatively, anhydrobiosis may induce a stress response through the release of certain physiological by-product (i.e. stress hormones, for instance glucocorticoids) in those individuals that makes them less attractive (Leary and Baugh 2020).

Despite the importance of condition-dependent chemical signalling, evidence linking female nutritional status and/or body condition to variations in chemical signalling remains limited, with only a handful of studies addressing this aspect. For instance, in moths, pheromone production diminishes in females under sugar-induced stress, enabling males to assess phenotypic variation among females (Foster and Johnson 2011). Similarly, starvation significantly reduces the release of body pheromones in the wasp spider (Weiss and Schneider 2022a). Costs associated with chemical signalling have been unveiled through trade-offs in resource allocation, where energy allocated to one function compromises investment in others. For instance, in spiders, pheromone quantity is the sole predictor of female attractiveness to males, and pheromone signals intensify with improved body condition (Weiss and Schneider 2022b). Condition-dependent chemical signalling is also evident in males; glands of undernourished

males contain fewer pheromone precursors in cockroaches (Clark *et al.* 1997), while female mealworm beetles exhibit a preference for pheromones from well-fed males (Rantala *et al.* 2003).

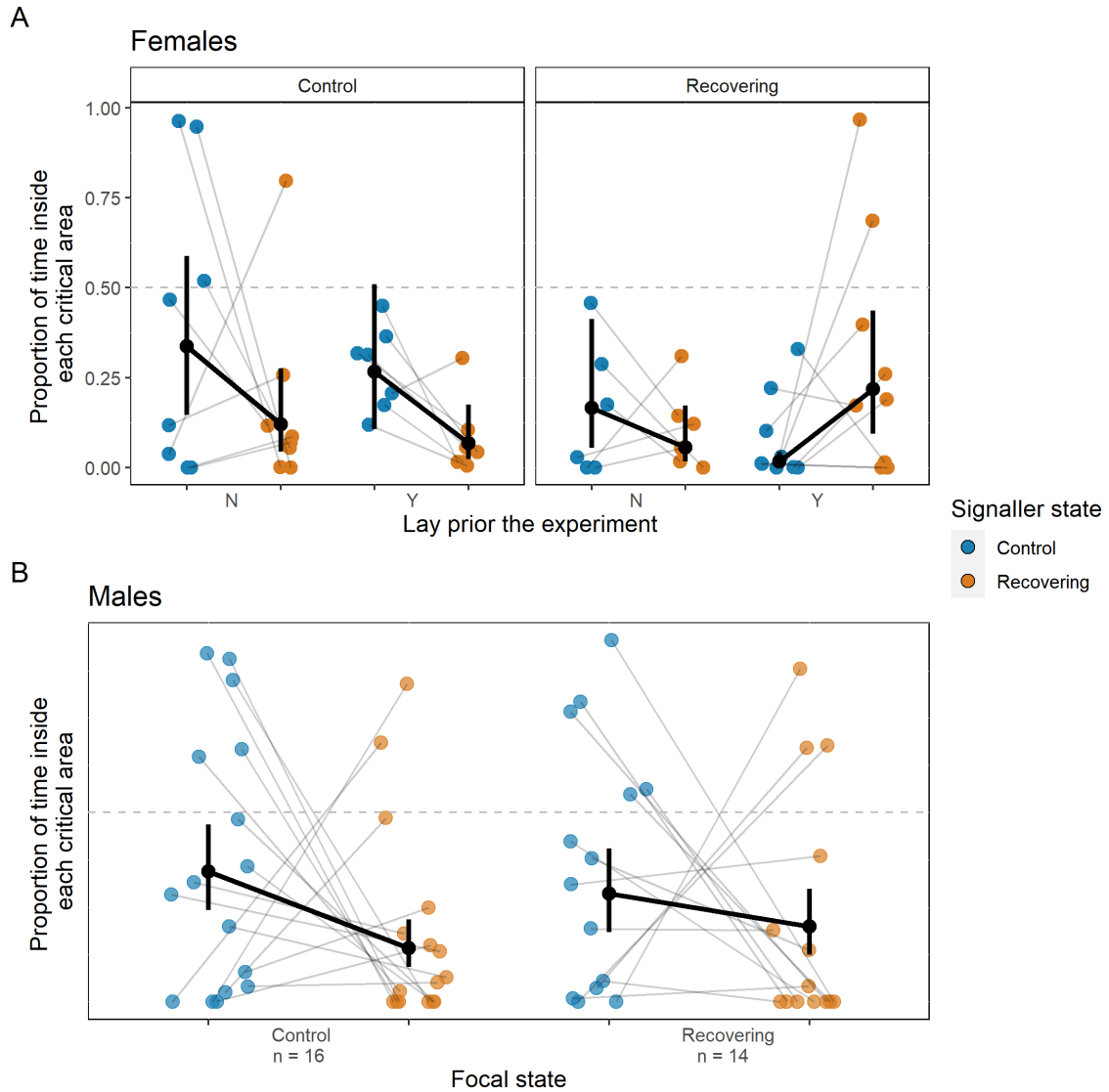


FIGURE 6 **Mate preferences for control vs. anhydrobiosis-recovering individuals in *Macrobiotus annewintersae* (II)** A. Proportion of the time spent inside each critical area by females focal (non-crossers are excluded). Additionally, the analysis considers the reproductive history of females, specifically whether they laid eggs prior to the experiment (a proxy for sperm storage status). B. Proportion of the time spent inside each critical area by males focal (non-crossers are excluded). Black circles represent the means of posterior distributions of Bayesian GLMMs, whereas vertical lines represent the 95% high-density interval (HDI).

3.2 Peri-mating choice (III)

My observations offer a nuanced perspective on tardigrade mating dynamics, revealing intriguing patterns and behaviours that shed light on their reproductive strategies. Contrary to the rapid mating initiation and completion reported in certain tardigrade species (Sugiura *et al.* 2019), my findings demonstrate a varied mating success rate across the examined species (III). While in some species mating success rates were high (>75%), others exhibited lower rates (between 33% and 55%), underlining the complexity of reproductive interactions within tardigrade populations. Although the presence of SSO did not explain these observed patterns, they are consistent with observations reported in Vecchi *et al.* (2022), suggesting a significant role of females in regulating sperm transfer success through deliberate body movements. Despite these variations, a consistent pattern emerged across all species, reflecting previously observed behaviours in species that lay eggs freely into the environment (Sugiura and Matsumoto 2021b). Males typically engage in multiple courtship interactions with ready-to-mate females, including touching female cloacal area with their buccal region, a behaviour reminiscent of courtship rituals observed in other organisms (birds e.g. Davies 1983, Rowe *et al.* 2008, bats (e.g. Tan *et al.* 2009), reviewed in Eberhard 1990) which may facilitate genital stimulation or sperm transfer. For instance, male spiders tap females throughout mating, intensifying just before sperm transfer (Hrušková-Martišová *et al.* 2010, Peretti *et al.* 2021), a behaviour that also induces female quiescence. Additionally, male genital movement may play a role in post-copulatory sexual selection, stimulating female cryptic choice, as observed in spiders (Cargnelutti *et al.* 2021). This tapping behaviour potentially elucidates why male tardigrades touch females' cloaca. Unlike spiders, however, male tardigrades do not need to reduce the chances of being eaten by females. Interestingly, my study revealed no quantitative disparities in reproductive behaviour between species possessing SSOs and those without, suggesting a uniformity in mating behaviours across one family of tardigrades.

