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Chemical communication in the spider *Pisaura mirabilis*

Dissertation
von

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Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Erklärung

Hiermit erkläre ich,

- dass die Dissertation weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
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Author Contributions

Publication I: Testing presence of directionality information in female spider silk trails through male trail-following behavior

CT, MB and TC conceived and designed the study; KÖU collected data for Experiment 1; ML and MB collected data for Experiment 2; MB collected data for Experiment 3; MJE and JOW provided logistics and assisted with data collection for Experiment 3; MB drafted the manuscript and analyzed data; CT and TC contributed to writing and editing. All authors have read, provided comments and agreed to the final version of the manuscript.

Manuscript I: Female spiders performing maternal care reduce silk-bound chemical signalling

CT and MB conceived and designed the study; MB collected data, drafted the manuscript and analyzed data; CT revised the manuscript. All authors have read, provided comments and agreed to the final version of the manuscript.

Publication II: Female state and condition-dependent chemical signaling revealed by male choice of silk trails

CT conceived and designed the study, and revised the manuscript; KÖU collected data; MB analysed the data; MB and KÖU drafted the manuscript. All authors have read, provided comments and agreed to the final version of the manuscript.

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Manuscript 2: Chemical signalling bound to silk of male nuptial-gifts does not depend on male feeding condition

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Shared first-authorship statement

I hereby declare that I am aware that the work in the paper/manuscript entitled

Female state and condition-dependent chemical signaling revealed by male choice of silk trails

of which I am shared first-author, will form part of the PhD dissertation by PhD student

Michelle Beyer

who made a

proportional contribution to data curation,

proportional contribution to methodology,

major contribution to the work in the analysis and visualization phase,

proportional contribution to the work in the writing phase.

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Summary

Chemical communication represents one of the most ancient and widespread forms of communication and has been extensively studied for more than a hundred years. Yet, for most of this period, research on chemical communication has been largely driven by traditional views on sex roles in reproduction, leading to biased literature that is skewed towards females chemically signalling and males detecting those signals. It was only in the last one to two decades that research slowly began to include investigations on male chemicals and females exerting choice based on those. While most of our understanding of chemical communication comes from studies on insects, other taxonomical groups, such as spiders, lag behind. Motivated by the need to expand our understanding on the functions of chemicals in a reproductive context for both sexes in this taxonomical group, I investigated in this thesis the influence of silk- and cuticle-borne chemical communication on male mate search and reciprocal assessment between male and female spiders. Specifically, I aimed to understand the role of silk- and cuticle-borne chemicals during inter-sexual communication in the web-less hunting spider *Pisaura mirabilis*, a species known for its peculiar mating behaviour that involves male offering of nuptial-gifts to the female.

Mate search is essential part of reproduction and particularly challenging in solitary animals, such as cursorial spiders, that live widespread in their habitats. Trails, which are paths in the environment for example created by pheromone deposition on a substrate, represent one of the most efficient and target-oriented means for mate search. Specifically, trails increase the chances of finding mates compared to random search, especially if these trails convey directionality information that indicate the movements of the trail producer. Yet, trail directionality is rarely investigated and poorly understood. In Chapter 1, I investigated presence of directionality information in female spider silk trails by exposing males to unidirectionally deposited female silk, and scoring male trail-following direction with reference to female movement. I further explored potential influences of female phenotypic traits on male trail-following behaviour. I found overall lack of general directional trail-following (i.e., males following trails in female direction). However, males followed trails more often when they were unwashed (opposed to washed with solvents to remove putative chemicals) or sourced from females that were relatively larger (opposed to smaller) than the male. These results suggest context-dependent male directional trail-following, possibly reflecting male mate choice, and likely based on chemical trail attributes assessed through probing of silk lines that convey information on female quality.

Male mate choice has traditionally been overlooked, with mate preferences and choices largely attributed to females only. However, when there is large variation in female quality and/or when mating is costly for males, as for costly spider nuptial-gifts, selection should favour male discriminatory abilities and choice. Female reproductive potential varies with female developmental and reproductive state, and/or body condition, and males are expected to exert mate choice towards females that are of higher reproductive value. In Chapters 2 and 3, I studied male behavioural responses in the form of courtship investment or trail-following, when sequentially or simultaneously exposed to silk sourced from females varying in their phenotypic states. Females signal their developmental and reproductive state, but not mating state, as well as their body condition. Males adjusted their courtship investment and exerted trail-following choices based on these female states. Specifically, males courted adult females more often than juvenile or adult ones, preferred high-condition females over low-condition ones, and did not differentiate between mated and unmated females, whereas males decreased their courtship towards females that carried their fertilized egg-sacs. Such differentiated male behavioural responses might reflect a male strategy in light of the high costs of courtship.

In the context of mate assessment, male chemicals are rarely viewed as secondary sexual traits that potentially affect female mating decisions. However, male chemicals are also important for reproduction as they potentially affect female mate choice either by reliably reflecting male underlying qualities or by manipulating the female into matings. In Chapters 4 and 5, I explored the presence and variation of male chemical signalling via the nuptial-gift and its role in female mate choice. For this purpose, I observed female mate acceptance behaviours to chemical properties of male gift silk, and investigated whether these behaviours are influenced by male phenotypic traits. I found that chemicals appear to be present on the silk covering male nuptial-gifts, shown by higher female acceptance of gifts with unwashed opposed to silk washed with solvents. However, there was no variation in mass-dependent silk-borne chemical signalling, as females accepted gifts produced by males of high and low mass equally likely. While male silk-borne chemicals prime female responses in mate choice, females may base their mate choice on directly accessible mate traits instead of silk-borne ones, most likely due to the male's ability to use silk to mask non-nutritive gift contents and cheat females into mating in this system.

Overall, this dissertation provides important knowledge on chemical communication in a reproductive context and without the limitations of traditional views on fixed sex roles. I show that an impressive variety of conditions and innate states influence both chemical signalling and mate choice, with the latter itself presenting in many facets ranging from courtship investment, trail-following decisions to direct mate acceptance. Both sexes in my model species assess and produce chemicals, consequently influencing mate choice decisions in the other sex. My work highlights the importance of conducting research unobstructed by sex-biases, as only such unbiased research offers to opportunity to fully understand inter-sexual chemical communication.

General Introduction

Chemical communication is considered the most ancient and widespread form of information transmission, and its role in animal social interactions and reproduction has been acknowledged for over a century (Darwin 1871; Wyatt 2003; Bradbury and Vehrencamp 2009). Chemical information transfer occurs between a sender that is signalling and a receiver that is responding by altering its behaviour (Wilson 1975). Such information transfer has evolved either only on the side of the receiver, with information being sent inadvertently as a chemical cue (e.g., age, size), or on sides of both sender and receiver, with information evolved to be transmitted in form of chemical signals, such as pheromones (Maynard Smith and Harper 2003; Wyatt 2003, 2014). Pheromones are chemicals evolved for intra-specific communication, with one individual emitting the pheromone and a conspecific receiving and behaviourally or physiologically responding to it (Karlson and Lüscher 1959). For example in bees and termites, pheromones emitted by the queen elicit courtship display in males or affect development by inhibiting or activating ovary growth of workers in the colony (Hoover et al. 2003; Oi et al. 2015). Information transmission can be substrate-borne, requiring physical contact between the emitting source and receiver, such as with urine traces in male mice that upon direct contact invoke both female attraction and associative learning with respect to the odour of an individual male (Roberts et al. 2010), or mucous trails in molluscs that carry information about the trail producer’s location and characteristics (e.g., sex, size) (Ng et al. 2013). Information transfer can also be air-borne, requiring no direct contact, as for example in garter snakes in which courtship is terminated by exposure to an air-borne scent from mating conspecifics (Shine and Mason 2012), or in case of air-borne scent marks present in many mammals, providing information about characteristics such as identity or sexual receptivity (e.g., Michael et al. 1971; Kimura 2001; Rekwot et al. 2001; Gómez-Baena et al. 2023).

Chemical cues and signals cover a wide range of important functions in reproduction, forming the basis of both mate localization and mate assessment as they can effectively convey information about the sender’s characteristics and internal states, including the species, population, sex, identity, location, genetic quality, immune competence, age or sexual receptivity (Rantala et al. 2002; Johansson and Jones 2007; Wyatt 2010; Ng et al. 2013). The emission of such chemicals is often sex-dependent. In many species, there is a bias for males investing more in mate search and courtship than females (Fromhage et al. 2016), usually resulting in the females sending information as part of their advertisement and the males having evolved sensory detection mechanisms to understand such information. This female bias is also mirrored in the literature, with most studies focusing on female chemical signalling (Kittredge and Takahashi

1972; Johansson and Jones 2007; Keller-Costa et al. 2015; Coombes et al. 2018), leaving male chemicals much unattended. However, male chemical signalling also plays an important role for mate search, courtship and mating, and it has the potential to be shaped both by sexual selection through female choice, if chemicals reliably reflect the male's underlying condition (Steiger and Stökl 2014), and by sexual conflict, if males manipulate females into matings beyond female reproductive interests (Arnqvist and Rowe 2005).

In many arthropod species, chemical communication is seen as the main channel of information transfer (Butler 1967; Greenfield 2002; Ando et al. 2004; Cardé and Millar 2004; Hoffmann et al. 2006), together with vibratory signalling (Hill 2008; Cividini and Montesanto 2020). Chemical cues and signals in arthropods can be emitted directly into the air (e.g., odour plumes of moths (Weatherston and Percy 1977)), they can be attached to the sender's cuticle (e.g., cuticular hydrocarbons (Howard et al. 2003; Howard and Blomquist 2005; Blomquist and Ginzel 2021)) or deposited onto a substrate (Pompanon et al. 1997; Cooper and King 2015). Particularly vast knowledge on chemical communication has been acquired in insects, with more than 3,000 chemically identified pheromones, of which more than 1,000 are identified in lepidopteran (butterflies, moths) and hymenopteran (bees, ants, wasps) species alone (Wheeler and Duffield 2019; Petkevicius et al. 2020). Partly due to its major importance for pest control management, for many insect pheromones there also exists profound understanding of the biosynthetic pathways (Tillman et al. 1999; Blomquist 2003; Ando et al. 2004; Keeling et al. 2004), production sites, such as Dufour's, labial, mandibular, anal or sternal glands (Wheeler and Duffield 2019) and reception structures, such as single-pored trichoid (i.e., hair-like) sensilla for contact-chemoreception ("taste"/gustation), and wall-pores or multiporous sensilla for airborne chemical reception ("smell"/olfaction), both present in antennae (Meinecke 1975; Hunger and Steinbrecht 1998; Steinbrecht 1999). Behavioural responses to many known pheromones are also well-studied in insects (Shorey 1973; Witzgall et al. 2010; Yew and Chung 2015).

Contrasting to the breadth of knowledge accumulated in insects, our understanding of chemical communication in spiders lags behind. Research in spiders most frequently confirms chemical communication through behavioural studies (Prouvost et al. 1999; Papke et al. 2001; Roberts and Uetz 2004; Gaskett 2007; Henneken et al. 2015; Scott et al. 2015; Beyer et al. 2018). As discussed below, physiological (i.e., biosynthetic) and/or mechanistic (i.e., secretion/production) pathways of chemical communication remain instead less explored (Schulz 2013; Trabalon 2013; Fischer 2019). In spiders, large part of chemical communication is taking place via the means of chemicals attached to the animal's cuticle or silk, with most research focussing on the latter (Gaskett 2007). Silk consists of protein fibres (Foelix 2010) and is produced by every species of spider, creating an excellent substrate for chemical communication that is available at any given time and space, allowing for continuous chemical information transmission while at the same time avoiding the risk of attracting predators or parasitoids (i.e., insects whose larvae live inside a host as parasites, eventually killing it) to the spider’s body (van Baarlen et al. 1996). Similar to other taxonomic groups, there also exists a sex-bias in spider literature, leading to much knowledge on the chemical composition of female silk functioning to attract mate searching males, while chemicals in male silk remain poorly explored (Scott et al. 2018a).

Motivated by the need to expand our understanding of the functional role of chemicals in a reproductive context for both sexes in this taxonomical group, I investigated in this thesis the influence of silk-borne chemical communication on male mate search and reciprocal mate assessment between male and female spiders.

Chemical communication in spiders via silk and body cuticle

Due to their solitary lifestyle, often limited vision and widespread distribution in their habitats, chemical communication is commonplace in many spider species, making them an excellent model for investigating the role of chemical communication in different aspects of reproduction (e.g., Anderson and Morse 2001; Tichy et al. 2001; Gaskett 2007; Henneken et al. 2017; Fischer 2019). Many spiders emit chemicals from their cuticle and/or silk that can either be transmitted via air-borne transmission or upon contact (Gaskett 2007). A cuticle is covered by surface-lipids that primarily function as protection against water loss and desiccation (Howard 1993). Cuticular hydrocarbons represent a major surface lipid class that is also often involved in chemical communication, specifically in insects (Singer 1998). While hydrocarbons are also important for spider chemical communication, spiders produce a wide variety of cuticular compounds including esters and ethers that allow, for example, the production of reliable signals that are not affected or masked by prey cuticular hydrocarbons (Schulz 2013). In spiders, cuticular compounds are essential both for inter-species recognition and intra-species communication. Upon contact, they allow the discrimination between conspecific and prey (Kullmann 1972), or conspecific and sibling-species (inter-species recognition) (Trabalon et al. 1997). However, they also serve family recognition during maternal brood care, or colony and/or group recognition in social groups (intra-species communication) (Trabalon et al. 1996; Trabalon 2013). Additionally, cuticular chemicals also induce courtship behaviour (Roland and Rovner

1983; Jackson 1987; Papke et al. 2001; Gaskett 2007) and transmit information on female hunger state (Trabalon 2011) and/or sexual receptivity (Prouvost et al. 1999). From a distance, cuticular chemicals also serve as long-range air-borne attractants of conspecifics during mate search (Miyashita and Hayashi 1996).

While cuticular surface lipids are important for chemical communication in spiders, silk is also a well-known vector for chemical signals, and a large body of research focusses on silk-borne chemical communication (Gaskett 2007). The addition of chemicals to silk allows spiders a continuous chemical emission without the necessity of active pheromone emission from glands which may hinder other activities such as prey capture (Schulz 1997). Many female spiders aid mate searching males by adding air-borne chemicals to their webs to attract mates from a distance (Witt 1975; Ross and Smith 1979; Jackson 1987; Chinta et al. 2010; Fischer et al. 2021), or by adding contact chemicals either to their web that upon contact with the silk induce exploration, or to their silk lines left during movements (namely, draglines) to induce silk line-following, for example, by stimulating the male (Dijkstra 1976; Tietjen and Rovner 1980; Bell and Roberts 2016). Many known chemicals in spiders also serve the purpose of sexually stimulating the other sex before and/or during courtship, for example, leading to increased courtship intensity (Blanke 1973; Witt 1975; Pollard and Jackson 1982; Jackson 1987; Baruffaldi et al. 2010; Scott et al. 2018b). Additionally, spiders that signal chemically provide information on their characteristics and states, including size, hunger state, body condition, age and mating state, setting the base for mate assessment for the information receiver (Riechert and Singer 1995; Rypstra et al. 2003; Stoltz et al. 2007; Xiao et al. 2009; Baruffaldi and Andrade 2015; Henneken et al. 2015).