Close-range contact pheromones are implied in triggering courtship behaviour. Studies on close-range courtship behaviour of true bugs reveal specific behavioural steps, from contact to copulation, indicating that behavioural postures and chemical stimuli are involved in successful mate recognition (e.g. Borges *et al.* 1987, Žunič *et al.* 2008). Cuticular hydrocarbons (CHCs), renowned for their pivotal protective function and contribution to desiccation resistance in numerous insect species (Ferveur 2005), have emerged as significant pheromones (Blomquist and Bagnères 2010). Investigations into the role of CHCs have revealed their importance in sex recognition, facilitating the identification of potential mates (Singer 1998). Furthermore, their diverse functions in sexual communication within species suggest robust sexual selection pressures on CHCs, with both males (e.g. Chenoweth and Blows 2005, Peterson *et al.* 2007) and females (e.g., Rundle *et al.* 2009, Ingleby *et al.* 2013) demonstrating

preferences for specific CHC profiles, which serve as indicators of mate quality and attractiveness (e.g. Grillet *et al.* 2006, Jennings *et al.* 2014), mating status, and receptivity to mating (e.g., Blomquist and Bagnères 2010). Additionally, CHCs can be exchanged between males and females during mating interactions (Yew and Chung 2015). While the presence and potential role of CHCs remain unknown in tardigrades, their involvement could be crucial in tardigrade courtship behaviour. CHCs potentially serve as essential signals for copulatory attempts, thus elucidating why successful matings typically involve tactile interactions in tardigrades. This underscores the necessity for further investigation into the presence and function of CHCs in tardigrades, potentially unveiling novel insights into their mating behaviour and reproductive strategies.

Furthermore, copulation serves as a potential avenue for transmitting cues that influence reproductive decisions and outcomes in both sexes (Wedell *et al.* 2002). Males across various species, whether practising internal or external fertilisation, commonly release their spermatozoa accompanied by seminal fluid, a secretion produced by accessory sex glands (e.g. Wigby and Chapman 2004, Poiani 2006). Notably, in *Drosophila*, seminal fluid elicits profound morphological and physiological changes in the female reproductive tract upon copulation (Mattei *et al.* 2015). However, the investigation of traits governing ejaculate quality – encompassing individual sperm cell phenotypic attributes such as size, shape, and motility, as well as non-sperm components like seminal fluid – has received disproportionately less empirical scrutiny compared to sperm numbers (Simmons and Fitzpatrick 2012, Fitzpatrick and Lüpold 2014, Evans and Lymbery 2020). The potential release of seminal fluid alongside spermatozoa in tardigrades remains an intriguing yet unexplored aspect of their reproductive biology. Further research in this area holds promise to uncover new perspective into the reproductive strategies employed by tardigrades.

3.3 Post-mating choice (II, III)

3.3.1 Gamete recovery from stress-event

The ability of organisms to withstand environmental stressors, such as desiccation, is a remarkable display of their adaptability. Moss, which is found in various ecosystems, can lose over 50% of its water content in one day due to environmental stressors such as desiccation (Robinson *et al.* 2000). This phenomenon is worsening due to the effects of global warming (Li *et al.* 2021). In this context, tardigrades residing within moss ecosystems exhibit a remarkable survival strategy. They undergo a transformative tun formation to endure prolonged dry periods, a process known as anhydrobiosis (Wright *et al.* 1992). However, it is still unclear how tardigrades recover their reproductive capabilities after rehydration, which is a fascinating mystery in tardigrade biology. My investigation into the post-anhydrobiotic reproductive dynamics of tardigrades unveils intriguing insights into gamete recovery from stress-induced

desiccation. Contrary to expectations, the presence of females does not accelerate the speed of sperm recovery in male tardigrades following one-week anhydrobiosis, compared to when males are isolated. This observation underscores the unique physiological mechanisms governing tardigrade sperm motility recovery, which diverge from established paradigms in mammalian reproductive biology. While extensive research has elucidated the detrimental impact of oxidative stress on sperm motility in mammals (Varisli *et al.* 2009, Aitken *et al.* 2016, Kurkowska *et al.* 2020, Ritchie and Ko 2021, Gualtieri *et al.* 2021), the mechanisms underlying sperm recovery from stress in invertebrates remain largely unexplored (Gallo *et al.* 2016, Esposito *et al.* 2020). Evidence from numerous taxa suggest that the germline and its associated reproductive physiology is susceptible to thermal stress, particularly high temperature (reviewed in Walsh *et al.* 2019). For instance, the number of sperm within an ejaculate in the flour beetle is significantly reduced following a simulated heat-wave exposure (Dickinson 2018), highlighting the vulnerability of sperm to environmental stressors. Similarly, the process of resuming biological activity post-rehydration poses significant challenges in mammals, often resulting in the failure of dried cells to regain functionality (Saragusty and Loi 2019). It is also worth noting that tardigrades do not tolerate prolonged exposure to warm water, with findings indicating significantly higher temperature tolerance in anhydrobiotic tardigrade tuns compared to their active state (e.g. Neves *et al.* 2020). Therefore, unravelling the mechanisms facilitating efficient sperm motility recovery in tardigrades holds immense significance across various domains, including agriculture, medicine, and the conservation of endangered species.

My findings indicate that previous mating and sperm storage combined with the anhydrobiotic state affects the laying behaviour of female tardigrades (Fig. 7). We observed that the reproductive outcomes of recovering females paired with recovering males, irrespective of their sperm storage status, were characterized by a notable absence of egg-laying activity. Furthermore, a substantial proportion of recovering females paired with control males exhibited similar reproductive behaviour, suggesting a mutual impairment of gamete functionality hindering the fertilization process. While studies on oocyte preservation and recovery following desiccation have predominantly focused on vertebrates (Paynter and Fuller 2007, Hochi *et al.* 2011, Gil *et al.* 2014, Iussig *et al.* 2019, Comizzoli *et al.* 2022), the mechanisms governing cellular recovery in tardigrades remain a subject of conjecture. Nevertheless, the upregulation of heat soluble proteins (CAHS and SAHS) emerges as a plausible avenue for future exploration, potentially unravelling the molecular underpinnings of tardigrade resilience to environmental stressors (Møbjerg and Neves 2021, Arakawa 2022). Leveraging cutting-edge techniques such as single-cell (Sun *et al.* 2022) or spatial transcriptomics (Gleason and Burton 2015) holds promise for elucidating the intricate mechanisms underlying gamete recovery in tardigrades, offering unprecedented insights into the resilience of life in the face of adversity.

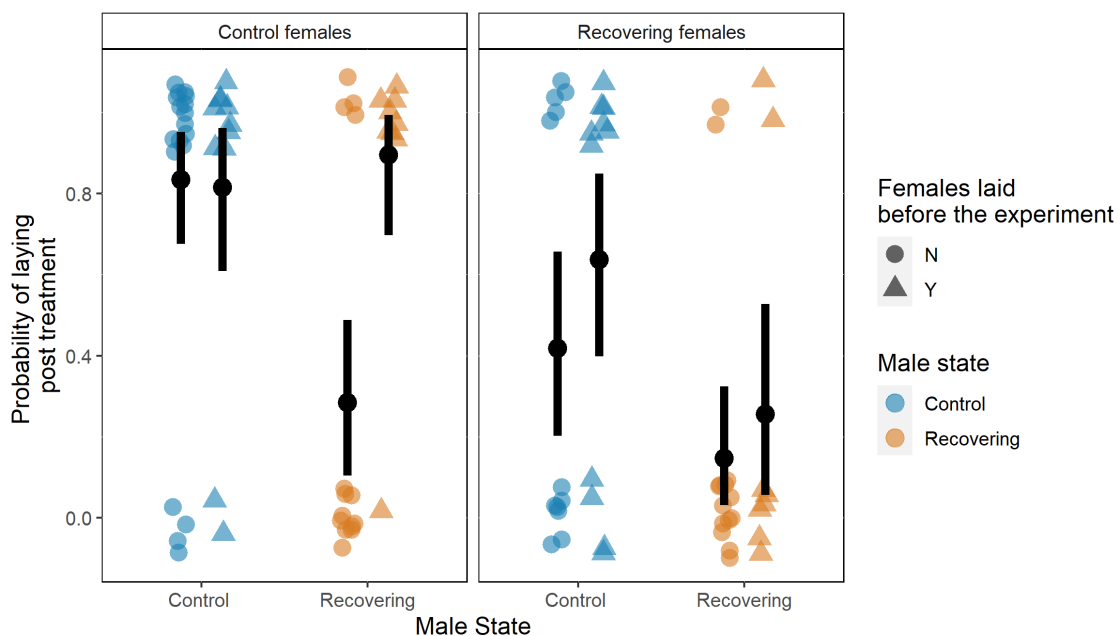


FIGURE 7 **Probability of oviposition post-treatment for control vs. anhydrobiosis-recovering *M. annewintersae* (II)**, considering the reproductive history of females (i.e. whether females laid prior to the experiment, a proxy for sperm storage state). Black circles represent the means of posterior distributions of the Bayesian GLMMs, whereas vertical lines represent the 95% HDI.

3.3.2 Role of the sperm storage organ in tardigrades

My investigation into tardigrade reproductive strategies highlights compelling advantages conferred by the presence of a sperm storage organ (SSO). Notably, there is a stark contrast in the reproductive outcomes between females with and without access to stored sperm. Initially, my findings demonstrate that control females, presumed to possess a depleted SSO, exhibited a notable lack of egg-laying behaviour when paired with anhydrobiosis-recovering males carrying immotile sperm (II, Fig. 7). In contrast, the majority of females with previously stored sperm from prior matings, successfully laid eggs when paired with anhydrobiosis-recovering males (II, Fig. 7), reaffirming the resilience of sperm stored within female tardigrades during anhydrobiosis events, as supported by earlier research (Vecchi et al., unpublished).

Moreover, my investigation indicates a notable temporal advantage associated with the presence of an SSO. While no significant delay in the onset of egg-laying was observed among species possessing an SSO, females lacking this organ typically started ovipositing within a day after mating (III). In contrast, those with an SSO exhibited a minimum latency of at least two days, suggesting a strategic delay likely associated with the utilisation of stored sperm for fertilisation post-copulation. However, it is worthy to note that despite my concerted efforts to conduct post-mating assessments within a 24-hour timeframe, interruptions to this protocol occasionally occurred. As a result, there may have been incomplete documentation of oviposition timing during the

weekly post-mating event monitoring. The recorded timing may have been longer than the actual time it took for females to lay their first batch. Furthermore, my longitudinal observations reveal an extended reproductive capacity in species with an SSO, as evidenced by the ability to lay multiple clutches over a two-month period (III). In contrast, species lacking an SSO typically laid eggs within the first week (however, due to limitations of the observation the protocol, it remains uncertain whether a single or multiple oviposition occurred within the first week). Nonetheless, this observation underscores the sustained egg-laying activity facilitated by stored sperm. These reproductive delays observed in species with an SSO parallel documented phenomena in other organisms (Birkhead and Møller 1993, Yushin and Kosaka 2013, Orr and Zuk 2014, Dhillon *et al.* 2020, Levine *et al.* 2021, Shankar *et al.* 2022) and likely stem from the strategic utilisation of stored sperm for fertilisation following mating. For instance, comparable patterns of post-mating egg-laying latency have been reported in species featuring an SSO, such as *Drosophila melanogaster*, where females can utilise stored sperm for fertilisation up to three weeks post-mating (Neubaum and Wolfner 1999). My findings underscore the adaptive advantage conferred by the presence of a sperm storage organ (SSO) in tardigrades, showcasing a form of bet-hedging strategy. Bet-hedging involves sacrificing immediate reproductive success for increased survival or long-term reproductive success by spreading efforts across different conditions or time periods (Simons 2011). The SSO allows tardigrades to strategically store sperm, enabling them to delay fertilization until environmental conditions are favourable or until they encounter a suitable mate. This bet-hedging strategy enhances reproductive success, even in unpredictable or challenging environments, contributing to our understanding of tardigrade reproductive biology and highlighting the broader significance of SSO functionality in shaping reproductive strategies across diverse organisms.

3.4 Limitations and future directions

Tardigrades are fascinating creatures, but the lack of previously established experimental methods to conduct behavioural ecology research made working with them quite challenging. In my research, I faced several methodological hurdles, including developing a choice and mating arena adapted to their small size, and ensuring an adequate sample size.

In order to obtain the final choice chamber, I tested various designs. Ultimately, transparent walls, agar-coated arenas and bottom-light sources proved the most effective.

Since mating behaviour and preferences in other taxa have been shown to change after previous matings (Moore and Moore 2001, Martin and Hosken 2002, Mack *et al.* 2003, Mautz and Sakaluk 2008), virgin individuals were used in study I. However, finding enough individuals of both sexes from hatchlings that were raised individually proved to be a daunting task. Isolating hundreds of eggs from

the main culture over a period of several weeks was necessary to obtain the 15 to 20 focal individuals of each sex (I). Therefore, for subsequent studies (II, III), I used non-virgin individuals instead, which made it easier and faster to get more sexually mature individuals. I could assume that all individuals taken from the main culture had had a mating experience. A previous study found that randomly selected females from the main culture had stored sperm (Vecchi *et al.* 2022). I also ensured to isolate the largest and sexually mature individuals from the main culture (e.g. with visible eggs for females). Finally, to control for individual mating history, they were kept isolated for at least two weeks, which should be enough time to virtually empty female sperm reserves (Vecchi *et al.* 2022) and promote keenness to (re)mate. However, this isolation period had drawbacks, such as egg-reabsorption and a higher mortality risk. This required me to isolate twice the number of individuals from the main culture than the one used in the experiments. A further sample size issue arose in study III. Despite randomly allocating mating pairs, most showed no interest in mating: over 135 videos (using 300 individuals) were recorded to obtain about 50 successful matings.

Moving forward, there are several promising avenues for future research in the field of tardigrade behavioural ecology. Firstly, further exploration into refining experimental methodologies tailored specifically to tardigrades is warranted. Building upon the insights gained designing the mate choice chambers, continued efforts to optimize experimental setups could enhance the accuracy and reliability of behavioural studies in these organisms. Additionally, given the challenges encountered in obtaining sufficient sample sizes, developing strategies to increase the efficiency of tardigrade collection and maintenance would be beneficial. This could involve investigating alternative culturing techniques or resolving sex-determination in those organisms.

Despite these challenges, my research contributes to our understanding of tardigrade behaviour, reproduction, and evolution. Nonetheless, key questions remain unanswered regarding the underlying mechanisms of tardigrade mating behaviour.

My behavioural work is only the first step in the process of chemical ecology. Future studies could focus on elucidating chemical cues composition used by tardigrades in sex-discrimination and mate choice and test them in behavioural assays.

Study III primarily focuses on post-mating processes in tardigrades, particularly testing the role of the sperm storage organ (SSO) presence/absence in a comparative setting. The presence of an SSO in several of the tardigrade species used here hints at the possibility of females mating with multiple males. However, this aspect remained unexplored in my research. Future research could investigate the frequency and consequences of multiple mating events in females with an SSO, thereby elucidating post-copulatory sexual selection mechanisms. Exploring female cryptic choice or sperm competition will require the development of paternity assignment techniques.

4 CONCLUSIONS

This thesis offers an in-depth analysis of tardigrade reproduction, focusing on the intricate processes of sexual selection and evolutionary strategies. Through a thorough investigation of mate choice dynamics, including the preferences of both males and females, it uncovers novel and fascinating insights into intersexual communication and behavioural responses among tardigrades, examining them across the three key stages of mate choice: before, during, and after mating.

In the pre-mating (pre-copulatory) stage, Study I focus into intersexual communication in tardigrades, while Study II explores condition-dependent mate choice. I examined different types of chemical signals and their respective impacts on sex-discrimination and mate choice (I), revealing intriguing insights into the intricate dynamics underlying mate choice in this understudied phylum. Notably, males exhibited a discernible preference for females based on waterborne signals, while females show relative neutrality (I). Moreover, I found that, as expected, control males and males in recovery preferred control females (II), aligning with the assumption that males would prioritise mating with control females due to their potential advantages in terms of reproductive success (Bonduriansky 2001, Edward and Chapman 2011) and that anhydrobiosis is costly to females at the pre-mating stage. Intriguingly, both control females and females in recovery who had not laid eggs before the experiment, displayed similar preferences for control males, whereas recovering females who had laid eggs prior to the experiment preferred recovering males (II). This underlines the pivotal role of female mate choice in shaping reproductive dynamics among tardigrades.