Contrasting to the extensive knowledge of behavioural responses elicited by chemicals, there persists a lack of knowledge on identification, exact production sites (i.e., pheromone producing tissues) and perception structures for spider chemicals. The number of structurally identified pheromones in spiders remains small, with pheromones (and often their biosynthetic pathways) identified in approximately a dozen species out of the spider families Pholcidae, Araneidae, Linyphiidae, Theridiidae, Agenelidae and Ctenidae (Schulz and Toft 1993; Papke et al. 2001; Xiao et al. 2009; Chinta et al. 2010; Jerhot et al. 2010; Schulz 2013). The low number of identified pheromones may partly be explained by the low economic impact of spiders (especially compared to insects), experimental difficulties during the identification process (e.g., rearing of large numbers of animals not always easy or possible), small amounts of produced pheromones that can be acquired from cuticle or silk, and lack of chemical expertise (Schulz 2004, 2013; Gaskett 2007).

Unlike insects that typically synthesize pheromones in specific pheromone producing glands (Ando et al. 2004; Wheeler and Duffield 2019), such glands are not present in spiders. Instead, it is hypothesized that pheromone production in spiders occurs in one (or multiple) of the up to seven different types of silk glands (number depending on phylogenetic family, species and sex, with female orb-weavers possessing the highest number of silk gland types) that produce structurally and functionally different silk types (Vollrath 1992; Schulz 2004; Garb 2013). However, to date, there only exists one study that successfully investigated the origin of pheromone production in a spider species, tracing the synthesis of the female-specific contact pheromone of the spider *Steatoda grossa* back to the posterior aggregate silk gland (Fischer et al. 2022).

Spiders perceive contact (i.e., non-volatile) chemicals in the environment through hair-like tip-pore sensilla on the tips of their legs and pedipalps (i.e., modified appendages) (Foelix 1985). However, spiders are lacking separate physiological structures such as multiporous sensilla that are used for perception of air-borne (i.e., volatile) chemicals in insects (Forster 1980; Foelix 1985; Forster et al. 1987; Anton and Tichy 1994; Müller et al. 2020). Despite this lack, many behavioural studies clearly demonstrate that spiders are able to sense air-borne chemicals and, thus, are able to smell (Blanke 1973; Papke et al. 2001; Gaskett 2007; Uhl and Elias 2011; Uhl 2013). Consequently, recent research assumes that both volatile and non-volatile chemicals are perceived through tip-pore sensilla (Müller et al. 2020). However, this dual chemoreception is yet to be verified.

Regardless of our poor understanding of the sensory mechanisms, chemicals are essential in all steps of reproduction in spiders (Huber 2005): they play a major role in attracting mates from a distance as they allow the localization of solitary mates that are widely dispersed in their habitats, they transmit information crucial for induction of courtship, largely influencing the outcome of courting and mate acceptance, and are decisive for mate assessment as they convey individual characteristics to the signal receiver that are important indicators of quality, ensuring an increase of fitness when choosing the most suitable mates while lowering risks and costs of reproduction by avoiding unsuitable matches.

Spider chemical trails as means for mate search

Trails are found in many social insects and molluscs, as well as in reptiles and mammals (Wilson 1962; Gehlbach et al. 1971; Jeanne 1981; Jamon 1994; Nieh et al. 2004; Ng et al. 2013; Czaczkes et al. 2015) and likely represent the most potent and target-oriented means for finding mates across taxa. Trails are paths in the environment, created by a sequential or continuous act during movement (e.g., pheromone or urine deposition, repeated movement following the same path) that either physically alters the environment (e.g., game trails, cut-out trails in vegetation in leaf-cutter ants (McCaffery 1976; Howard 2001)) or adds chemical cues for both the trail producer (e.g., a trail to the burrow (McFaruume 1980)) and conspecifics (e.g., transmission of information about sex or reproductive state (Gehlbach et al. 1971; O'Donnell et al. 2004)).

Apart from gaining information on the trail producer's characteristics, an individual encountering a trail of a prospective mate has to choose a direction in which to proceed. While a correct choice leads the trail-follower closer to finding a mate, a wrong choice takes the animal further away from its goal. Consequently, any information that aids an individual in correctly determining the direction in which to follow such a polarized trail embedded with directionality information, provides benefits to both trail producer and follower (Cook and Cook 1975; Tietjen 1977; Rosengren and Fortelius 1987; Nieh et al. 2004). While trail-following is a common feature employed for mate localization by a large variety of animals throughout many taxa, embedded trail directionality has been described in only few species (Gehlbach et al. 1971; Tietjen and Rovner 1980; Jackson et al. 2004; Nieh et al. 2004; Ng et al. 2013) and its respective trail encryption is understood in even fewer cases (Ford and Low 1984; Jackson et al. 2004). For example, female garter snakes encrypt trail polarity during their movements by sequentially touching the surface of an ambient object (e.g., with their anterolateral body side) with pheromone secreting glands (Ford and Low 1984). Contrasting, ants create non-random bifurcation angles in their pheromone trail networks, providing every bifurcation with directional information (Jackson et al. 2004).

The large discrepancy between occurrence of trail-following and embedded trail directionality, might be explained through variation in species communication modalities, spatial distributions, population densities and life history. For example, trail directionality is not expected, when the costs for trail deposition including embedding of directionality information, in terms of energetic costs (i.e., production and/or secretion of substances) or ecological costs (i.e., increased visibility to predators), override the benefits of finding the trail producer. However, the limited evidence for trail directionality could also result from the small number of empirical studies mainly focusing on molluscan mucous trails and the pheromone trails of ants, leading to bias in the literature (Rosengren and Fortelius 1987; Jackson et al. 2004; Ng et al. 2013; Czaczkes 2015).

Cursorial web-less spiders represent an excellent system to investigate directionality in silk trails (with trails consisting of silk and added chemicals), due to their solitary lifestyle and almost ubiquitous use of silk lines accompanied with often reciprocal communication between the sexes. However, trail directionality has only been investigated in a handful of species (Dijkstra 1976; Tietjen 1977; Anderson and Morse 2001; Bell and Roberts 2016) and been found in just two species of wolf spiders, *Lycosa rabida* and *L. punctulata* (Tietjen 1977). The encryption mechanism of directionality information in spider silk trails remains unknown. Contrasting to trail-following, which is hypothesized to be induced chemically, trail directionality is thought to be encoded structurally, possibly using so-called attachment discs, silk structures anchoring the silk line to a substrate (Apstein 1899; Dijkstra 1976). Trail-following animals might be able to assess differences in silk tension surrounding the area of an attachment disc (Wolff and Herberstein 2017; Wolff et al. 2021), by using their legs or pedipalps both additionally possessing specialized chemo-sensing structures (Foelix 2010; Müller et al. 2020). Indeed, trail-following in many spider species is often accompanied with extensive probing of and pulling on silk threads through the male's pedipalps (Tietjen 1977; Tietjen and Rovner 1980). While trail-following and its implications on animal orientation are generally well-studied, directionality and especially the mechanisms for encoding directionality in trails remain almost completely unexplored.

Variation in female chemical signalling and male mate assessment

One of the purposes of chemical communication is to influence mate choice, for example, by providing information such as a signaller's reproductive quality to the information receiver, allowing the restriction of the set of potential mates (Wiley and Poston 1996). While traditionally females were considered to be choosy for the most suitable partners due to their limited number of costly eggs (Bateman 1948), there is increasing evidence for male choosiness (Bonduriansky 2001; Byrne and Rice 2006; Edward and Chapman 2011; Fitzpatrick and Servedio 2018). Specifically, male choosiness is predicted, when there is large variation in female quality and/or when reproduction is very costly for males (Andersson 1994; Kokko and Monaghan 2001; Herberstein et al. 2002; Andersson and Simmons 2006; Kvarnemo and Simmons 2013). Examples for costly reproductive aspects entail risky mate search (Berger-Tal and Lubin 2011), energetically demanding courtship (Woods et al. 2007), nutritional donations (Lewis and South 2012), or parental care (Sargent et al. 1986). Male choosiness also depends on male individual quality, with high- and medium-quality males exerting stronger mate choice than low-quality ones (Pollo et al. 2022). By asserting mate choice towards mates that impose lower costs, males can reduce costs associated with reproduction, for example, by choosing unmated or satiated females that pose lower cannibalism risks (Herberstein et al. 2002; Baruffaldi and Andrade 2015). In order to increase male fitness, selection should favour males that are able to discriminate between females varying in their reproductive value, for example, by using traits that are indicative of female quality, often reflecting female fecundity (e.g., body condition or size) (Bonduriansky 2001; Jones et al. 2001; Katvala and Kaitala 2001).

From the female perspective, variation in chemical release may lead to fitness benefits. Chemical cues and signals can be indicators of an individual's internal state, such as developmental or mating state, as they vary in their produced quantity (i.e., amount of components) or quality (i.e., the ratio of their components) due to associated changes in the signaller's internal chemistry (Johansson and Jones 2007; Thomas 2011). Sexually immature females should limit their chemical advertisement in order to prevent attraction of unwanted suiters, whose mating attempts could lead to costly sexual harassment (Arnqvist and Rowe 2013). Upon reaching sexual maturity and receptivity, females should start production of chemicals that convey information on female fertility and attract prospective mates to secure matings and avoid reproductive failure (Rhainds 2010). After a female mated, it may switch to halt emission of chemical attractants and/or to emit chemicals that repel mates (Thomas 2011; Weiss and Schneider 2022a) in order to avoid costs of superfluous matings (Arnqvist and Rowe 2013). Interestingly, specifically in spiders, it is at this time point – up to when a female has mated – at which most research of variation in chemical signalling stops (e.g., Riechert and Singer 1995; Roberts and Uetz 2005; Gaskett 2007; Stoltz et al. 2007; Eberhard et al. 2021) (but see, e.g., (Baruffaldi and Costa 2010)), disregarding the female's reproductive time beyond that point, including egg-laying, egg-sac production and maternal care. Female spiders should cease or limit chemical signalling after egg-sac production in order to avoid sexual harassment by males attracted during this phase of intensifying maternal investment with many female spiders performing some form of parental care (such as tending egg-sacs, or guarding them on webs), essential for the success of the brood (Xiao et al. 2009; Ruhland et al. 2016). Similarly, once this phase of maternal care is over, females should re-commence their chemical signalling and attract males once again, for example, to replenish diminished sperm stores (Perampaladas et al. 2008). Yet, female chemical communication between the sexes during maternal care remains unknown.

Aside from developmental and reproductive variation, female chemical signalling can depend on the female's underlying body condition. While pheromone production was, for a long time, assumed to be a low-cost signal (Wyatt 2014), an increasing amount of studies show considerable physiological costs on the signalling individual (Foster and Johnson 2011; Harari et al. 2011; Henneken et al. 2015), suggesting that chemical signalling could function as an honest indicator of the signaller's underlying quality and/or body condition (Zahavi 1975; Johansson and Jones 2007). Costs of chemical production can consist of constraints on energetic resources linked to the individual's body condition, as pheromone production may make use of available resources inside the animal (Blomquist et al. 2018). Nutrition also plays a key role in chemical production as amino acids and some fatty acids from the diet are often used as precursors for pheromones (Tillman et al. 1999; Blomquist et al. 2018; Blomquist and Ginzel 2021). Examples for variation in female chemical signalling linked to nutrition and/or body condition can be found across many taxa, for example, in moths (Jaffe et al. 2007; Foster and Johnson 2011), flies (Adams and Nelson 1990), spiders (Henneken et al. 2017b; Weiss and Schneider 2022b), reptiles (Shine et al. 2003), and mammals (Leon 1975). Further suggestive of costs of pheromone production are trade-offs in resource allocation (Stearns 1992), with chemical advertising reducing the survival of, for example, female moths (Harari et al. 2011).

While chemical signalling is often closely linked to physiological innate states such as developmental or reproductive state, chemicals can also be emitted strategically. A strategic emission/production of chemicals allows the signaller to adjust their chemical investment to reproductive needs that might change over time and space. For example, chemical production can be adjusted in response to social context, such as presence of (low-courting) prospective mates (Havrilak et al. 2015; Rehermann et al. 2016) or of competing signallers (Holdcraft et al. 2016). Chemical investment can also depend on physiological conditions, such as perceived risk of mating failure, with individuals increasing their signalling the closer they are to producing unfertilized eggs, equivalent to reproductive failure (Cory and Schneider 2016; Weiss and Schneider 2022a).

Despite the focus on female-produced chemicals and male discrimination based on these chemicals described above, male chemicals are also crucial for reproduction and form the base of female mate assessment by providing information on quality traits such as genetic background, immunocompetence or condition (Johansson and Jones 2007; Steiger and Stökl 2014).

Variation in male chemical signalling and female mate assessment

Selecting a suitable mating partner is often essential for female fitness by influencing the number and quality of her offspring, as females are seen as the sex investing more in reproduction as they produce large (costly) gametes and often provide some form of maternal care (Bateson 1983). Consequently, female choosiness is expected to reduce some of these costs associated with reproduction. As female reproductive needs vary over time and space, the degree of female choice is expected to change accordingly. For example, female choosiness depends on female age with young females being more choosy (Gray 1999; Pervez et al. 2004; Mautz and Sakaluk 2008), body condition and/or diet with high-condition and/or satiated females being more choosy than low-condition ones (Hebets et al. 2008; Moskalik and Uetz 2011), or male density with higher choosiness in high densities (Lehmann 2007). Female mating state also influences female choosiness (Richardson and Zuk 2023), with unmated females being less choosy and more receptive than mated ones as a choosy unmated female risks reproductive failure by remaining unmated (Rhainds 2010). Similarly to sexual selection in males, selection should favour females that are able to discriminate between males varying in their underlying qualities.

Interestingly, the majority of the research on spiders focuses on chemicals produced by females, with male-produced chemicals remaining much less explored. Male chemicals, as learned from research on insects, have several known functions. They can consist of aggregation or attraction pheromones as they attract females or both sexes, often using volatile male-specific compounds (Landolt 1997; Schlyter and Birgersson 1999; Dickens et al. 2002; Zhang et al. 2002; Lacey et al. 2004; Ejima 2015; Stökl and Steiger 2017). Male chemicals also function both as aphrodisiacs as they increase female receptivity for mating (Baker and Cardé 1979; Grillet et al. 2006; Ejima 2015) and as anti-aphrodisiacs, reducing female attractiveness to subsequent courting males after mating (Seidelmann and Ferenz 2002; Schulz et al. 2008; Estrada et al. 2011). Male-produced chemicals are also subject of female mate choice, conveying information on species-belonging, for example, in moths with females preferring the chemicals of conspecific males (Hillier and Vickers 2004), or immunocompetence, with female meal-worm beetles preferring males with better immunocompetence (i.e., encapsulation rate against novel antigens) (Rantala et al. 2002). Chemical production in males is also linked to nutrition and/or body condition, with examples being found in male moths (Harari et al. 2011; Gonzalez-Karlsson et al. 2021), beetles (Rantala et al. 2003; Ming and Lewis 2010), cockroaches (South et al. 2011), and flies (Johansson et al. 2005).

While in spiders to date only a single male pheromone has been chemically identified (Xiao et al. 2010), an increasing number of research shows behavioural evidence of putative pheromone production in males from seven species across five families (Scott et al. 2018a). Some of the chemicals produced by male spiders serve functions similar to those elicited by female silk-borne pheromones as they can induce female courtship behaviour (Ross and Smith 1979) or facilitate orientation (Roland and Rovner 1983; Suter and Hirscheimer 1986). However, most of the known male chemicals in spiders are suspected to serve as aphrodisiacs by increasing the female's acceptance to mate (Scott et al. 2012), as anti-aphrodisiacs by decreasing female attractiveness towards other males after the first mating (Scott et al. 2015), and to reduce cannibalism risks by inducing a state of quiescence (Gering 1953; Xiao et al. 2015) or lower aggression in females (Aisenberg et al. 2008). As male chemicals can influence female reproductive behaviour, for example, by increasing female willingness to re-mate, such chemicals have potential to lead to sexual conflict, with males forcing matings beyond female reproductive interests (Tuni et al. 2020). Therefore, investigating their role in a mating context remains key to advance our understanding of spider inter-sexual communication.

Thesis aims and study system

In this thesis, I aimed to shed light on the role of silk- and cuticle-borne chemical communication in male mate search and reciprocal assessment between both male and female spiders. Consequently, I conducted a number of empirical studies to improve our understanding of the functional role of chemicals in reproduction in this taxonomical group, investigating inter-sexual communication without traditional assumptions on sex roles.
Specifically, this dissertation aims to provide insights into:

1) **The presence of directionality information in female spider silk trails in the context of male mate search**
   The use of trails is widespread among animal taxa, but adding directionality information that allow trail-following individuals to follow trails correctly to their source is rarely investigated and poorly understood. Exposing males to unidirectionally deposited female silk, and scoring their trail-following direction with reference to the female movements, allows uncovering of present directional information aiding males in their mate search. Addressing variation in female phenotypic traits can further uncover potential female influences on male trail-following behaviour (Chapter I: Beyer et al. 2023, accepted in Behavioral Ecology and Sociobiology).

2) **Variation in female chemical signalling and its role in male mate choice**
   Female reproductive potential varies with their developmental and reproductive state, and/or body condition. Hence, males should exert mate choice towards females that are of higher reproductive value. Studying male behavioural responses when sequentially exposed to silk sourced from females varying in their phenotypic states indicates male differential

effort during courtship (Chapter II: Beyer et al., Manuscript unpublished). Testing male trail-following preferences in a binary-choice setup towards silk trails of females varying in their phenotypic states allows to unveil male choice decisions during mate search (Chapter III: Beyer et al. 2023, published in Behavioral Ecology).

3) **The presence and variation of male chemical signalling and its role in female mate choice**

While research in spiders traditionally focusses on female chemicals and male mate assessment thereof, male chemicals are also critical for reproduction, as they potentially affect female mate choice by reliably reflecting a male's underlying condition or via manipulation. Investigating female behavioural responses to chemical properties of male silk allows uncovering presence of male silk-borne chemicals (Chapter IV: Beyer et al. 2021, published in Biology Letters). Addressing variation in male phenotypic traits can further elucidate influences on female mate acceptance (Chapter V: Beyer et al., Manuscript unpublished).

The spider Pisaura mirabilis as a study system

The European nursery web spider *Pisaura mirabilis* (Pisauridae) is a web-less hunting spider and represents an ideal system to study chemical communication between the sexes in a reproductive context. *P. mirabilis* is common and can be found in grasslands and deciduous woodlands throughout the Palearctic region, covering wide ranges, for example, in Europe (Clerck 1757). Male *P. mirabilis* are mainly known and investigated for their nuptial-gifts – food donations consisting of silk-wrapped prey – that are essential for mate acceptance and mating as they are offered by the male during courtship and consumed by the female during copulation (Nitzsche 1988; Drengsgaard and Toft 1999; Stålhandske 2001; Tuni and Bilde 2010; Albo et al. 2011b; Tuni et al. 2013; Ghislandi et al. 2017, 2018).

In Southern Germany, the spider's life cycle is annual: spiderlings hatch in summer, hibernate as juveniles during winter, reach adulthood during spring and reproduce in summer/autumn. Females carry their eggs in their mouthparts (i.e., chelicerae) for about three weeks and do not feed during this period (Austad and Thornhill 1986). After this time, the egg-sac is attached to vegetation and a nursery web is spun around it. While the female is guarding the web, it might begin to feed again; additional matings are also possible, though, rare (Austad and Thornhill 1986). In the laboratory, the development to adulthood can be accelerated and winter hibernation can be skipped by providing a warm environment (approx. 20–25 °C) (Tuni and Bilde 2010), enabling the experimental use of two generations of spiders within one year.

In this species, silk is used by males for gift construction, and both sexes leave silk lines during movements. Such draglines may be key for finding potential mates during the breeding season when gift-carrying males are found wandering in the field (Ghislandi et al. 2018). Female silk lines are likely to contain chemicals that upon direct contact (Beyer et al. 2018) induce male courtship behaviour in form of tremulations of their opisthosoma (i.e., vibrations), leg rubbing and gift-wrapping (Lang 1996; Bilde et al. 2007; Eberhard et al. 2021). Chemicals in female silk lines are also known to transmit information on female developmental state, with males courting adult females more than subadults (Eberhard et al. 2021). Contrasting, males do not differentially invest in females that vary in their mating states, courting unmated and mated females equally (Tuni and Bilde 2010; Eberhard et al. 2021).

Male silk around the prey of nuptial-gifts has several functions as it facilitates keeping of the mating position and prolongs copulation duration, and consequently sperm transfer, by extending female feeding duration (Lang 1996; Drengsgaard and Toft 1999; Bilde et al. 2007; Andersen et al. 2008; Albo et al. 2013). The production and transport of nuptial-gifts is associated with high costs for males as they are confronted with lost foraging opportunities in order to donate their prey to females (Albo et al. 2009), costs of silk production (Lang 1996; Albo et al. 2011a), as well as costs of movement (Prokop and Maxwell 2012) and metabolism associated with carrying the gift during mate search (Prokop and Okrouhlík 2021). However, males can reduce these costs by strategically using silk in order to hide non-nutritive gift contents, such as insect exoskeletons (e.g., resulting from the male feeding on the prey itself) or plant seeds, leading to males cheating the female into matings (Ghislandi et al. 2017). Females, on the other hand, derive both nutritional benefits from gift consumption (e.g., earlier oviposition) (Bilde et al. 2007; Toft and Albo 2015), and indirect (genetic) benefits from mating with multiple partners (e.g., increased egg-hatching success) (Tuni et al. 2013).

While, as stated above, there is evidence for variation in female chemical signalling regarding their developmental state, research investigating male trail-following of female silk lines and male mate choice for trails of females varying in their reproductive value (developmental, reproductive and/or body condition) is lacking. Similarly, male responses in the form of courtship effort towards silk-borne signals of females during maternal care (i.e., tending for their egg-sacs) remain unstudied. Despite there being scarce evidence for male-produced chemicals in other spider species, it remains unknown whether *P. mirabilis* males use chemical information attached to their gift silk, possibly in order to convey their qualities and/or manipulate females into mate acceptance.

Chapter I

Publication I: Testing presence of directionality information in female spider silk trails through male trail-following behaviour

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ORIGINAL ARTICLE

Testing presence of directionality information in female spider silk trails through male trail-following behaviour

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Abstract

Mate search is challenging for solitary species. Trails represent a particularly potent, target-oriented means for finding mates, as trail-following increases encounter rates between individuals compared to random search. Embedding directionality information into the trail allows individuals to follow trails correctly to the source. Yet, directionality remains poorly understood. Spiders deposit trails during locomotion consisting of silk-lines and substrate-borne chemicals. We conducted multiple experiments to test whether female silk-trails convey directionality information, whether directionality is chemically or structurally encoded and depends on female phenotype. We also examined whether males interact with silk in a way that suggests information gathering. We exposed males of the cursorial spider *Pisaura mirabilis* to female trails deposited unidirectionally, and scored their trail-following direction (i.e. same as or opposite to the females'). Tests were repeated after washing trails with a solvent to remove putative chemicals, and by sourcing silk from females of different feeding regimes. While we found little evidence for male directional trail-following, we did find that unwashed trails were more likely to be followed than washed trails. Similarly, trails of relatively larger females were more likely to be followed correctly than those of smaller females. Males extensively probed and pulled on silk lines with their appendages, suggesting gathering of chemical and tactile information. Taken together, results suggest that directional trail-following is selected for only under specific contexts in this species. Chemical attributes of trails may convey information on female quality, with directional trail-following reflecting male mate choice in a system characterized by costly male nuptial-gifts.

Significance statement

In the context of male mate search, following conspecific trails increases the chances of encountering a mating partner, especially if trails provide information about the direction the conspecific went. Yet, trail directionality remains poorly understood. Female spiders deposit silk trails as they walk. We overall show that males follow trails directionally only under a specific context. Males were more likely to follow correctly when trails were unmanipulated (compared to being washed with solvents to remove chemicals) and when they were deposited by relatively larger females (compared to smaller ones). Chemical attributes of trails may potentially indicate directionality, while decoding movement direction from trails of larger females may reflect male preferences for females of higher reproductive value.

Keywords Trail-following · Directionality · Spider · Pisaura mirabilis · Chemical communication · Silk

Introduction

Finding a mating partner is essential for animal reproduction, and potentially challenging for solitary species with widely dispersed individuals in the population (Bell 1990). Animals rely on multiple cues (i.e. incidental sources of information) and signals (i.e. information evolved to be communicated and to change the behavior of the receiver) during mate search (Bradbury and Vehrencamp 2009; Stevens 2013). Long distances between senders and receivers are often covered through auditory signals, as bird songs (Hinde 1971; Todt and Naguib 2000) and cricket calls (Forrest 1982), or by producing long-range airborne chemicals, as odor plumes of female moths (Butt and Hathaway 1966; Weatherston and Percy 1977). Vibrational (Rovner and Barth 1981; Virant-Doberlet and Cokl 2004) and visual signals (Rutowski et al. 2001) are instead generally used for mid- to close-range mate localization. Animals can also use trails for mate search that are deposited on the substrate and accessible to conspecifics. Such trails can consist of chemical information,

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Extended author information available on the last page of the article

such as pheromones, which are chemicals emitted to alter a conspecific's behavior (Karlson and Lüscher 1959)). Trails can also be created through physical alterations of the environment, for example by cutting trails into the vegetation for eased locomotion as done by leaf-cutter ants (e.g. Howard 2001)). Trails represent perhaps the most potent and target-oriented means for finding mates and food sources across taxa (e.g. social insects (Wilson 1962; Jeanne 1981; Nieh et al. 2004; Jarau 2009; Bordereau and Pasteels 2010; Czaczkes et al. 2015), reptiles (Gehlbach et al. 1971; Cooper and Vitt 1986), molluscs (Cook and Cook 1975; Ng et al. 2013; Vong et al. 2019) and mammals (Jamon 1994; Harmsen et al. 2010)). By adding specific information to a trail (e.g. pheromones), the trail producer can inform a following conspecific about its sex, mating status or body condition, allowing both advertisement of the producer and assessment by the trail follower (e.g. Gehlbach et al. 1971; Edwards and Davies 2002; O'Donnell et al. 2004; Baruffaldi et al. 2010). Apart from extrapolating information on the phenotypic characteristics of its producer, when encountering a trail, an individual has to choose a direction in which to proceed. While a correct choice results in a high probability of finding a mate, a wrong choice takes the animal further away from its' goal. Thus, any aid to the individual in determining the direction to follow such as polarized trails embedded with directionality information, provides benefits (Cook and Cook 1975; Tietjen 1977; Rosengren and Fortelius 1987; Nieh et al. 2004). Embedded trail directionality, appears to be rare as it has been described in only few species (e.g. Gehlbach et al. 1971; Tietjen and Rovner 1980; Jackson et al. 2004; Nieh et al. 2004; Ng et al. 2013), and its respective encryption in the trail is understood in even fewer cases (Ford and Low 1984; Jackson et al. 2004). For example, snakes can encrypt trail polarity by sequentially touching a specific surface of ambient objects (e.g. anterolateral) with pheromone secreting glands during movements (Ford and Low 1984). Ants instead create non-random bifurcation angles in their trail networks, allowing the polarity of a trail to be read at every bifurcation (Jackson et al. 2004). The occurrence of directionality information in trails may reflect variation in species communication modalities, spatial distributions, population densities and life history. Trail directionality is, for example, not expected when the costs of trail deposition including embedding of directionality information, in terms of energetic costs (i.e. production and/or secretion of substances) or ecological costs (i.e. increased visibility to predators), override the benefits of finding the trail producer. The limited evidence for trail directionality may, however, also result from a small number of empirical studies focusing on few taxonomical groups (e.g. molluscan mucous trails and pheromone trails of ants) (Rosengren and Fortelius 1987; Jackson et al. 2004; Ng et al. 2013; Czaczkes et al. 2015), leading to bias in the literature. Hence, overall trail directionality remains poorly understood.

Spider silk lines, consisting of proteinaceous silk fibers and other substances (i.e. pheromones) (Foelix 2010), are a medium for mate localization. Females of many web-building spiders add airborne pheromones to their webs to attract mating partners from a distance (Witt 1975; Ross and Smith 1979; Jackson 1987; Chinta et al. 2010; Fischer et al. 2021). Direct contact with the female's web often induces explorative behavior and/or positive chemotaxis in the male, further aiding mate search (Suter and Renkes 1982; Watson 1986; Suter et al. 1987). During movements, web-less cursorial spiders release long silk lines (i.e. draglines) with a stabilizing function (van der Kraan and Richter 1970). Chemical and tactile properties of draglines are known to stimulate male sexual behaviors (Jackson 1987; Barth 1993; Chinta et al. 2010; Beyer et al. 2018; Eberhard et al. 2021) and to induce random mate search (Hegdekar and Dondale 1969; Yoshida and Suzuki 1981; Taylor 1998). Draglines represent trails, as males that follow these silk lines can greatly increase their chances of finding a mate compared to random search (Tietjen 1977; Anderson and Morse 2001;

Bell and Roberts 2016; Scott et al. 2019). Yet, trail directionality has been investigated in a handful of spiders (Dijkstra 1976; Tietjen 1977; Anderson and Morse 2001; Bell and Roberts 2016) and only been found in two species of wolf spiders, *Lycosa rabida* and *L. punctulata* (Tietjen 1977). The encryption of directional information in spider trails has yet to be decoded. While trail-following is hypothesized to be induced chemically, trail directionality itself is thought to be encoded structurally in spider silk, possibly with the help of so called attachment discs, silken structures that anchor the silk line to the substrate (Apstein 1899; Dijkstra 1976). Spiders might be able to assess differences in silk tension in the area surrounding the discs (Wolff and Herberstein 2017; Wolff et al. 2021) by using their legs or pedipalps (i.e. a modified pair of appendages), which additionally possess specialized chemo-sensing structures (Foelix 2010; Müller et al. 2020). Indeed, trail-following is described as being accompanied by extensive probing of silk threads through the male's pedipalps (Tietjen 1977; Tietjen and Rovner 1980).