These findings represent significant advancements in our understanding of tardigrade reproductive behaviour, particularly within the pre-mating phase, laying a foundation for future inquiries to identify the specific chemical compounds mediating intersexual communication. Contrary to previous research emphasising male mate choice and competition, my findings illuminate the potential significance of female preferences, particularly regarding physiological resilience and adaptability of a prospective mate. Females may

favour males capable of swift recovery from extreme environmental conditions or more tolerant to stress, a trait that may confer reproductive advantages in harsh ecological contexts, as it is the case in other organisms (Ravi *et al.* 2013). Moreover, my investigation suggests a potential interplay between anhydrobiosis—the ability to enter a dormant state in response to desiccation—and mate choice, hinting at complex interactions between reproductive strategies and environmental pressures. Future inquiries should delve into the trade-offs associated with sperm storage in females and its implications for reproductive success and fitness.

In the peri-mating phase, Study III reveals a uniform mating behaviour among tardigrade species despite a diverse spectrum of mating success rates. I observed consistent patterns of male courtship rituals involving tactile interactions with females, reminiscent of behaviours in other organisms (Davies 1983, Peretti *et al.* 2021). The potential role of close-range contact pheromones, akin to cuticular hydrocarbons in insects (Peterson *et al.* 2007, Thomas and Simmons 2009, Ingleby 2015, Hare *et al.* 2022), suggests a crucial role for chemical signalling in tardigrade courtship. Further investigation into these chemical cues could offer more insights into their mating behaviours and reproductive strategies of tardigrades. Additionally, the transmission of cues through copulation, known to occur in *Drosophila* (Poiani 2006, Wigby *et al.* 2020), presents another avenue for understanding reproductive decisions and outcomes in tardigrades that yet remains largely unexplored.

My investigation into post-mating processes (II, III) in tardigrades sheds light on their remarkable resilience and adaptability to environmental stressors. I provide valuable observations regarding gamete recovery following stress-induced desiccation. Despite the absence of accelerated sperm recovery in the presence of females, my findings underscore unique physiological mechanisms governing tardigrade reproductive dynamics. Furthermore, my work highlights the significant advantages conferred by the presence of a sperm storage organ (SSO) in tardigrade reproduction. Females with stored sperm from prior matings, paired with infertile males (i.e. recovering from anhydrobiosis) successfully laid eggs (II), reaffirming the resilience of sperm stored within female tardigrades during anhydrobiosis events, as supported by earlier research (Vecchi *et al.*, unpublished). Moreover, females with SSOs demonstrated a sustained reproductive capacity, as seen in other species with a sperm storage organ (Orr and Zuk 2014, Pascini and Martins 2017) (III). These findings not only deepen our understanding of tardigrade reproductive biology but also underscore the broader significance of SSO functionality in shaping reproductive strategies across diverse organisms. While my research provides valuable insights, avenues for future investigation remain, including the exploration of multiple paternity and female cryptic choice.

In summary, this thesis represents a pioneering effort to unravel the mechanisms of intersexual communication, mate choice dynamics, and evolutionary strategies in tardigrades. Through innovative experimental approaches and a comprehensive taxonomic breadth, this research illuminates previously unexplored aspects of tardigrade reproductive biology, setting the

stage for future studies to delve deeper into the diversity of these fascinating microorganisms. Additionally, unravelling the intricacies of sexual selection processes holds far-reaching implications for various fields, including ecology, evolutionary biology, and conservation. By elucidating the factors driving reproductive success and mate choice, this research contributes to our broader understanding of the complex interactions between individuals within populations and ecosystems. Furthermore, the insights gleaned from this study have implications beyond tardigrade biology. For instance, tardigrade ability to survive extreme conditions, such as desiccation, could inspire the development of novel technologies for the preservation and storage of biological materials. Understanding the factors influencing reproductive success in tardigrades can also inform conservation strategies aimed at preserving these resilient microorganisms and their habitats. By identifying key environmental cues and behavioural responses, conservation efforts can be tailored to better protect tardigrade populations, particularly in vulnerable ecosystems.

Overall, this thesis represents a significant step forward in our understanding of tardigrade reproductive biology and its broader evolutionary significance. By laying the groundwork for future investigations and highlighting the creative use of experiments to uncover hidden biological mechanisms, this research contributes to a growing body of knowledge on the diversity and resilience of tardigrades in the face of environmental challenges.

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RÉSUMÉ (RESUME IN FRENCH)

Les perspectives multiples sur la sélection sexuelle et les stratégies évolutives dans l'exploration de la reproduction des tardigrades

Darwin a introduit le concept de sélection sexuelle pour expliquer l'évolution et l'émergence des caractères sexuels secondaires. Ce concept suggère que certains individus sont en compétition pour l'accès à un partenaire (souvent les mâles, connue sous le nom de sélection intrasexuelle), tandis que d'autres ont des préférences pour le sexe opposé basées sur des traits désirables (typiquement les femelles, connue sous le nom de sélection intersexuelle ou de choix du partenaire). La sélection intersexuelle implique plusieurs étapes (pré-, péri- et post-copulation), depuis la détection des partenaires potentiels jusqu'aux interactions post-accouplement qui influencent le succès de la fécondation. Alors que les premières recherches se sont concentrées sur les signaux émis par les mâles et les préférences des femelles, les études récentes mettent l'accent sur la compréhension de la sélection sexuelle dans les deux sexes, ce qui permettrait d'améliorer notre compréhension de la dynamique de l'évolution entre les espèces.

Le choix du partenaire implique la capacité de sentir, de percevoir et d'évaluer les signaux émis par les partenaires potentiels avant de prendre une décision comportementale et/ou physiologique. Souvent, ces caractéristiques ont été modifiées à des fins de communication, comme des ornements colorés ou des comportements de séduction. Pour communiquer, trois modalités sensorielles principales existent : la vision, l'audition et la chimioréception, qui comprend l'odorat et le goût. Les caractéristiques que les individus expriment et les choix qu'ils font lors de la sélection intersexuelle sont étroitement liés à leur environnement. Les changements dans les conditions environnementales, tels que les fluctuations de température ou l'exposition à des polluants, peuvent affecter l'attractivité des traits utilisés dans la sélection des partenaires et les comportements associés à la séduction. Des études récentes suggèrent que des facteurs tels que la saisonnalité, les niveaux hormonaux et le contexte social jouent un rôle crucial dans la décision de choisir un partenaire. En outre, la force du choix du partenaire varie considérablement d'une espèce et d'un individu à l'autre, influencée par les coûts et les avantages associés à la sélection d'un partenaire, y compris les dépenses de temps et d'énergie, ainsi que la réduction potentielle du succès reproductif par le rejet de partenaires potentiels. La condition physique d'un individu et les facteurs liés à son historique de vie influencent également la dynamique du choix d'un partenaire.

Les tardigrades sont de minuscules organismes qui vivent dans la mousse ou le lichen. Ils présentent des similitudes avec les nématodes et les arthropodes. Malgré leur petite taille, ils présentent des comportements complexes et une anatomie bien développée, notamment un cerveau et un système digestif simples. Les tardigrades se reproduisent rapidement et grandissent grâce à un processus de mue. Leur résilience et leur capacité à survivre à des conditions

extrêmes sont des qualités remarquables des tardigrades. Pour résister à la dessiccation, ils subissent un état réversible appelé anhydrobiose, au cours duquel ils rétrécissent (tun) et subissent des changements moléculaires. Cependant, malgré des études approfondies sur leurs capacités de survie, notre compréhension des tardigrades, notamment en termes de communication, reste limitée. Les tardigrades se reproduisent tout au long de leur vie adulte. Les femelles pondent des œufs soit librement, soit à l'intérieur de leur exosquelette. Les processus de fécondation varient d'une espèce à l'autre, certaines observations suggérant une fécondation interne. Cependant, de nombreux aspects de la reproduction des tardigrades, tels que la sélection des partenaires et le stockage des spermatozoïdes, restent mal compris. Cela offre des opportunités de recherche pour découvrir leur biologie complexe et leurs stratégies de reproduction.

Ma thèse porte sur la reproduction des tardigrades en examinant la sélection sexuelle et les stratégies évolutives au cours des trois phases clés du choix du partenaire : pré-, péri- et post-copulatoire. Elle se concentre spécifiquement sur l'impact du stress et des facteurs environnementaux sur ces décisions. Elle comble une lacune de la littérature en prenant en compte de manière égale les mâles et les femelles dans le processus de sélection du partenaire. Pour ce faire, j'ai utilisé des individus issus de cultures en laboratoire de cinq espèces de tardigrades étroitement apparentées vivant dans la mousse. Parmi ces espèces, trois possèdent un organe de stockage du sperme chez les femelles, tandis que les deux autres n'en ont pas.