With their solitary lifestyle and the almost omnipresent use of silk lines for reciprocal communication between the sexes (Beyer et al. 2018; Eberhard et al. 2021), cursorial spiders, such as the nursery-web spider *Pisaura mirabilis*, represent an extremely promising system for investigating directionality in silk trails. This species is well-known for the male's food donations to the female (i.e. nuptial gifts) that are crucial for mate acceptance and mating (Nitzsche 1988; Stålhandske 2001; Albo et al. 2011; Ghislandi et al. 2017). Draglines of females are embedded with tactile chemicals (Beyer et al. 2018) that induce male courtship behavior (Eberhard et al. 2021) and silk-wrapping of nuptial gifts (Bilde et al. 2007; Albo et al. 2011; Ghislandi et al. 2017; Magris and Tuni 2019). These also communicate female developmental state (Eberhard et al. 2021) and body condition (Beyer et al. unpublished) to the male. During the mating season, males and females actively move around the vegetation (Ghislandi et al. 2018) and would benefit from depositing silk trails with directionality information to increase their encounter rates. Due to the costs of gift construction associated to lost foraging opportunities, silk production and carrying costs (Lang 1996; Albo et al. 2011; Prokop and Okrouhlík 2021)), males are particularly expected to exploit information left in the environment by female trails to orientate and locate females during mate search. From the female perspective, being found by a male not only ensures multiple matings but also leads to foraging benefits due to the nutritional value of nuptial gifts (Tuni et al. 2013; Toft and Albo 2015). Embedding directional information in trails may also likely depend on the female's phenotype, as in spiders, silk line structural properties such as density and size (Vollrath 1999) as well as chemical signaling bound to silk (Weiss and Schneider 2022a; Weiss and Schneider 2022b), may depend on the releasers' body mass. Although a formal test for directionality is lacking, when given a choice, *P. mirabilis* males preferentially follow silk trails deposited by females in high body condition, compared to those of low-condition females (Beyer et al. unpublished). These findings may suggest chemical and/or structural differences in silk trails that trigger a differential response in mate searching males. Male trail-following may ultimately reflect mate choice decisions, with female body mass, size and condition (i.e. a trait reflecting both body mass and size) indicating higher fecundity in arthropods (Bonduriansky 2001; Leather 2018) including spiders (Danielson-Francois et al. 2002; Johnson et al. 2014), and hence females of higher reproductive value. Not least, trail-following may also be affected by the male's own individual phenotypic characteristics, (body condition, mass or size), known to affect the strength of choice (Pollo et al. 2022) , or on those of both interacting sexes.

Here, we hypothesize that i) female silk trails provide directional information to males and that such information is either chemically or structurally mediated; ii) silk trails provide information on female phenotypic characteristics (body mass, size and condition) that affect directional trail-following in males, and iii) males assess silk line properties (e.g. chemicals and/or silk tension) using their appendages. Hence, we conducted three experiments. In a first experiment, we placed males in the middle of a silk trail unidirectionally deposited by a female, and scored whether males moved in the same or opposite direction to female movements during trail deposition. In this experiment, we also included a treatment, where putative chemicals of trails were removed by washing with a solvent. If trails provide directional information, males are expected to move in the same direction the silk was deposited by females. If this information is solely chemically transmitted, directional information should be lost when trails are washed, whereas if it is mediated by silk structure, males should move in the direction of the female regardless of trail washing. In a second experiment, we manipulated female body condition through differential feeding regimes, and tested whether male movements in the same or opposite direction to female movements during trail deposition depend on female body mass, condition and size. If trail deposition reflects female phenotypic characteristics, males should be more likely to move in the direction of females with high trait values, representing more fecund mating partners. Finally, in a third experiment, we analyzed high-speed video recordings of males in contact with female trails surrounding the area of silken attachment discs. If males perform leg and pedipalp movements on silk lines, these might indicate male sensory assessment of trails, such as silk tension assessment by pulling and chemical assessment by probing silk.

Experiment 1. Do female silk trails convey directional information to males, and is this chemically or structurally mediated?

Methods – Experiment 1

Animal collection and rearing

Juvenile and subadult *Pisaura mirabilis* of both sexes (*n* = 19 females, *n* = 31 males) were collected in Planegg-Martinsried near Munich (Germany) during spring 2021. They were brought to the lab of the Ludwig-Maximilians-University of Munich, where they were individually placed into transparent plastic vials (9 cm height, 5 cm diameter) that were covered with foam lids. Animals were reared at room temperature (approx. 23 °C) and under natural photoperiod (15 h light: 9h dark). To maintain high humidity, vials contained moss that was sprayed with water on feeding days. Spiders were fed three times a week, depending on their age, using either 15 fruit flies (*Drosophila melanogaster*) for juveniles, or two cricket nymphs (*Gryllus bimaculatus*) or two house flies (*Musca domestica*) for (sub-)adults. Prey type for (sub-)adult animals varied due to logistic reasons. To minimize diet-dependent variation in chemical composition of silk (Craig et al. 2000; Tso et al. 2005) and silk-borne chemicals (Henneken et al. 2015; Henneken et al. 2017), each spider was tested on a single day (reduction of within-individual variation) and, on a feeding day, in which all (sub-)adult spiders received the same prey type (reduction of between-individual variation). Vials were inspected daily for moulted exoskeletons to control for spider maturation to adulthood. Individuals were tested approximately 2 weeks after their molt to adulthood, and were of similar age to minimize putative age-dependent variation in sexual signaling known in other spider species (e.g. Baruffaldi and Costa 2010; Cory and Schneider 2016). All animals remained unmated through the course of the experiments.

Experimental setup

To test for directional trail-following, we used a testing corridor (60 cm length x 12 cm height x 7 cm width) (Fig. 1) made of polystyrene foam, with all internal surfaces covered with brown parcel tape (Tesapack 64014) to ease cleaning. Two holes (5 cm diameter) were cut in both side walls of the device for the female to enter and exit, and one in the middle of one long side (center hole) of the device through which the focal male could enter. Each hole could be closed with a fitting foam lid to prevent spiders from exiting the device prematurely.

Female trails were obtained by allowing sexually mature females to enter the testing device on one side and move freely to the opposite end without changing direction, hence reliably depositing a unidirectional single trail consisting of silk and possibly female body odor (experiment 1a). In cases where the female changed direction or stopped moving, the female was removed, the device cleaned with ethanol (EtOH; extra pure, Carl Roth, Germany) and dried using cotton cloth before repeating the trial. Once the female left the device through the exit hole on the opposite side to where it had entered, it was returned to its housing vial. Female silk lines were visible to the observer. The direction of the female's movement (left to right or vice versa) was randomized before each trial to account for directional biases. A male, not carrying a nuptial gift, was introduced through the center hole of the device immediately after female removal and his movements (left to right or vice versa) observed. A trial was considered completed when the male reached one of the holes for exiting of the device.

We included a washed treatment (experiment 1b), where, after the female had walked through the device, the inside of the device was sprayed with 96 % EtOH to remove putative chemicals on the silk and on the device's substrate (left from the female's body). Ethanol was chosen as it is able to dissolve both

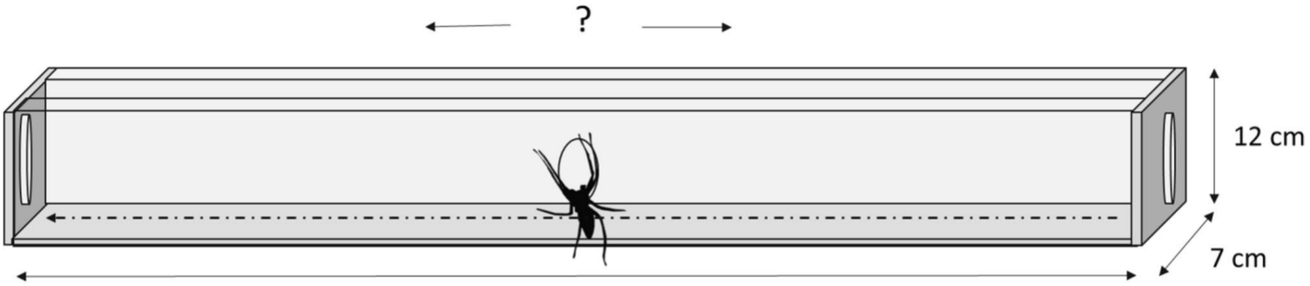

Fig. 1 Schematic representation of the testing corridor with the male spider encountering the single unidirectionally deposited female silk trail (dashed line) via the center hole in the long side of the device, and moving either in the same direction as female (indicated by the arrow at the end of the silk line) or the opposite towards one of the exit holes

polar and (weakly) non-polar substances, due to its polar (hydroxyl (OH)) and non-polar (ethyl (C2H5)) groups. While it might not be able to remove all silk-borne chemicals, especially complex lipids such as long-chained glyceryl ethers that are found in/on silk (Chinta et al. 2010; Schulz 2013; Gerbaulet et al. 2022), we would still expect to dissolve a variety of (organic) compounds known to function as spider pheromones such as some alkanes found in Araneus diadematus (Schulz 2013; Fischer 2019). After spraying the ethanol, the device was left to dry completely (approx. 5-10 min) before the male was introduced into it. We also carried out an additional control treatment (experiment 1c) that consisted of allowing males to enter empty cleaned devices, to investigate whether male directional decisions are subject to directional biases, with males preferably choosing a specific direction in which to proceed. This control treatment was repeated three times for each male. To test for potential effects of washing treatments on silk structure we inspected draglines washed with polar (water) or non-polar (pentane) solvents using scanning electron microscopy (experiment 1d), and, as reported in the Online Resources, results suggest no visible structural differences between washed and unwashed draglines, confirming results of a previous study (Shao and Vollrath 1999) (Online Resource, Fig. S1, S2, and Table S1).

Each male (n = 31) was tested five times using an unwashed trail (experiment 1a), a washed trail (experiment 1b), and three times no-trail controls (experiment 1c) in a randomized order and on a single day. Each male was exposed to silk sourced from the same female during unwashed and washed-trail treatments. Due to a low available number of females (n =19) of similar age range (i.e. 13-16 days from final moult), some females were used with multiple males. Each trial was video recorded using a webcam (Logitech HD Pro Webcam C920) fixed at a distance of 40 cm over the testing device, and connected to a laptop. After each trial, males were returned to their housing vials, and the device was cleaned using EtOH and dried before re-use. Body mass of males and females was measured to the nearest 0.01 g using a digital scale (Kern PKT, Kern & Sohn GmbH, Germany) before testing (hence, if present, nuptial gifts were gently removed from males using forceps), and prosoma width was measured as a proxy for spider body size to the nearest 0.01 mm using electronic calipers (Aerospace, China) once experiments were completed.

Scoring of male trail-following

Behavioral parameters were scored from the videos using the event-logging software BORIS (v7.10.5) (Friard and Gamba 2016). We scored which end of the device was reached by the male (same as or opposite to female movements) and measured the total amount of time the male spent in the device, from entering the device to touching one of the exit holes on the sides, as an indicator of the duration of male assessment of cues in the environment. We additionally noted the occurrence of directional turns, defined as a 180° turn of the animal (yes/no) before the male touched one of the exit holes of the device to explore their function as potential indicators of accessibility of directional information. To minimize observer bias, blinded methods were used when all behavioral data were analyzed by keeping the observer naïve to the identities and treatments of the spiders observed in the videos.

Statistical analyses

To test the hypothesis that female silk trails provide directional information to males (experiment 1a), we conducted a binomial test, testing the proportion of male choices in the correct direction (i.e. same direction as the silk was deposited by the female). To test whether directional information is chemically mediated and to account for the data structure involving multiple use of animals, we fitted a binomial generalized linear mixed-effects model (GLMM), including male movement in the correct direction as response variable, treatment (unwashed and washed trails from experiment 1a and b) and testing order (to account for increasing male experience with the testing device) as fixed effects. To account for multiple use of spiders (i.e. to avoid pseudo-replication), spider identities of each sex were added as random effects. Finally, we conducted binomial tests to test for male biases in moving consistently in one specific direction (experiment 1c) both over the course of the experiment and on specific days (see Online Resource).

In addition to our main questions described above, we conducted four additional explorative models to test whether i) males that spent more time assessing the environment and/or performing directional turns are more likely to follow trails correctly (i.e. in the direction deposited by the female), ii) female phenotypic characteristics (body mass, condition and size) influence male directional trail-following, with males following correctly high trait-females and effects being lower in the washed treatment due to removal of chemically encoded information on female phenotype, iii) male phenotypic characteristics (body mass, condition and size), and iv) relative differences between female and male phenotypic characteristics influence male directional trail-following, with males following correctly females with relatively higher relative trait differences. Body condition was calculated using a residual body condition index, defined as the residuals of a linear regression of body mass on size (i.e. prosoma width) (Jakob et al. 1996). Detailed description of statistical models are reported in the Online Resource.

All numeric variables were grand-mean-centered by subtracting each value by the variable's overall mean to ease biological interpretation and standardized using the standard deviation of the variable. Statistical analyses were performed using R (version 4.1.1, R Core Team 2021). Binomial generalized linear mixed-effect models (GLMMs) were applied using the "stan_glmer" function, which included the simulation of posterior distributions based on 4000 iterations (package "rstanarm" (Goodrich et al. 2020). Model fit was visually assessed based on the model's fitted values (goodness of fit graph) and the distribution of residuals. The statistical significance of fixed effects was inferred from the Bayesian 95% credible intervals (CI) associated with the mean parameter estimate (β) with effects being considered significant in the frequentist's sense when the 95% CIs did not overlap zero (Nakagawa and Cuthill 2007).

Results – Experiment 1

Within each treatment, males did not display a significant preference for choosing the same direction as the female (binomial test: unwashed treatment: p = 0.28, 61 %, 95 % - CI: 42 % - 78 %, 19 out of 31; washed treatment p = 0.15, 35 %, 95 % CI, 19 % - 55 %, 11 out of 31) (Fig. 2). Males chose the same direction as the female that deposited the trail significantly more often when the trail was unwashed in comparison to when it was washed with EtOH (Table 1). Testing order did not influence the response significantly (Table 1).