Mes résultats suggèrent que les tardigrades peuvent faire la distinction entre les sexes, les mâles montrant une préférence pour les femelles (I), ce qui confirme des observations antérieures sur le comportement de recherche de partenaires chez ces organismes. J'ai également découvert que les femelles s'associent brièvement mais de manière comparable avec des individus des deux sexes (II). Mes résultats renforcent les recherches antérieures indiquant que la diffusion de signaux chimiques transportés par l'eau est cruciale dans la communication intraspécifique des tardigrades dans un contexte de reproduction. Mes recherches indiquent également que les tardigrades peuvent être capables de discerner l'état physiologique d'un individu à travers ces mêmes signaux chimiques, influençant ainsi leurs choix (II). En fait, les mâles et les femelles (témoins) s'associent de préférence à des individus du sexe opposé qui ne sont pas en train de se remettre d'une anhydrobiose. Des études sur d'autres organismes, tels que les papillons de nuit et les araignées, ont montré que la production de phéromones diminue dans des conditions de stress, ce qui indique des compromis potentiels dans l'allocation des ressources entre la production de phéromones et d'autres fonctions physiologiques.

Contrairement aux observations précédentes, les taux de réussite des accouplements chez les tardigrades sont variables, et la présence d'organes de stockage du sperme n'a pas permis d'expliquer ce constat. Malgré cela, le comportement reproductif est similaire chez les espèces testées, les mâles s'engageant dans de multiples interactions de parade nuptiale. Ce comportement, qui consiste notamment à toucher la région cloacale de la femelle,

ressemble aux rituels de cour observés chez d'autres espèces et pourrait faciliter la réceptivité de la femelle ou le transfert de sperme. De plus, mon étude n'a révélé aucune différence quantitative du comportement de reproduction entre les espèces avec ou sans organe de stockage du sperme, suggérant une cohérence dans ce comportement au sein de ces membres de la famille.

J'ai constaté que la présence de femelles auprès de mâles se remettant d'une semaine d'anhydrobiose n'accélère pas le rétablissement de la motilité de leurs spermatozoïdes. Si l'impact du stress oxydant sur la motilité des spermatozoïdes chez les mammifères est bien documenté, les mécanismes sous-jacents chez les invertébrés tels que les tardigrades restent largement inexplorés. La compréhension de ces mécanismes pourrait avoir des implications significatives, de l'agriculture aux efforts de conservation des espèces. En outre, j'ai observé que l'historique d'accouplement combiné à l'état anhydrobiotique affecte le comportement de ponte des femelles tardigrades. Les femelles récupérant de l'anhydrobiose, quel que soit leur état de stockage du sperme, en contact avec des mâles dans le même état, ont montré peu d'activité de ponte. Cette altération mutuelle de la fonction des gamètes entravant la fécondation suggère des pistes de recherche intrigantes, révélant potentiellement la résilience moléculaire des tardigrades au stress environnemental.

Mon étude sur les stratégies de reproduction des tardigrades met en lumière les avantages offerts par la présence d'un organe de stockage du sperme (SSO). Les observations révèlent des résultats reproductifs distincts entre les femelles avec et sans SSO, soulignant l'importance de sa fonctionnalité. Les femelles tardigrades témoins et dont j'ai assumé que leur organe de stockage du sperme était vide, qui sont entrées en contact avec des mâles en convalescence dépourvus de sperme mobile, ont pu pondre des œufs, contrairement aux femelles dont le sperme avait été stocké auparavant. Ceci met en évidence la résistance du sperme stocké chez les femelles tardigrades pendant l'anhydrobiose. En outre, mes observations longitudinales révèlent également une capacité de reproduction étendue chez les espèces pourvues d'un organe de stockage du sperme. Cependant, des recherches supplémentaires sont nécessaires pour explorer des aspects tels que les accouplements multiples et le choix des femelles cryptiques, qui pourraient fournir des informations plus approfondies sur la dynamique de la reproduction des tardigrades et les stratégies évolutives, améliorant ainsi notre compréhension de leur résilience dans des environnements difficiles.

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ORIGINAL PAPERS

I

FIRST EVIDENCE OF SEX-SPECIFIC RESPONSES TO CHEMICAL CUES IN TARDIGRADE MATE SEARCHING BEHAVIOUR

by

Justine Chartrain, K. Emily Knott, Łukasz Michalczyk & Sara Calhim 2023

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RESEARCH ARTICLE

First evidence of sex-specific responses to chemical cues in tardigrade mate searching behaviour

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ABSTRACT

Chemical cues are widely used in intraspecific and interspecific communication, either as substances deposited in the substrate or as molecules diffused in water or air. In tardigrades, an emerging microscopic study system, chemical communication and its role in reproduction are poorly known. Here, we assessed sex differences in the detection of (a) short-range diffusing signals and (b) deposited cue trails during the mate-searching behaviour of freely moving virgin male and female *Macrobiotus polonicus*. We tracked individual behaviour (a) in simultaneous double-choice chambers, where live conspecifics of each sex were presented in water and (b) of freely moving pairs on agar without water. We found that males, but not females, preferentially associated with opposite-sex individuals in trials conducted in water. In contrast, neither sex detected nor followed cues deposited on agar. In conclusion, our study suggests that mate discrimination and approach are male-specific traits and are limited to waterborne chemical cues. These results support the existence of Darwinian sex roles in pre-mating behaviour in an animal group with virtually non-existing sex differences in morphology or ecology.

KEY WORDS: Chemical communication, Waterborne signal, Deposited cue, Semiochemicals, Sex discrimination

INTRODUCTION

Semiochemical communication is widespread and used by animals across multiple contexts, from finding food to attracting mates (Wyatt, 2014). Behavioural responses to conspecific chemical cues, and specifically sex discrimination, have been investigated in a wide range of taxa, both aquatic and terrestrial (Dunham and Oh, 1992; Bouchard, 2001; Cooper and Pérez-Mellado, 2002; Park et al., 2004; Hutter et al., 2011; Stamps and Shaw, 2019; Kudo et al., 2022). Importantly, sexually mature animals that fail to discriminate between the sexes and/or species incur a reproductive cost (Burdfield-Steel and Shuker, 2011; Lerch and Servedio, 2022). To find and/or attract a mate, animals can use two main types of chemical signals: (i) diffusing signals and/or (ii) trail deposited on the substrate. For instance, male copepods can find females using pheromone plumes that receptive females produce as trails or clouds (Bagoien and Kiørboe, 2005; Kiørboe et al., 2005). Similarly, both male and female apple snails can follow opposite-sex trails, and, in

addition, males are attracted to waterborne sex pheromones of females (Takeichi et al., 2007).

Most studies examining the role of pheromones in sexual selection have focused on diffusing chemical signals used in long-range mate attraction and mate recognition (Johansson and Jones, 2007). Furthermore, there is a strong taxonomic bias towards insects and mammals (Brennan and Keverne, 2004; De Pasqual et al., 2021), and a focus on organisms with obvious male bias sexual dimorphism sensory organs and searching behaviour (Naka, 2018). However, such knowledge is non-existent or very limited for several animal groups, particularly micrometazoans such as the phylum Tardigrada. Tardigrades are a microscopic animal phylum categorized in the superclade Panarthropoda (Yoshida et al., 2017; Jørgensen et al., 2018) yet are largely neglected in behavioural ecology and evolutionary research. Even though individuals must be surrounded by water to be active, they colonised terrestrial environments, such as mosses and lichens, thanks to their cryptobiotic adaptations (Møbjerg and Neves, 2021). They have relatively simple sensory organs: some species present light-sensitive eyes, but all have body segments containing cells with characteristics of mechanoreceptors and chemoreceptors (Møbjerg et al., 2018). Sexual dimorphism is mainly limited to a female bias in body size, with very few reported examples of secondary sexual characters (Gašiorek et al., 2019, 2021) and parental care (Pilato et al., 2006). Of the ca. 1500 tardigrade species described so far (Degma et al., 2023), mating behaviour has been described for only 10 species (Sugiura and Matsumoto, 2021b), with only one showing secondary sexual dimorphism. Moreover, detailed records of both pre-mating and mating phases have been described for only four species, of which none show secondary sexual characters (Bingemer et al., 2016; Sugiura et al., 2019; Bartel and Hohberg, 2020; Sugiura and Matsumoto, 2021a). Together, these studies suggest a crucial role of semiochemicals in intraspecific sexual contexts (i.e. mate attraction and sperm transfer) through diffusing cues. In addition, a recent study showed that tardigrades could detect deposited chemical cues (trail) in interspecific (i.e. predator–prey) contexts (Meyer et al., 2020). In that study, prey avoided areas previously occupied by predators while the latter preferred the areas occupied by prey.