Males in repeated control (no-trail) treatments did not show directional bias over the course of the experiment: individual males did not preferentially choose the same direction opposed to varying directions during their three no-trail trials (within individual bias; binomial Test: p = 0.28, 39 %, 95 % - CI: 22 % - 58 %, 12 out of 31) and all males did not choose a specific direction (right or left) in the testing device when exposed to female trails (between individual bias; binomial test: p = 0.47, 46 %, 95 % - CI: 35 % - 56 %, 42 out of 92). Model results were retained after excluding a single biased day from the data (see Online Resource Table S2).

When exploring other factors, such as male behaviors during the trial (time spent in the device, occurrence of turns) and male and female phenotypic traits (body mass, condition and size) that may affect male directional decisions, we found the following: i) the total time spent in the device and 180° directional turns did not explain variation in directional male trail-following (Online Resource Figure S3, S4, Table S3); ii) males were more likely to follow trails in the same direction as the female laid them when the female had high body mass and condition (Online Resource Table S4, Fig. S5) but not large size (Online Resource Table S4). The significant effect of the washing treatment was retained, yet the effect of female body mass was significantly smaller in the washed than the unwashed treatment; iii) male body mass, condition and size, did not affect directional trail following (Online Resource Table S5), iv) nor did the relative difference in mass or size between the sexes (Online Resource Table S6, S7).

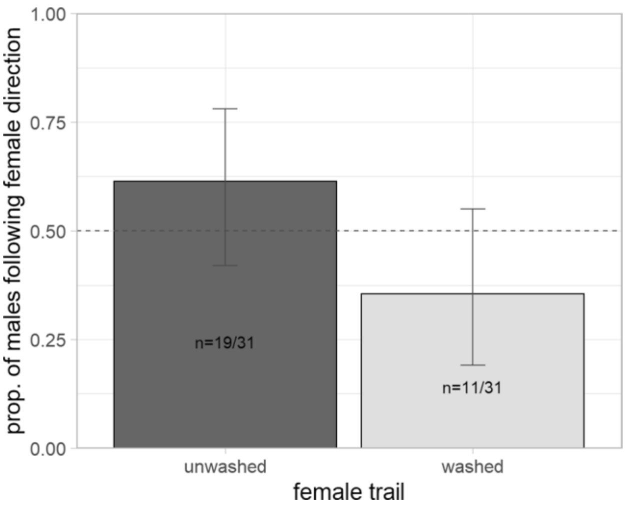

Fig. 2 Likelihood of males following the trail in the same direction as deposited by the female when trails are unwashed (experiment 1a) and washed with solvents (experiment 1b). The dashed line indicates random choice. Error bars represent 95% credible intervals of the binomial test. Numbers within bars = number of males following in female direction out of all males within treatment. Directional trail- following was significantly higher in unwashed trails

Table 1 Estimated effect sizes and 95% credible intervals (CIs) of the GLMM (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including treatment (washed and unwashed trails) and testing order (test number) ($n = 62$)

	Directional trail-following
Fixed effects	**β (95% CI)**
Intercept*	0.55 (−0.28, 1.44)
Treatment (washed)[a]	**− 1.23 (− 2.37, − 0.12)**
Test number[b]	0.10 (−0.50, 0.71)
Random effects	**σ^2 (95% CI)**
Male ID	0.44 (0.00, 2.28)
Female ID	0.28 (0.00, 1.66)

Significance is shown in bold

*Reference category; estimate for treatment (unwashed) and mean values of remaining fixed effects

[a] Difference between reference category and treatment (washed)

[b] Mean centered and standardized using the standard deviation within all trials

Experiment 2. Do silk trails provide information on female phenotypic characteristics (body mass, size and condition) that affect directional trail-following in males?

Methods – Experiment 2

Animal collection and rearing

Juvenile and subadult *P. mirabilis* of both sexes ($n = 153$) were collected during spring and autumn 2022 in the same locality as described for experiment 1. For animals caught in spring, room temperature and natural photoperiod were on average 25 °C and 15 h light: 9h dark. To ensure a comparable room temperature for animals reared in autumn/winter, vials were placed on heating mats (approx. 23 °C). The natural photoperiod for these animals was 10 h light: 14 h dark. The seasonal differences between the two cohorts of spider were statistically addressed (see below).

To obtain females varying in body mass, we applied a differential feeding treatment to which a randomly chosen subset of females was assigned to as soon as they reached adulthood, for a total of two weeks. The low-fed regime was implemented by providing spiders once a week with one housefly (n = 48 females), and the well-fed feeding regime by providing 1 fly three times a week ($n = 53$ females). Well-fed females had a significantly higher body mass (two-sample *t*-test: $t = -8.20$, d.f. = 112.76, $p < 0.001$; mean mass ± s.e. in mg: well-fed: 116.00 ± 2.56, $n = 58$; low-fed: 85.79 ± 2.65, $n = 57$) and body condition than low-fed females (two-sample *t*-test: $t = -7.18$, d.f. = 111.48, $p < 0.001$; mean body condition residual index ± s.e.: well-fed: 12.46 ± 2.47, $n = 58$; low-fed: -11.17 ± 2.17, $n = 57$). In autumn, well-fed females were also significantly larger in size than low-fed females, leading to overall larger well-fed females (two-sample *t*-test: $t = -2.42$, d.f. = 106.90, $p = 0.02$; mean size ± s.e. in mm: well-fed: 3.59 ± 0.04, $n = 58$; low-fed: 3.48 ± 0.03, $n = 57$).

Experimental setup

To test for male directional trail-following of trails from high-fed (experiment 2a) and low-fed females (experiment 2b), we used the testing corridor and experimental approach previously described (Fig. 1). As the time spent in the device for trail deposition varied greatly between females, we measured and standardized the time spent in the device in a subset of females ($n = 35$) to control for possible variation in trail deposition (e.g. quantity of deposited silk or putative chemicals): if a female did not exit the device after 60 seconds, a paintbrush was tapped against the top of a side wall behind the female's position to induce forward movement. We additionally carried out a single-run control treatment in an empty device (experiment 2c), testing males without female silk trails as described for experiment 1.

Each male ($n = 52$) was tested three times using the trail of a well-fed female trail (experiment 2a), of a low-fed female trail (experiment 2b), and a no-trail control (experiment 2c) in a randomized order and on a single day. Due to a low available number of females of similar age range of 13-16 days from final molt, some females (n = 13) were used with multiple males. Each trial was video recorded using a webcam (Logitech HD Pro Webcam C920 or Logitech BRIO 4K) fixed at a distance of 40 cm over the testing device, and connected to a laptop. After each trial, males were returned to their housing vials, and the device was cleaned using 96 % EtOH and dried before re-use. Body mass and size of males and females was measured as described for experiment 1.

Scoring of male trail-following

We scored which end of the device was reached by the male (same as or opposite to female) either from the videos using the event-logging software BORIS (v7.10.5) (in spring) or by direct observations (in autumn). As for experiment 1, observer bias was minimized using blinded methods when all behavioral data were analyzed.

Statistical analyses

As done in experiment 1, we first used the data to test the hypothesis that female silk trails provide directional information to males by conducting a binomial test within each treatment (experiment 2a and 2b).

To test the hypothesis that male trail-following is influenced by female phenotypic traits (body mass, size and condition) (experiment 2a and 2b), with males being more likely to move in the direction the trail was deposited in the case of females with high-trait values representing more fecund partners, we fitted three distinct models with each of the female traits (either body mass, body size or residual body condition index) and testing order as fixed effects. Spider identities of each sex were added as random effects to account for repeated measurements. As the season of experiment conduction (spring vs. autumn) did not explain variation in the data (see Online Resource, Table S8), we removed it from the model simulations. To test whether male phenotypic traits (male body mass, condition and size) affect male movements on trails of high-fed and low-fed females, we expanded the models described above by fitting three distinct models, each with one of the male traits (either body mass, body size or residual body condition index). To account for phenotypic effects of both sexes, we repeated these models including the relative trait difference, calculated as the relative difference between male and female body mass and size (female value divided by male value). Values of the variables body mass, size, condition and trial number were grand-mean-centered by subtracting each value by the variable's overall mean and standardized using the standard deviation of the variable, to ease biological interpretation of the model output.

Finally, we investigated male directional bias over the course of the experiment and on specific days (experiment 3c) by using a binomial test to compare the number of males choosing each direction during individual experimental days. As a single experimental day showed male directional bias (binomial test: $p = 0.07$, 88 %, 95 % - CI: 47 % - 100 %, 7 out of 8), analyses were repeated with a dataset excluding this biased day.

Statistical analyses were performed using R (version 4.1.1) and as described for experiment 1.

Results – Experiment 2

Within each feeding treatment, males did not display a significant preference for choosing the same direction as the female (binomial test: well-fed: p = 0.36, 43 %, 95 % - CI: 30 % - 57 %, 25 out of 58 trials; low-fed: p = 1.00, 51 %, 95 % - CI: 37 % - 64 %, 29 out of 57 trials). Testing order did not significantly affect the response (Table 2). Males did not significantly differ in the likelihood of following trails in the same direction deposited by a high-mass (i.e. well-fed) compared to a low-mass (i.e. low-fed) female (Table 2 model a), nor by larger females or females in higher body condition (Table 2 models b and c).

Male likelihood to follow in female direction was not significantly influenced by either male body mass, size or condition (Online Resource Table S9). While male likelihood to follow in female direction was also not significantly influenced by the relative mass differences between the sexes, males were significantly more likely to follow in female direction, if the female was of relatively larger size than the male (Figure 3, Table 3). This significant effect was also found for data collected in spring, but not in autumn (Online Resource Table S11).

The control (no-trail) treatment (experiment 2c) showed no directional male bias over the course of the experiment: individual males did not preferentially choose a specific direction (right or left) in the testing device when exposed to female trails (binomial test: p = 0.16, 60 %, 95 % CI: 46 % - 72 %, 36 out of 60 for the left direction in the device). When excluding the biased day from the data and repeating the model simulations, results were retained (see Online Resource Table S12).

Table 2 Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition in experiment 2, including female body mass (model a), size (model b) and residual body condition index (model c), accounting for testing order (test number) (n = 115). Significance is shown in bold

	Directional trail-following – female phenotype		
	Model a) mass	Model b) size	Model c) condition
Fixed effects	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Intercept*	−0.13 (−0.62, 0.33)	−0.14 (−0.62, 0.32)	−0.13 (−0.62, 0.32)
Test number^{a}	0.43 (−0.03, 1.07)	0.42 (−0.03, 1.03)	0.40 (−0.07, 0.99)
Female trait^{a}	−0.07 (−0.58, 0.39)	0.29 (−0.18, 0.84)	0.40 (−0.64, 0.31)
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**	
Male ID	0.16 (0.00, 0.91)	0.17 (0.00, 0.96)	0.16 (0.00, 0.93)
Female ID	1.11 (0.00, 7.12)	1.07 (0.00, 6.74)	0.96 (0.00, 0.93)

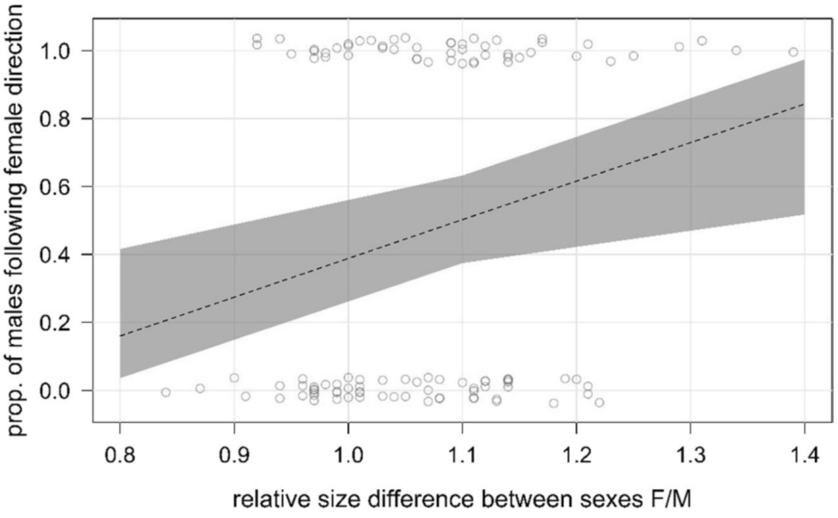

Fig. 3 The effect of the relative difference in body size between males and females on the male's probability to follow trails in the same direction laid by females in experiment 2 ($n = 115$), with values > 1 indicating relatively larger females and values < 1 indicating relatively smaller females compared to male size. Males are significantly more likely to follow females that are relatively larger than they are. The dashed line represents the regression line, grey areas the 95 % credible intervals and circles the relative body size difference of the individual male and female pairings in the trials

Table 3 Estimated effect sizes and 95% credible intervals (CIs) of the GLMM (binomial) testing for predictors of the male likelihood of following trails in the same direction moved by females during trail deposition, including testing order (test number) and relative difference in female and male body mass (model a) and size (model b) as fixed effects and spider identity as random effect ($n = 115$)

	Directional trail-following – relative trait difference between sexes	
	Model (a) mass	Model (b) size
Fixed effects	**β (95 % CI)**	**β (95 % CI)**
Intercept*	-0.47 (-2.26, 1.36)	-6.10 (-12.31, -1.15)
Test number[a]	0.44 (-0.01, 1.01)	0.40 (-0.06, 1.04)
Relative trait difference[b]	0.28 (-1.18, 1.72)	**5.56 (0.94, 11.33)**
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	0.15 (0.00, 0.84)	0.14 (0.00, 0.83)
Female ID	0.91 (0.00, 5.41)	0.81 (0.00, 6.64)

*Reference category; estimate for mean values of fixed effects

[a] Mean centered and standardized using the standard deviation within all trials

[b] Value calculated by dividing female by male trait value

Experiment 3. Do male spiders interact with silk in a way that suggests information gathering?

Methods – Experiment 3

Animal collection and rearing

In spring 2021, juvenile and subadult *Pisaura mirabilis* of both sexes were collected in Greifswald (Mecklenburg-Vorpommern, Germany). They were brought to the lab of Greifswald University, where they were individually placed into transparent plastic vials (9 cm height, 5 cm diameter), covered with foam lids. Animals were reared at room temperature (approx. 23 °C) and under artificial light (three UV lamps, Exo Terra Repti Glo 10.0, 10 % UV-B, 33 % UV-A, 20 Watts) set to natural photoperiod. To maintain high humidity, vials had their bottom removed and were placed upside down in water-filled trays. Spiders were fed twice a week using either a common green bottle fly (*Lucilia sericata*) or 2 small crickets (*Acheta domesticus*, *Gryllus assimilis*), with spiders receiving the same prey type on a respective feeding day. To accelerate development, animals were placed inside a climate chamber for seven days (25 °C, 67 % humidity).