Chemical signals can serve as honest or deceptive indicators of quality, age or other reproductive factors and may be used to compete for mates (Johansson and Jones, 2007). Typically, the female transmits signals, and the male responds, leading to a potential blind spot in research regarding female response to pheromones (Hare and Simmons, 2019). To address this, it is vital to study both sexes' reactions to chemical cues and their impact on mate-searching behaviour, which will also contribute to a broader understanding of the evolution of these signals.

Using the gonochoristic tardigrade *Macrobiotus polonicus* (Pilato et al., 2003), we tested the role of both waterborne diffusing signals and deposited cue trails (in water-free

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environments) in sex discrimination and mate searching behaviours of both females and males. As our study system shows limited sexual dimorphism in sensory morphology and ecology, we hypothesised that (i) both females and males would preferentially associate with the opposite sex, and that (ii) both sexes could detect and follow an opposite-sex deposited-trail cue.

MATERIALS AND METHODS

Culture

We used two strains of a laboratory culture of the moss-living eutardigrade *Macrobotus polonicus* [strain AT.002 (Stec et al., 2021) and IT S218]. The second strain was obtained from a moss sample collected in Anzola Emilia, Bologna, Italy (44°34'08.2"N, 11°10'44.1"E) in December 2019. Females of this species lay eggs freely in the environment (Pilato et al., 2003) and have a sperm storage organ (Vecchi et al., 2022). The mating behaviour of *M. polonicus* has not been described fully; however, we have observed sperm release similar to that observed by Sugiura et al. (2019) in closely related species.

Tardigrades were kept in 5 cm diameter plastic Petri dishes with a scraped bottom (to aid motility) filled with mineral water and were fed *ad libitum* with algae (*Chlorococcum hypnosporum* and *Chlorella* sp.; Sciento UK) and rotifers (*Lecane* sp.) or nematodes (*Panagrellus pycnus*) according to what was available in culture, inside a climate chamber at 16°C, on a 2 h:22 h light:dark cycle. Half of the medium was partially changed weekly. Thanks to the transparent cuticle, the sex and reproductive state of *M. polonicus* can be determined non-invasively using light microscopy ($\times 400$ magnification).

All individuals used for this study were virgins. Virgin individuals were obtained by isolating eggs and then rearing hatchlings individually in 3 cm diameter plastic Petri dishes, kept in the same conditions as the main cultures. We determined the sexual maturity of individuals by observing motile sperm and mature oocytes in the male and female gonad, respectively. Each trial was conducted with individuals from the same strain (experiment 1: $n=23$ trials with AT.002 and $n=10$ with IT S218; experiment 2: $n=16$ trials with AT.002 and $n=11$ with IT S218).

Experiment 1: waterborne (diffusing) cues

We used 33 focal individuals (16 females and 17 males) to test whether tardigrades could differentiate the sexes using diffusing signals. We created a choice chamber arena (Fig. 1A) consisting of a 4-well slide (Ibidi, Gräfelfing, Germany) and two fishing lure rings (4 mm inner diameter). The arena surface was coated with 250 μ l of 1.5% agar (BD BACTO™ Agar) with the placement of the lure rings following a printed scheme in which their edges were at an 8 mm distance from each other, and each was 4 mm from the centre of the arena. When the agar was solidified, we added 1 ml of mineral water to flood the arena so chemical cues could diffuse within the chambers. This setup allowed us to provide the focal individual only with diffused chemical cues from the signallers as they could neither see nor touch them. The signallers were able to move freely within the lure area. Some of them were used twice for two different focal individuals (one of each sex), with at least 12 h of interval between the two trials (i.e. the next day). Different arenas were used for every trial conducted on the same day, and all arenas were thoroughly cleaned at the end of each day.

We placed the signallers inside the lure rings in darkness (swapping female and male sides between trials) and let them habituate for 30 min. Afterwards, we added the focal individual in the centre of the arena and filmed its behaviour for 30 min.

A stereomicroscope with a diffused bottom light linked to a camera (and the S-EYE software) was used. Position tracking was done automatically (see 'Video analysis', below). The preferential association was assessed as latency to cross, and time spent within the critical area of the opposite sex signaller.

Experiment 2: trail-deposited cues

Individuals used in the first experiment were randomly grouped in mixed-sex pairs (both focals and signallers). We used 27 couples to test whether tardigrades could follow a trail-deposited cue. The individuals were placed in the same type of Ibidi chamber slides as in experiment 1, but without water and the fishing lure rings. An ultra-thin continuous water film coated agar and the tardigrades, which allowed them to move freely in the arena but limited any chemical cue to the tardigrades' trails. We used the same microscope and camera setup as in experiment 1. After starting the recording, we placed the first individual and waited (ca. 5 min) for it to move. Then, we added the second individual and continued recording for 30 min. The order of individuals (male versus female) was alternated between trials. Position data for both individuals were obtained automatically (see 'Video analysis', below). In addition, we documented in detail the number and type of all observed interactions (i.e. bumps and following behaviour).

Video analysis

To obtain the position of the focal individuals during each trial during both experiments, we analysed the videos in R v.4.2.0 with the function 'trackR' from the package 'trackR' (<https://swarm-lab.github.io/trackR/>). The tracking was checked, and tracking position errors fixed with the function 'trackFixer' of the same package.

To determine whether the focal individuals were inside a critical area (dashed lines, Fig. 1A) for the waterborne-diffused cues, we applied a distance-to-centre function to the output (xy coordinates) given by trackR. For the trail-deposited cue experiment, we calculated the distance between individuals' paths every 3 s. Only those that differed by less than one body length distance from each other were considered to overlap (i.e. perpendicular overlap was excluded). We then assessed the proportion of time the paths of both animals overlapped.

Statistical analyses

All statistical analyses were conducted in R v.4.2.0 (<http://www.R-project.org/>). We ran generalised linear models using the 'brm' function from the 'brms' package v.2.17.5 (Bürkner, 2017).

In the diffusing signal experiment, for the latency to cross the first critical area, the predictor was the sex of the focal individual, whereas, for the proportion of time spent inside a critical area, the predictor was included as four types of dyad interactions (focal–signallers): female–female, female–male, male–female and male–male. The ID of the focal individual was included as a random variable. The focals that did not cross any critical area were removed from the analysis in both models. To test whether the path of one sex overlapped more often with the path of the other, we used sex as a predictor of the proportion of time an individual was on top of the other one's trace. We included trial ID as a fixed effect for the trail data as the couples could interact. To assess whether the absence of water affected motility, we compared the distance travelled in both experiments, where the focal individual's sex was the predictor. We used default priors for each response distribution type: binomial (logit link) distribution for the proportion of time spent inside each critical area and the proportion of time an individual was on top of