Experimental setup

To study male movements on silk trails, we used a walking corridor (30 cm length x 5 cm width x 7 cm height) made of transparent acetate sheets. Female trails were obtained by letting a female walk unidirectionally through the walking corridor. To ensure deposition of attachment discs, the corridor was tilted by 40°, resulting in the female slightly slipping while walking, inducing disc deposition to secure itself. After the female reached the end of the corridor, it was returned to its housing vial and the male was introduced to the starting point of the trail. A total of 11 males were tested multiple times (up to 5) using silk lines sourced from 9 females (total trial number $n = 22$).

Trials were video recorded using a manually post-triggered high-speed camera (MIRO LC 320S), connected to a laptop, with 100 mm Macro (Canon) fixed at a distance of 25 cm below the transparent runway and the field of view of the camera was set to the area surrounding attachment discs (n = 20). In two additional trials, the camera was fixed above the runway, with the field of view set to the beginning of the silk thread. The video resolution was of 250 frames per second. To ensure continuous illumination during the recording, two direct current flashlights (Heider CFX Super Power) were fixed in 10 cm distance below and next to the device at 120° and 40°, respectively. Spiders did not appear to be disturbed by these lights and no noticeable behavioral changes were observed.

Scoring of male movement

Every video (n = 22) contained 2 real-time seconds worth of high-speed material, resulting in recordings of approximately 90 seconds duration. One video was excluded from further analyses due to the male not moving (total number of videos n = 21). These were scored manually by one observer (MB). Silk lines and attachment discs were visible from videos. Movements of male appendages (legs and pedipalps) in respect to silk lines and attachment discs were observed to determine if and how males make physical contact to the silk.

This data was used to thoroughly describe the probing behavior of males when coming in contact with female dragline silk, no statistical analyses followed.

Results – Experiment 3

We were able to detect three distinct male behaviors: i) in 91 % of the trials (19 out of 21) males tapped the substratum with the tips of their pedipalps; ii) from those trials in which males came into direct contact with a silk line (n = 19), in 42 % of the trials (8 out of 19) males pulled on the silk line, guiding it either towards their body (Fig. 4a) or their pedipalps (Fig. 4b), using the tarsal claws of one of the 1st, 2nd or 3rd pair of legs legs; iii) silk lines were also commonly probed by males (16 out of 19 trials; 84 %) by sliding along parts of it using the tips of one of the 1st pair of legs (7 out of 16 trials; 44 %), or by placing the silk line on the ventral side of one of the pedipalps (pedipalpal cymbium) and guiding the silk line along it (15 out of 16; 94 %). This was observed both from ventral and dorsal views of the animals (Fig. 4c/d). In none of the video recordings, males made direct physical contact with the attachment discs.

Videos of the above mentioned behaviors can be viewed on Figshare (Beyer et al. 2023).

Discussion

In this study, we investigated whether trails, consisting of silk lines and/or body odor, of females of the hunting spider Pisaura mirabilis carry directionality information that aids male mate search. We further examined whether directionality is chemically or structurally encoded and depends on female phenotypic characteristics (body mass, condition and size). We found that overall males did not directionally follow female trails. However, these results varied in different experiments. Males were more likely to follow trails correctly when they were unwashed, compared to when they were washed with a solvent to remove putative chemicals. Similarly, males were more likely to correctly follow trails when they were deposited by females that were relatively larger in size. We also examined whether males interact with silk in a way that suggests information gathering, and indeed describe extensive probing and pulling on silk lines with their appendages. Taken together, while our findings on male trail-following behavior provide no overwhelming evidence for directional trail-following, our results that males are more likely to follow non-washed trails correctly, and follow trails of relatively larger females lead us to conclude that directional trail-following might be present in this species, but facultative and fragile.

Contrasting to our expectations, *Pisaura mirabilis* males did not generally follow trails in the direction they were deposited by females. One possible explanation is that potential costs of encoding directionality information in a silk trail, such as production/secretion of directionality inducing chemicals or structural components, or increased predation or parasitism risk (van Baarlen et al. 1996; Wignall and Taylor 2009; Fei et al. 2023), may outweigh the benefits of eased mate finding. Alternatively, directionality information in female trails is not necessary in this species. Despite not being territorial, spiders occur in relatively patchy clusters during the mating season (personal observation), which may drastically increase encounter rates between prospective mates, even when following trails in a random direction. Although field studies report males actively roaming the environment in the process of mate finding (Ghislandi et al. 2018), we

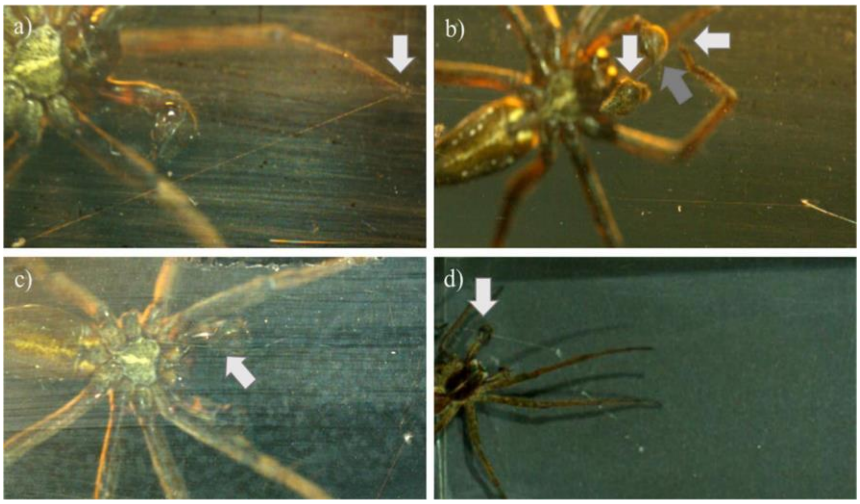

Fig. 4 Video stills showing a male *P. mirabilis* filmed either from below (a-c) or above the transparent runway (d): a) pulling on a silk thread with its first front leg (light grey arrow), b) pulling on a silk thread with its front leg and a pedipalp (light grey arrows) while gliding along the line with the remaining pedipalp (dark grey arrow), c) probing of a silk thread with a pedipalp in ventral view (light grey arrow), d) dorsal view of probing of a silk thread with a pedipalp (light grey arrow)

lack studies on the mechanisms and cues used. While our males did not follow female trails directionally, we cannot fully exclude that our findings are driven by the artificial test environment. It is also possible that directional information is present in female silk trails, but males choose specific scenarios in which to follow a trail, for example when sensing the trail was deposited by a female of high reproductive value, or males are unable to decode present directional information.

Nevertheless, given the higher proportions of males correctly following the trail when these remained unwashed, chemical attributes of trails may provide a potential means for conveying directionality information. Despite little being known about the chemical sensing and communication of *P. mirabilis*, for spiders in general it is suggested to occur via contact-chemoreception through cuticular structures (i.e. sensilla) that are abundant on spider appendages, including pedipalps (Foelix 2010; Keil 2012). Hence, the observed probing both via legs and pedipalps in our high-speed video recordings, may allow males detection of putative pheromones present in the silk or on the substrate (Bristowe and Locket 1926; Kaston 1936; Jackson 1983; Humbel et al. 2021). Gathering of information for initiating and possibly furthering directional trail-following was also suggested to play a role in two species of wolf spiders (Tietjen 1977), shown to repeatedly pull and probe the silk lines with their front legs and pedipalps. The exact mechanism for directionality in these species remains unknown. The author excluded the presence of a chemical gradient as the chemicals in these two species were stable over several weeks, reducing detection of fine-tuned differences in concentration - that would be necessary for reliable directional information - due to evaporation (Tietjen 1977). Directionality in pheromone trails has only been described in very few cases, such as in ants, where it is known to be embedded by using trail geometry based on trail-bifurcations (Jackson et al. 2004). In contrast, spiders cannot use the same types of mechanisms since they lack bifurcating trails and lack an end-goal, such as the nest and food sources in ants. Alternatively, spiders may make use of a gradient of contact pheromones which inactivates rapidly after release (Baruffaldi et al. 2010). Yet, despite the importance of contact silk cues being acknowledged in this species (Beyer et al 2018), the persistence of active chemicals in *Pisaura mirabilis* silk remains unknown.

The observed pulling of silk in the present work may also allow detecting variable tension around the silk attachment discs, necessary for inducing male directional trail-following (Tietjen 1977; Wolff et al. 2021). Position, angle and morphology of a disc could result in differences in silk tension in the area surrounding the discs (Wolff et al. 2021) as, for example, the different mechanical robustness of the disc with respect to its upstream (direction towards silk layer) or downstream (direction towards start of silk line) end could potentially be sensed by a trail-following individual (Wolff and Herberstein 2017). In order to assess differences in silk line tension, a male would be required to assess the tension around an immobilized portion of it by using its appendages. Explanations relying on chemical and structural (i.e. silk tension) assessment are not mutually exclusive; chemical signals may be coupled with mechanical orientation signals that are used in concert to find potential mates. While there is a potential that washing of the silk lines with ethanol could have also affected structural and mechanical properties of the dragline – that potentially play a role in trail directionality cues, we believe this to be unlikely. Any impact on the silk material would be global, i.e. any existing relative differences in upstream versus downstream draglines would have been maintained. Some spider silk types are known to transition into a rubbery state upon contact with water, thereby shrinking and increasing its diameter – a process called supercontraction (Liu et al. 2005; Stengel et al. 2020), and silk structure may also change after treatment with solvents (Beyer et al. 2021). However, our ultrastructural inspection of silk draglines (namely, major ampullate silk type) washed with solvents did not reveal visible structural changes in comparison to unwashed silk, such as an increase in silk diameter (see Online Resources). The observed effect of trail washing on the prevalence of directional trail-following behavior is thus most likely explained by the removal of chemical cues only. Further research and especially chemo-analytical tests (e.g. chromatography) are needed to verify removal of chemical information by washing with solvents.

Our study also revealed an effect of female phenotype on correct trail following of males, as males followed trails in the direction of females that were relatively larger in size – and avoided the direction of females that were relatively smaller – than themselves. Interestingly, in our first experiment, when exploring a potential role of female phenotypic traits, we found that the likelihood of male *P. mirabilis* to directionally follow females was conditional on female body mass, with 90 % of the males following heaviest females (i.e. females of the quantile with highest absolute body mass) (Online Resource). In our second experiment, when formally testing for the effects of variation in female phenotypic traits (body condition, mass and size) obtained through experimental feeding manipulations, on male directional trail-following, we failed to confirm these results. We believe results regarding the effect of female mass from experiment 1 warrant cautious interpretation, as, given the homogenous feeding conditions that females were given during rearing, natural variation in female body mass was overall low, leading to few high-mass females driving such significant effect. On the contrary, the experimental procedure adopted in experiment 2, where female body mass and condition were experimentally manipulated through differential feeding regimes, coupled with a high sample size, is likely to be much more reliable.

The finding that male directional trail following did not depend on female body mass, condition or size remains, however, puzzling. We have hypothesized mass- and /or condition-related information to males stemming from varying amounts or composition of chemicals embedded in, or added to, the silk (Baruffaldi et al. 2010; Henneken et al. 2015; Henneken et al. 2017; Weiss and Schneider 2022a), or from thicker silk threads (Vollrath 1999). Given the costs associated with nuptial gifts we have also hypothesized selection favoring male discriminatory abilities of female cues. If information carried by female trails correlates with the female's reproductive value, males should move towards females of higher body mass, condition and size to safeguard their heavy investment in mating (Bonduriansky 2001). *Pisaura mirabilis* females of larger size and mass are known to be more fecund (Austad and Thornhill 1986; Stålhandske 2001; Pandulli-Alonso et al. 2022). In arthropods, male mate choice is often based on female fecundity (Bonduriansky 2001; Edward and Chapman 2011), a trait that is generally positively correlated with female body mass (or size) (Leather 2018), with mass itself being able to reflect recent food intake and subsequently the likelihood and timing of reproduction (e.g. egg laying) (Stoltz et al. 2010). In addition, in spiders, information on female mass is also used by males as a proxy for female satiation to assess the risk of cannibalism, with females of low mass (and thus low satiation) posing a greater risk than those of higher mass (Baruffaldi and Andrade 2015). *Pisaura mirabilis* females are more likely to cannibalize males when starved (Toft and Albo 2016). In one of our recent studies, we show that *Pisaura mirabilis* males discriminate between silk of females varying in their body condition, with males exerting preference for silk of females in higher compared to lower condition (Beyer et al. unpublished data). While said study was conducted in a binary choice set up, with males simultaneously being exposed to the trails of a high- and low-condition female, and did not include tests of directionality, it does show that there is variation in female silk and that males can and do in fact assess female phenotype exclusively from their trails.

Males were more likely to follow correctly relatively larger females, suggesting that – to some degree – males perceive relative differences in female phenotypes via their trails, and these are indicative of female movements. It is possible that male *P. mirabilis* can only probe the diameter of the silk line to infer female characteristics, and that larger (but not heavier) females produce larger silk threads. This would explain the effect of relative size, but not relative mass, on trail-following, despite evidence from orb-weavers indicating no effect of body size (but an effect of mass) on diameter of radial silk threads (Vollrath and Kohler 1996). The perception of relative phenotypic differences would require individuals to self-assess their own size in relation to the size of another, an ability widespread in intra- and especially inter-sexual encounters (i.e. male-male conflicts) in arthropods (e.g Taylor and Elwood 2003; Briffa 2008), including spiders (e.g Wells 1988; Schaefer and Uhl 2003; Taylor and Jackson 2003). Such relative size-dependent self-assessment is already hypothesized in female *P. mirabilis* that allow longer copulations to relatively smaller males (Prokop 2006). Relatively smaller males also gain relatively higher paternity shares (Matzke et al. 2022). Their advantage may be explained by higher agility (Blanckenhorn 2000) and potentially higher mechanical compatibility in entering the mating position (Dufour 1844; Masly 2012; Xia et al. 2023). If male *P. mirabilis* use a putative self-assessment ability in relation to the female's size, larger females may be preferred and followed. A perception of potential incompatibility may drive male avoidance of trails deposited by smaller females whose genitalia (e.g. epigynal opening) are either difficult to reach or enter with a comparatively large males' pedipalps due to females being comparatively too small, likely consequently resulting in reduced success of copulation and/or insemination (Schick 1965). In contrast, the perception of compatibility might increase male likelihood to follow trails of females that are relatively larger, e.g. due to eased access to the female's genitalia. We can only speculate whether mechanisms involve the interaction between relatively smaller male sensory appendages in smaller males and enhanced chemical and/or structural trail properties of larger females signaling directionality. We note, that despite having a large sample size, the body sizes in this study represent natural variation, with a median (and mean) relative size difference of male-female testing pairs of approximately 1, as males and females in this species are of similar size. Further research might be necessary in which body size in male and female testing pairs is manipulated, especially since strength of male choice regarding female quality (e.g. large size) is known to be higher for medium- and high-quality males (Pollo et al. 2022).