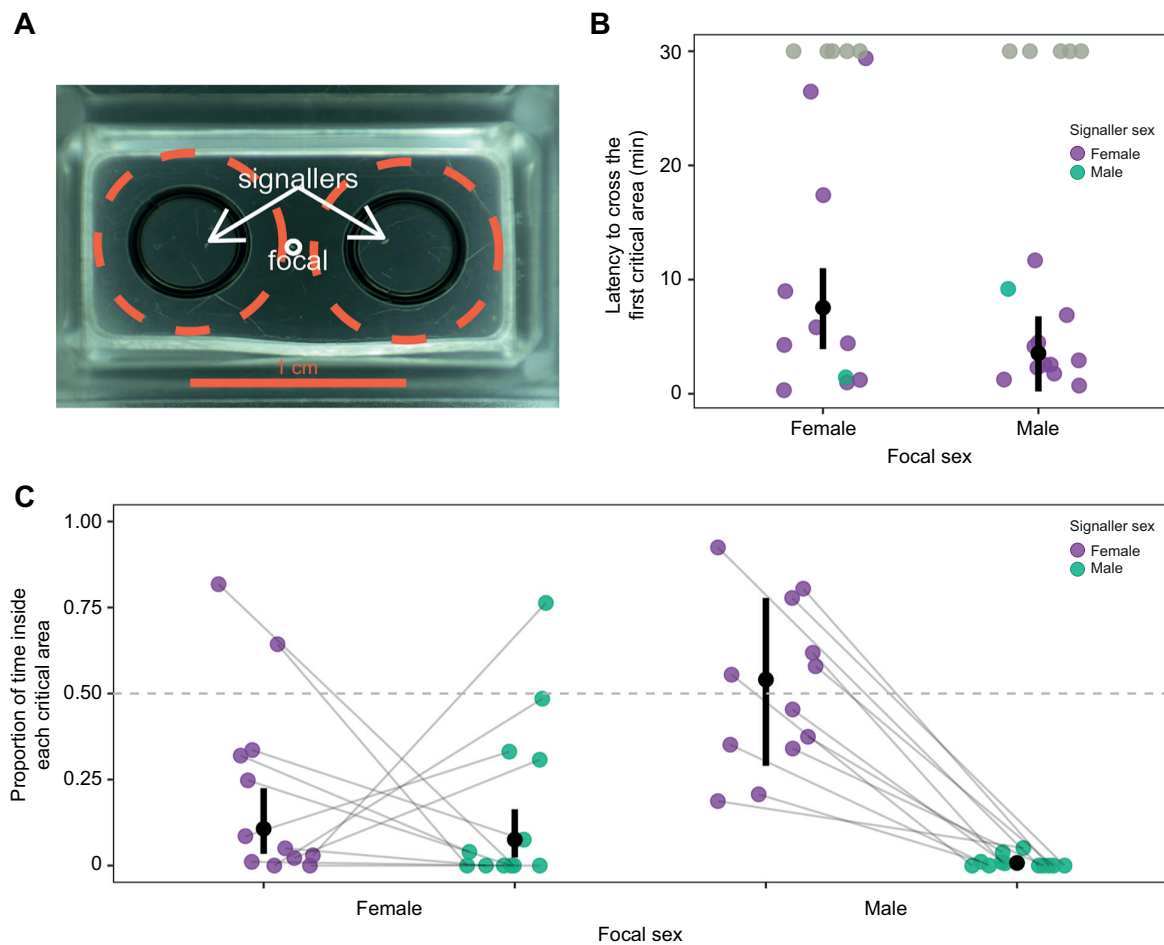


Fig. 1. Experiment 1: waterborne (diffusing) cues. (A) Photograph of the arena (choice chamber) setup from above (total size 21.6×11.4 mm, lure ring chambers 4 mm inner diameter). Dashed lines indicate the critical area. (B) Latency to first cross a side for focal females ($n=16$) and males ($n=17$); five individuals of each sex (grey) did not spend time inside a critical area (non-crosser). (C) Proportion of time spent inside each critical area ($n=11$ females, $n=12$ males, non-crossers are excluded). Black circles represent means of posterior distributions, whereas vertical lines represent the 95% high-density interval (HDI).

another one's path, Gaussian for the latency to cross the first critical area, and the distance travelled. The results are presented as back-transformed Bayesian posterior means and 95% high-density interval (HDI) using the 'mean_hdi' function from the 'tidybayes' package v.3.0.2 (available from Zenodo: <https://zenodo.org/record/7933163>), computed from $n=4$ well-converging and uncorrelated chains with $n=60,000$, 100,000 and 15,000 saved iterations, respectively.

RESULTS

Experiment 1: waterborne (diffusing) cues

In total, 10 individuals (5 females and 5 males) did not cross any critical area, and their latency to choose was recorded as 30 min (i.e. the maximum duration of the trials; Fig. 1B). The first choice in both sexes was biased towards female signallers except for one focal individual of each sex (Fig. 1B). The latency to first cross a critical area was not significantly associated with the sex of the focal individual ($n=23$, Bayesian mean estimate [95% HDI]: females latency=7.54 min [3.91, 10.99 min]; males latency=3.54 min [0.20, 6.77 min]) (Fig. 1B).

Females spent a very short but similar time next to either sex, whereas males showed a strong attraction to female signallers

(female $n=12$, mean of the proportion of time spent next to female [95% HDI]=0.107 [0.034, 0.22]; next to male=0.08 [0.023, 0.164]; males $n=12$, mean of the proportion of time spent next to females [95% HDI]=0.540 [0.290, 0.778]; next to males=0.008 [0.002, 0.018]) (Fig. 1C).

Experiment 2: trail-deposited cues

The proportion of time an individual spent on top of the other one's path was low (i.e. less than 1% of the experiment duration), and there were no differences between the sexes in this trait ($n=27$, mean of the proportion of time a female was on top of a male's path [95% HDI]=0.009 [0.007, 0.011]; a male was on top of a female's path=0.006 [0.005, 0.008]) (Fig. 2A).

Although we did not observe trail following for either sex, we discovered other behaviours. First, individuals were randomly encountering each other, which we describe as a contact. We noted which sex was initiating the contact (i.e. the individual colliding into the other). Second, individuals followed each other after these collisions (see Movie 1 and Fig. 2B). Half of the couples ($n=15$ of 27) collided at least once. In total, 24 contacts were recorded, with 1.6 contacts on average per couple (from 1 to 4 contacts in one trial).

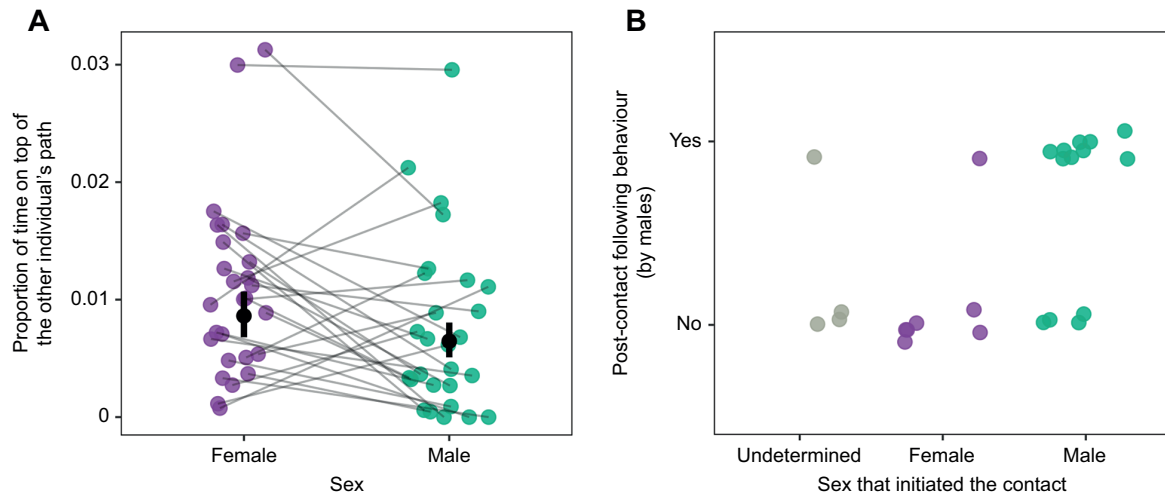


Fig. 2. Experiment 2: trail-deposited cues. (A) Proportion of time when the paths of the two individuals overlapped ($n=27$ trials). Black circles represent means of posterior distributions, whereas vertical lines represent the 95% HDI. Grey lines connect the two individuals used in the same trial. (B) Following behaviour observed according to the individual that initiated a contact during the trail experiment ($n=24$ recorded contacts). Only males showed this behaviour. The colour refers to the sex that initiated the contact.

Half of the contacts led to males moving together in tandem with females while maintaining physical contact. This behaviour was more often observed when the male had initiated the contact (Fig. 2B). Females never expressed this behaviour, and when they moved away from the male, they stopped and then started moving in another direction.

Lastly, we found no sex- or experiment-specific differences in activity level as measured by cumulative distance travelled (Fig. S1).

DISCUSSION

Signal type: waterborne (diffusing) versus deposited trail cues

Our results confirm previous work (Bartel and Hohberg, 2020) that diffusing waterborne signals are used in tardigrade intraspecific chemical communication in a reproductive context. However, we show, for the first time, sex differences in this context (see next section). We found no evidence that tardigrades can detect sex-specific cues deposited on the agar surface. This contrasts with what was found in a predator–prey deposited cue detection context (Meyer et al., 2020). However, that study differs from ours not only in context but also substantially in methodology: they used much larger taxa, with multiple individuals as signallers and focals, longer times to deposit cues on a substrate, and non-simultaneous response. Therefore, we cannot separate two potential explanations for our results: a lack of sex-specific signal or an inability to detect cues from a single signaller. Our observation of the interactions between individuals suggests that, in the absence of water, the detection of conspecifics is compromised. Only in half of the trials did the individuals interact, and of those, only one-third did so more than once (Fig. 2B). In addition, we did not observe any instances of trail-matching behaviour by either sex as assessed by the very rare (1%) spatial overlap of trails (Fig. 2A). Therefore, physical encounters appear to be random in the absence of water.

However, the behaviour observed after these physical encounters is insightful: only males followed females and mostly by maintaining physical contact (see Movie 1). In their natural habitat, the encounter rate can be quite variable: in another

Macrobiotus species, the number of sexually mature females showed a seasonal 7-fold change (Schuster and Greven, 2013). Therefore, males might have evolved this following behaviour to improve their mating opportunities. Furthermore, this behaviour might also be linked to the potential assessment of female receptivity and/or mating status using cues only detectable in close range. For example, it is well established that cuticular hydrocarbons (CHCs), in addition to their contribution to desiccation resistance (Hadley, 1981), have a key role in mate choice in the sister phylum to Tardigrada – Arthropoda (reviewed in Ingleby, 2015). Our current knowledge on tardigrade cuticle is mostly limited to interspecific morphological differences and their potential role in anhydrobiotic ability (reviewed in Czerneková and Vinopal, 2021). Unfortunately, the presence of CHCs in tardigrades (and their potential role in reproduction) remains unknown.

In many polyandrous species, males locate and establish a permanent association with immature females, i.e. pre-copulatory mate guarding (Parker, 1974). For example, male beetles are known to mount females for several hours during the day to defend the females from intruding males (Arakaki et al., 2004). Similarly, pre-copulatory mate guarding could explain our own observation of the males' following behaviour. However, in our experiments, females were ready to mate (i.e. eggs were visible in the ovary; see Poprawa et al., 2015), and not immature, as is usually the case in pre-copulatory mate guarding.

The biology of our study system can also explain the differences seen in cue-type responses. Tardigrades need a thin layer of water to be active, but mating in eutardigrades that lay free eggs requires that sperm be released into the environment, which then swim towards the female cloaca (reviewed in Sugiura and Matsumoto, 2021b). Thus, mating requires water to be present between individuals rather than just covering the cuticle. Therefore, it would not be surprising if pre-mating reproductive behaviours, such as sex discrimination or mate choice, are mediated by waterborne chemical cues and not by following a deposited trail cue. In fact, trail following is a widespread feature of chemical communication in aquatic environments, from crustaceans to fish (reviewed in Kamio et al., 2022).

Sex differences in response to waterborne signal

In accordance with previous mating observations (reviewed in Sugiura and Matsumoto, 2021b), our data quantitatively support male-biased mate-searching and mating initiation behaviours. When waterborne cues were available, males and females had a similar latency to approach one of the signallers (Fig. 1B), irrespective of the sex of the signaller (Fig. 1B). However, males spent significantly more time next to female signallers, whereas females showed no sex-based preference (Fig. 1C). These results suggest that tardigrades can discriminate between the sexes, with males preferentially associating with females.

There are many examples in the literature of males being attracted to opposite-sex chemical cues (Gomez-Diaz and Benton, 2013). In contrast, much less is known about the converse female response (Karlsson Green and Madjidian, 2011; Hare and Simmons, 2019). The latter might be a consequence of the focus on species with sexually dimorphic sensory apparatus (Naka, 2018), which is not the case in tardigrades (Møbjerg et al., 2018). In taxa without obvious sensory sexual dimorphism, both attraction (e.g. Passos et al., 2013) and insensitivity to male chemical signals were observed (e.g. Ratterman et al., 2009). This could be due to a lower female than male sensitivity to pheromones. Invertebrates, including insects and crustaceans, have specialised chemosensory organs, such as antennae, that detect environmental chemicals (Su et al., 2009). For example, male moths and beetles have larger and more complex antennae than females, which allow them to detect pheromones from farther away and with greater precision (Jourdan et al., 1995; Symonds et al., 2012). These differences in chemical sensory organs may be related to differences in reproductive behaviour and communication between the sexes in invertebrates. Unfortunately, most studies focus on female pheromones and male responsiveness to them (Doall et al., 1998; Marco et al., 1998; Winfrey and Fincke, 2017; Stamps and Shaw, 2019; Liu et al., 2022). Thus, the bias towards research on males and conspicuous visual ornaments has left a blind spot regarding female olfactory ornaments (Hare and Simmons, 2019).

Another non-exclusive explanation could be that females do not gain advantages from responding to chemical cues released by males but are equally attracted to both sexes via aggregating pheromones. These long-range pheromones are emitted by and attractive to both sexes (Wertheim et al., 2005). Individuals may aggregate for the benefit of living in a group; for example, by avoiding predation (Raveh et al., 2019), or aiding feeding (Tanaka et al., 2018) and reproduction (Roff et al., 2017). Aggregating pheromones are also essential for internally fertilising aquatic animals (Wyatt, 2014), and tardigrades could rely on them to find mates. For example, barnacle cyprids, the mobile larval form of barnacle, release a pheromone that attracts other cyprids to the location. This behaviour is thought to be an adaptation that increases the chances of successful settlement by bringing together many individuals in a small area. This pheromone also plays an essential role in the reproductive process by helping to gather conspecific cyprids in the same location to increase the future chances of fertilisation (Matsumura et al., 1998).

Conclusion

This study provides the first step in understanding intersexual communication in tardigrades by comparing distinct types of chemical signals, and the behavioural response of both sexes to them. Tardigrades were able to detect conspecifics using waterborne signals but did not respond to deposited trail cues. Moreover, females and males behaved differently: females showed no

sex-based preferences (if any), whereas males discriminated sex through waterborne signals, showing a strong preference to associate with females. Furthermore, in the absence of waterborne cues, males (but not females) can follow opposite-sex individuals, but only while maintaining direct body contact. Our results align with the very limited literature about reproductive behaviour, especially regarding the pre-copulatory stage, in tardigrades. Future work should focus on identifying the chemical compounds used in intersexual communication in this understudied phylum.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.C., S.C.; Methodology: J.C., S.C., Ł.M.; Validation: J.C.; Formal analysis: J.C.; Investigation: J.C.; Resources: Ł.M., S.C.; Data curation: J.C.; Writing - original draft: J.C., S.C.; Writing - review & editing: J.C., K.E.K., Ł.M., S.C.; Visualization: J.C.; Supervision: K.E.K., Ł.M., S.C.; Project administration: S.C.; Funding acquisition: S.C.

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Data availability

R code for the statistical analysis and the data related to it from this study are openly available in JYX: <https://doi.org/10.17011/jyx/dataset/88887>.

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II

THE COST OF BEING TOUGH: EFFECT OF ANHYDROBIOSIS ON MATE CHOICE AND REPRODUCTIVE SUCCESS IN A MACROBIOTID TARDIGRADE

by

Justine Chartrain, K. Emily Knott, Łukasz Michalczyk, Vilma Vaherto & Sara Calhim
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III

SPERM STORAGE ABILITY IS ASSOCIATED WITH OVIPOSITION BUT NOT MATING BEHAVIOUR: DIFFERENCES IN MACROBIOTID TARDIGRADES

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