To conclude, our tests on male trail-following provide no overwhelming evidence of general directionality in male movements during trail-following. Male spiders use silk trails as guides for movements, and the gathered chemical and tactile cues might encode information on female quality. Directionality appears to be present only under certain conditions, with chemical attributes of trails having a potential key role in providing males with size-dependent information on female movements. The natural history of this species (relatively clustered populations with high densities) may weaken mechanisms for embedding trails with directionality in females or detecting them in males, as these would require a costly sensory machinery in the face of increased predation and parasitism risks. These results contribute to the lack of empirical studies on directionality, and are key for advancing our understanding on mechanisms involved in animal communication and mate searching in free-living and solitary species of arthropods. Decoding of directional information remains an exciting venue for research. Finally, we emphasize the importance of accounting both for male and female interacting phenotypes to understand their influence on reproductive behaviors. Our findings that male trail-following is further influenced by indicators of relative size differences between the sexes points to a scenario of relative assessment potentially driven by male mate choice in a system characterized by high costs of mating via male nuptial gifts.

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Data availability All data generated or analyzed are included in the Supplementary information.

Competing interests The authors declare no competing interests.

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Testing presence of directionality information in female spider silk trails through male trail-following behavior

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Experiment 1.

Do female silk trails convey directional information to males and is this chemically or structurally mediated?

Experiment 1d: Silk ultrastructure investigation after washing treatment

To investigate structural changes in silk lines upon washing them with solvents, we collected silk lines by letting a female *P. mirabilis* walk over a piece of black paper cardboard (5 cm width x 15 cm length) to leave a single silk line. We let each female produce three silk lines, one for each treatment (unwashed, pentane-washed, deionized-water-washed). Once all three silk lines were collected, they were placed in individual petri dishes (15 cm diameter) under a fume hood. For the washing treatment, approximately 5 mL of the respective solvent (n-pentane, deionized water) were dropped onto the silk line using a disposable pipette, and left to dry for one hour (pentane evaporated after few seconds).

After drying, the silk was prepared for scanning electron microscopy by mounting it on aluminum stubs following established protocols (Ramírez, Ravelo, and Lopardo 2013). Briefly, the silk was placed on a double-sided carbon adhesive disc mounted on an aluminium stub by carefully folding the paper cardboard and catching the now mid-air suspended silk with the stub. Samples were sputter-coated with gold for 5 min without further fixation and examined at 1400 and 4000 magnification in an EVO LS10 scanning electron microscope (Carl Zeiss GmbH). Silk surface was inspected by eye and silk line diameter was measured by using one random measurement per silk line, using ImageJ (version 1.52).

No structural changes were visible between silk line-treatments (Figure S1). Silk line diameters did not vary significantly between treatments (one-way ANOVA: $F_{2,16} = 1.25$, $p = 0.31$; mean silk line diameter in µm ± standard error: unwashed: 1.17 ± 0.05, *n* = 6; pentane: 1.68 ± 0.29, *n* = 8; deionized water: 1.44 ± 0.22, *n* = 5) (Figure S2, Table S1).

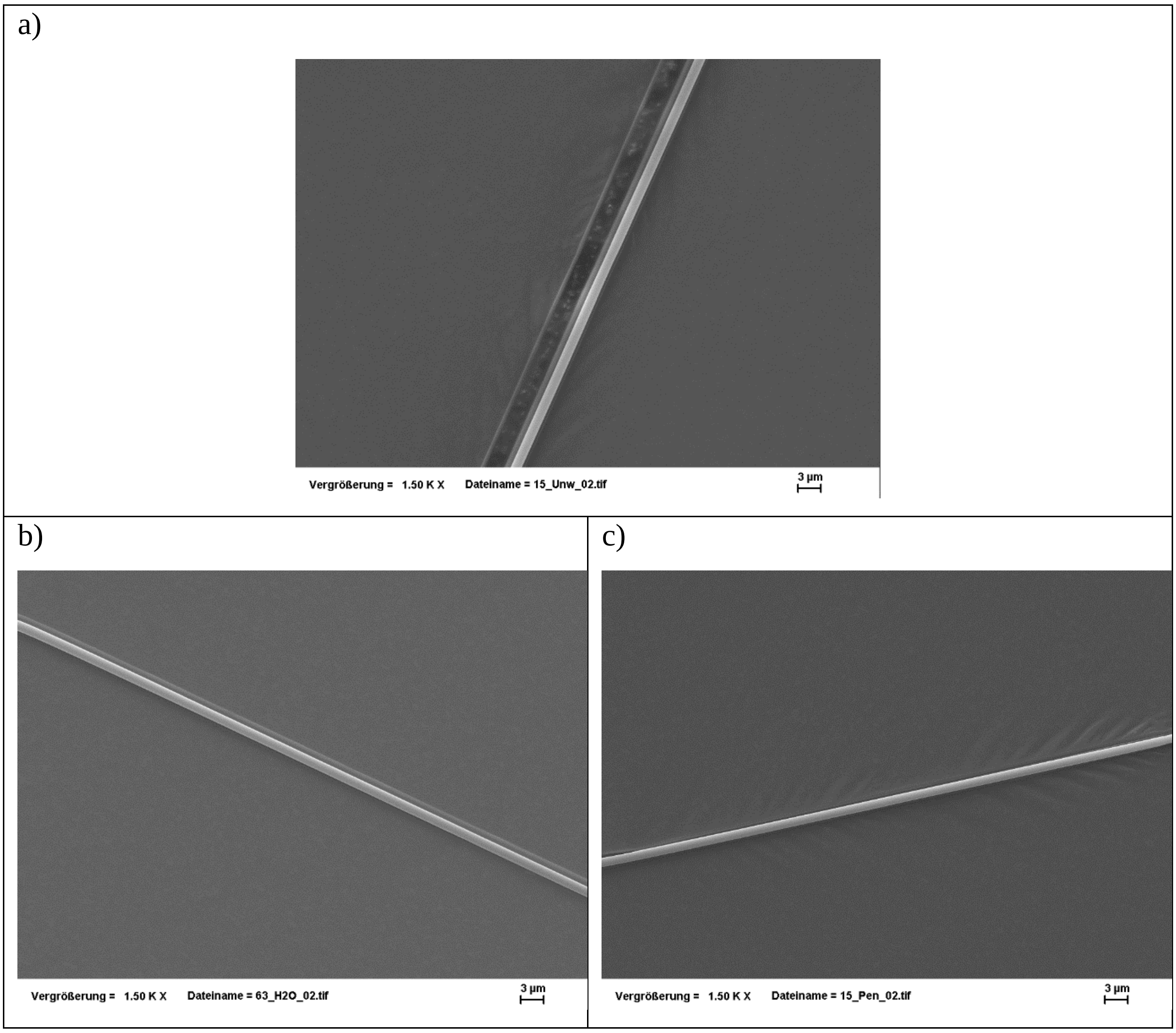

Figure S1. Ultrastructural images (1500x) of single female silk lines that are untreated (a), washed with deionized water (b) or washed with pentane (c).

Table S1: Post-hoc analysis (pairwise comparisons) of the estimates for differences in silk line diameter between different treatments using Tuckey's HSD-test.

Comparison between treatments	**95 % Confidence Intervals**		***p*-value adjusted**
	Lower	**Upper**	
unwashed-pentane	-1.34	0.32	0.28
unwashed-water	-0.66	1.21	0.73
water-pentane	-1.11	0.64	0.77

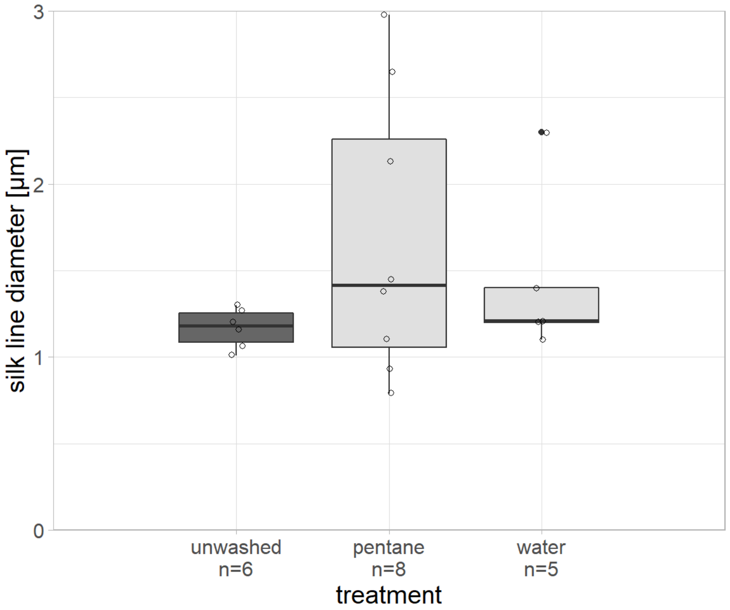

Figure S2. Silk line diameter in µm between treatments (unwashed: dark grey, washed: light grey). Open circles represent measurements for silk lines. No significant difference was found.

Experiment 1c: Details on tests for directional biases

We investigated within and between individual male directional bias both over the course of the experiment and on specific days by using binomial tests to compare i) the number of males always choosing the same vs. varying directions in their three control repetitions (within individual overall bias), ii) the number of males choosing the same or opposite direction as the female during the course of the experiment (between individual overall bias) and iii) the number of males choosing each direction during individual experimental days (between individual daily bias). As a single experimental day showed male directional bias (binomial test: *p* = 0.04, 83 %, 95 % CI: 52 % - 98 %, 10 out of 12), analyses were repeated with a dataset excluding this biased day. By repeating the model simulation excluding data from this specific day, the treatment effect was retained (Table S2).

Table S2: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMM (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition using a reduced dataset excluding the biased day, including treatment (washed and unwashed trails) and testing order (test number) in model (*n* = 54). Significance is shown in bold.

	Directional trail-following (reduced dataset)
Fixed effects	***β* (95 % CI)**
Intercept*	0.83 (-0.08, 1.91)
Treatment (washed)[a]	**-1.45 (-2.76, -0.28)**
Test number[b]	0.02 (-0.60, 0.66)
Random effects	**σ^2 (95% CI)**
Male ID	0.50 (0.00, 2.73)
Female ID	0.33 (0.00, 2.00)

*Reference category; estimate for treatment (unwashed) and mean values of remaining fixed effects; [a]Difference between reference category and treatment (washed); [b]Mean centered and standardized using the standard deviation within all trials

Exploratory models: i) testing the effect of male assessment behaviours

Here we tested whether males that spent more time assessing the environment and/or performing directional turns are more likely to follow trails correctly (i.e. in the direction deposited by the female). We investigated the effects of male trail assessment behaviors scored during the trials by adding i) total time spent in the device and ii) occurrence of turns, defined as a 180° directional change, as additional fixed effects to the model reported in the manuscript, resulting in the following model formula:

DirectionSameAsFemale ~ 1 + Treatment + Test + TimeInDevice + Turned +
(1|IdMale) + (1|IdFem).

In the unwashed treatment, 11 out of 31 males (35 %) undertook 180° directional changes, out of which 6 finally choose the same direction as the female that deposited the trail (54 %, 6/11) (Figure S3). Males did not significantly turn more often than expected by chance (binomial test: p = 0.15, 35 %, 95 % - CI: 19 % - 55 %, 11 out of 31). In the washed treatment, 4 out of 31 males (12 %) made directional changes, with 2 choosing the same direction the female deposited the trail (50 %, 2/4). Males did turn significantly less than expected by chance (binomial test: p < 0.001, 12 %, 95 % - CI: 3 % - 30 %, 4 out of 31).

Of all males that undertook directional turns, 9 turned more than once (60 %, 9/15), with a maximum of 16 turns for a single male in the washed treatment (Figure S4).

Neither the time spent in the testing device nor the occurrence of additional turns affected male likelihood of following female silk lines in the same direction as deposited. The treatment effect was retained (Table S3).

Figure S3. Proportion of males that undertook 180° directional turns for unwashed (dark grey) and washed (light grey) trail treatment. The dashed line indicates a proportion of turns of 0.5. Numbers above bars = number of turns per treatment.

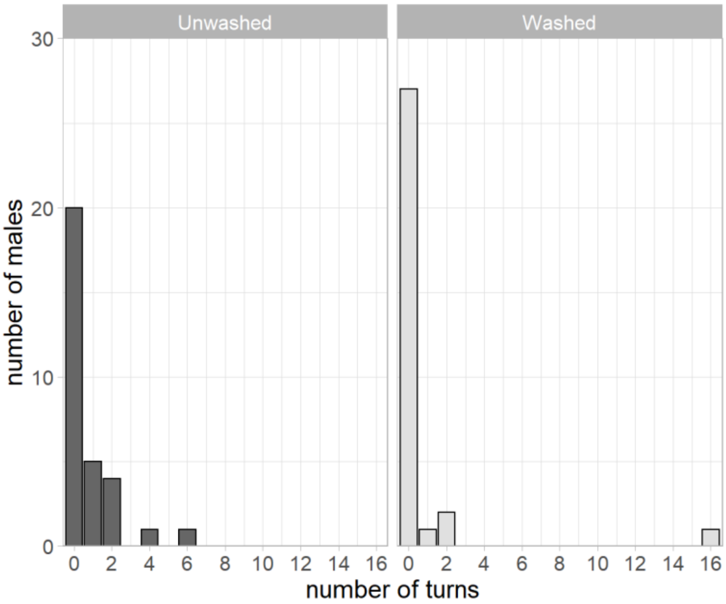

Figure S4. Number of turns with the frequencies of their occurrence for unwashed (dark grey) and washed (light grey) trails.

Table S3. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMM (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including treatment (washed and unwashed trails), testing order (test number) total time spent in the testing device, additional directional turns (*n* = 62). Significance is shown in bold.

	Directional trail-following
Fixed effects	***β*** **(95 % CI)**
Intercept*	0.54 (-0.50, 1.64)
Treatment (washed)[a]	**-1.25 (-2.49, -0.09)**
Test number[b]	-0.09 (-0.52, 0.70)
Time spent in device[b]	-0.08 (-0.83, 0.65)
Additional turns (yes)[c]	0.13 (-1.55, 1.87)
Random effects	**σ^2 (95% CI)**
Male ID	0.52 (0.00, 2.78)
Female ID	0.34 (0.00, 1.92)

*Reference category; estimate for treatment (unwashed), turned (no) and mean values of remaining fixed effects; [a]Difference between reference category and treatment (washed); [b]Mean centered and standardized using the standard deviation within all trials; [c]Difference between reference category and turned more than once (yes); [d]Difference between treatments (unwashed, washed) in the difference between female body mass

Exploratory models: ii) testing for the effect of female individual phenotypic traits

Here we test if female phenotypic characteristics (body mass, condition and size) influence male directional trail-following (high trait-females preferred by males) with the prediction of effects being lower in the washed treatment due to removal of chemically encoded information on female phenotype,

We investigated the influence of female body mass, body condition (measured as a residual index), and size, measured as prosoma width, on the probability of males to follow trails in the direction they were deposited by the female by fitting three models, each with one female trait, including treatment (washed and unwashed trails) and testing order (to account for increasing male experience with the testing device) as fixed effects. Spider identities of each sex were added as random effects to account for repeated measurements (i.e. to avoid pseudo-replication).

We found that males were more likely to follow trails in the same direction as the female laid them when the female had higher body mass (model A in Table S4, Figure S5). The significant effect of the washing treatment was retained, yet the effect of female body mass was significantly smaller in the washed than the unwashed treatment. The effect of the residual index differed significantly between treatments, while the treatment effect itself is reduced to a non-significant but strong tendency of males to follow female trails in the unwashed treatment more often than in the washed treatment (model B in Table S4). Male probability to directionally follow female trails was not significantly affected by female body size (model C in Table S4).

Table S4: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including treatment (washed and unwashed), testing order, and either female body mass (model A; $n = 62$), female residual body condition index (model B; $n = 50$) or female size (model C; $n = 50$), as well as the interaction between female trait and treatment. Significance is shown in bold.

	Directional trail-following		
	Model A) **Body mass**	**Model B)** **Body condition**	**Model C)** **Body size**
Fixed effects	**β (95 % CI)**		
Intercept*	0.78 (-0.20, 1.89)	1.00 (-0.18, 2.37)	0.93 (-0.24, 2.41)
Treatment (washed)[a]	**-1.49 (-2.80, -0.27)**	**-1.94 (-3.63, -0.41)**	**-2.01 (-3.96, -0.49)**
Test number[b]	0.03 (-0.61, 0.66)	0.08 (-0.74, 0.88)	0.39 (-0.41, 1.25)
Female trait[b]	**1.59 (0.43, 3.03)**	**1.55 (0.33, 3.01)**	0.58 (-0.55, 1.90)
Treatment (washed):Female trait[bc]	**-1.83 (-3.48, -0.44)**	**-1.52 (-3.15, -0.01)**	-1.48 (-3.35, 0.04)
Random effects	**σ^2 (95% CI)**		
Male ID	0.62 (0.00, 3.40)	0.98 (0.00, 5.56)	1.74 (0.00, 9.04)
Female ID	0.38 (0.00, 2.15)	0.47 (0.00, 2.90)	0.79 (0.00, 4.85)

*Reference category; estimate for treatment (unwashed) and mean values of remaining fixed effects; [a]Difference between reference category and treatment (washed); [b]Mean centered and standardized using the standard deviation within all trials; [c]Difference between treatments (unwashed, washed) in the difference between female body mass (A), residual body condition index (B) or body size (C)

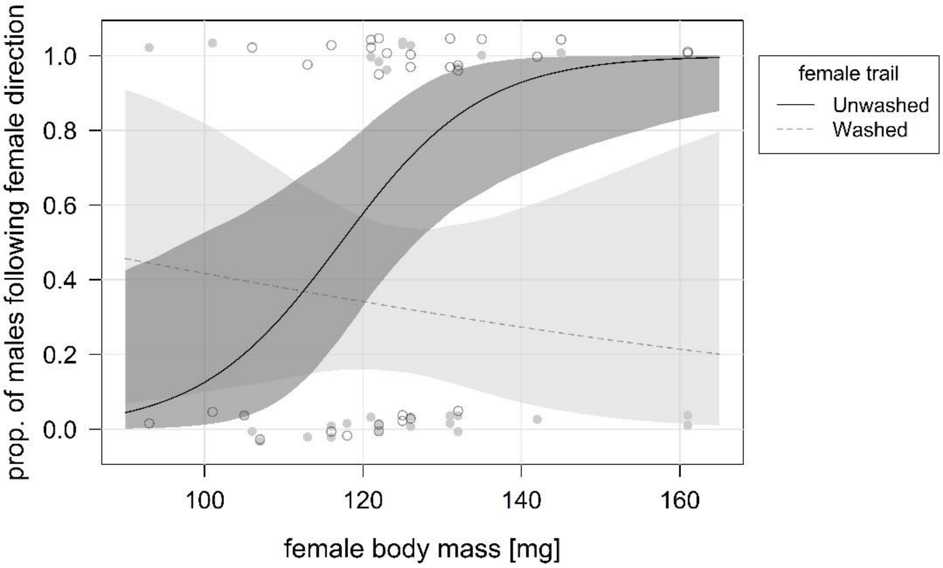

Figure S5. The effect of female body mass on the male's probability to follow trails in the same direction laid by females in each treatment (unwashed trail: dark grey, washed trail: light grey) in study 1 (*n* = 31 for each treatment). Males were more likely to choose the same direction of females of high body mass, when female trails were unwashed. Continuous and dashed lines represent regression lines, grey areas 95 % credible intervals and circles the body mass of the individual females in the trials of each treatment (open circles: unwashed, closed: washed).

Due to the sigmoidal appearance of the relationship between female body mass and the probability of males to follow a female (Figure S5), data (body mass and corresponding direction chosen) was separated into three thirds, comprised of females with lowest (*n* = 11; mean body mass in mg ± standard error: 110.63 ± 2.70 mg), medium (*n* = 10; 123.90 ± 0.59 mg) and highest (*n* = 10; 140.20 ± 3.79 mg) body mass, and a binomial test was applied to each quartile. Interestingly, in the highest-mass quartile, males followed in female direction in 9 out of 10 trials (binomial test: *p* = 0.02, 90 %, 95 % - CI: 55 % - 100 %, 9 out of 10). In contrast, males did not follow females of low (binomial test: *p* = 1, 45 %, 95 % - CI: 17 % - 77 %, 5 out of 11) or medium (binomial test: *p* = 1, 50 %, 95 % - CI: 19 % - 81 %, 5 out of 10) body mass.

Exploratory models: iii) testing for the effect of male individual phenotypic traits

Here we test if male phenotypic characteristics (body mass, condition and size) influence male directional trail-following with the prediction of …..

We investigated the influence of male phenotype (body mass, size, condition) on the probability of males to follow trails in the direction they were deposited by the female fitting three additional variations of model a, adding male mass and subsequently replacing the variable for male size and condition. Male phenotype (i.e. body mass, size or condition) did not significantly influence male likelihood to follow trails in female direction. The treatment effect was retained (Table S5, models A-C).

Table S5: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including treatment (washed and unwashed), testing order, and male body mass in model A) (*n* = 62), male residual body condition index B) (*n* = 52), and male body size in model C (*n* = 52). Significance is shown in bold.

	Directional trail-following		
	Model A) Body mass	**Model B) Body condition**	**Model C) Body size**
Fixed effects	***β* (95 % CI)**		
Intercept*	0.57 (-0.27, 1.48)	0.80 (-0.16, 1.89)	0.82 (-0.17, 1.91)
Treatment (washed)[a]	**-1.26 (-2.46, -0.15)**	**-1.55 (-2.89, -0.30)**	**-1.58 (-3.00, -0.27)**
Test number[b]	0.11 (-0.49, 0.70)	0.06 (-0.62, 0.75)	0.07 (-0.59, 0.75)
Male trait[b]	-0.11 (-0.80, 0.55)	0.22 (-0.54, 1.01)	-0.47 (-1.29, 0.26)
Random effects	**σ^2 (95% CI)**		
Male ID	0.51 (0.00, 2.95)	0.49 (0.00, 2.82)	0.47 (0.00, 2.67)
Female ID	0.32 (0.00, 1.94)	0.55 (0.00, 3.10)	0.54 (0.00, 3.14)

*Reference category; estimate for treatment (unwashed) and mean values of remaining fixed effects; [a]Difference between reference category and treatment (washed); [b]Mean centered and standardized using the standard deviation within all trials

Exploratory models: iv) testing for the effects of relative differences in male-female individual phenotypic traits

Finally, we combined both phenotypic traits from the sexes by calculating relative body mass, condition and size estimates (female value divided by male value), and replaced absolute trait values in the previous models.

We investigated the influence of the relative difference in female and male body mass and size male phenotype (body mass, size) on the probability of males to follow trails in the direction they were deposited by the female fitting two additional variations of model a, subsequently adding relative difference of mass and of size (dividing female values by male values). As the effect of the female phenotype was depending on the treatment, but male phenotype was not, we additionally fitted the abovementioned exploratory models only using data for the unwashed treatment.

Relative difference in female and male mass or size did not significantly influence male likelihood to follow trails in female direction. There was a significant difference between treatments (Table S6, models A and B). No significant effect was found when using the data for the unwashed treatment only (Table S7, models A and B).

Table S6: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including treatment (washed and unwashed), testing order, and relative difference in female and male body mass in model A) (*n* = 62) and relative difference in female and male body size in model B) (*n* = 42). Significance is shown in bold.

	Directional trail-following	
	Model A) **Body mass**	**Model B)** **Body size**
Fixed effects	**β (95 % CI)**	**β (95 % CI)**
Intercept*	-0.87 (-3.92, 2.13)	-4.41 (-17.31, 6.86)
Treatment (washed)[a]	**-1.27 (-2.46, -0.14)**	**-1.73 (-3.37, -0.25)**
Test number[b]	0.09 (-0.49, 0.68)	0.27 (-0.53, 1.11)
Relative difference in phenotype[c]	1.01 (-0.99, 3.14)	5.24 (-5.83, 18.15)
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	0.49 (0.00, 2.65)	0.66 (0.00, 3.82)
Female ID	0.27 (0.00, 1.58)	0.90 (0.00, 5.08)

*Reference category; estimate for treatment (unwashed) and mean values of remaining fixed effects; [a]Difference between reference category and treatment (washed); [b]Mean centered and standardized using the standard deviation within all trials; [c]Calculated by dividing female by male value

Table S7: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition using only data for the unwashed treatment, including testing order and relative difference in female and male body mass in model A) (*n* = 31) and relative difference in female and male body size in model B) (*n* = 21). Significance is shown in bold.

	Directional trail-following (reduced dataset)	
	Model A) Body mass	**Model B) Body size**
Fixed effects	***β*** **(95 % CI)**	***β*** **(95 % CI)**
Intercept*	1.39 (-5.48, 9.53)	-9.36 (-36.04, 12.78)
Test number[a]	0.57 (-0.82, 2.34)	1.47 (-0.11, 3.54)
Relative difference in phenotype[b]	-0.35 (-5.53, 4.52)	10.30 (-11.21, 36.88)
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	7.71 (0.00, 39.49)	3.86 (0.00, 22.89)
Female ID	1.83 (0.00, 10.48)	2.69 (0.00, 15.79)

*Reference category; estimate for mean values of fixed effects; [a]Mean centered and standardized using the standard deviation within all trials; [b]Calculated by dividing female by male variable value

Experiment 2

Do silk trails provide information on female phenotypic characteristics (body mass, size and condition) that affect directional trail following in males?

Experiment 2a: Effect of season

We explored the effect of season by adding the variable as random effect to our model. Season did not explain variation in the model output (Table S8).

Table S8: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMM (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition in study 2, including testing order (test number) and female body mass in model as fixed effects and spider identity and season as random effects (*n* = 115). Significance is shown in bold.

	Directional trail-following
Fixed effects	***β*** **(95 % CI)**
Intercept*	-0.13 (-1.20, 1.01)
Test number[a]	0.44 (-0.05, 1.11)
Female mass[a]	-0.08 (-0.58, 0.41)
Random effects	***σ²*** **(95% CI)**
Male ID	0.17 (0.00, 1.00)
Female ID	1.28 (0.00, 7.99)
Season	0.57 (0.00, 4.26)

*Reference category; estimate for mean values of fixed effects; [a]Mean centered and standardized using the standard deviation within all trials

Experiment 2a: Effects male body mass, size and condition

We investigated the influence of male traits (body mass, size and body condition) on the probability of males to follow trails in female direction by fitting two additional variations of our model presented in the manuscript.

Male likelihood to follow in female direction was not significantly influenced by either male body mass, size nor by male residual body condition index (Table S9, models a - c).

Table S9: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition, including male body mass (model a), size (model b) and residual body condition index (model c), accounting for testing order (*n* = 113). Significance is shown in bold.

	Directional trail-following – male phenotype		
	Model a) body mass	**Model b) body size**	**Model c) body condition**
Fixed effects	**β (95 % CI)**	**β (95 % CI)**	**β (95 % CI)**
Intercept*	-0.14 (-0.61, 0.31)	-0.38 (-1.07, 0.21)	-0.18 (-0.69, 0.29)
Test number[a]	0.44 (-0.02, 1.04)	0.42 (-0.03, 1.03)	0.42 (-0.04, 1.09)
Male trait[a]	-0.21 (-0.70, 0.24)	0.27 (-0.16, 0.75)	-0.03 (-0.54, 0.45)
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	1.01 (0.00, 6.14)	0.16 (0.00, 0.89)	0.17 (0.00, 0.95)
Female ID	0.15 (0.00, 0.81)	0.98 (0.00, 6.19)	1.30 (0.00, 8.35)

*Reference category; estimate for mean values of fixed effects; [a]Mean centered and standardized using the standard deviation within all trials

Experiment 2a: Effect of relative size difference depending on season

We investigated whether the influence of relative size difference between males and females occurred both for data collected in spring and autumn.

In spring, males were significantly more likely to follow females that were smaller than they were (Table S10, model A). This effect was not to be found in autumn (Table S10, model B).

Table S10: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition in study 2, including testing order (test number) and relative size difference (F/M) in spring, model a) (*n* = 78) and autumn, model b) (*n* = 35). Significance is shown in bold.

	Directional trail-following	
	Model A) Spring	**Model B) Autumn**
Fixed effects	**β (95 % CI)**	**β (95 % CI)**
Intercept*	-6.99 (-15.52, -0.53)	-5.61 (-18.69, 6.35)
Test number^a	0.41 (-0.14, 1.12)	0.65 (-0.37, 2.31)
Relative size difference^b	**6.40 (0.39, 14.33)**	5.05 (-6.01, 17.21)
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	0.20 (0.00, 1.19)	0.97 (0.00, 5.79)
Female ID	1.51 (0.00, 9.80)	2.92 (0.00, 19.06)

*Reference category; estimate for mean values of fixed effects; ^{a}Mean centered and standardized using the standard deviation within all trials; ^{b}Value calculated by dividing female size by male size

Experiment 2c: Effect of exclusion of biases on one day

Simulations of main models a and b were repeated excluding data collected on the biased day 24.05.2022.

Results in comparison to data not excluding the biased day were retained (Table S11).

Table S11: Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs (binomial) testing for predictors of male likelihood of following trails in the same direction moved by females during trail deposition in study 2 excluding the biased day, including testing order (test number) and female body mass in model A) (*n* = 100) and replacing female body mass with relative size difference between sexes in model B) (*n* = 98). Significance is shown in bold.

	Directional trail-following (reduced dataset, biased day)	
	Model A)	**Model B)**
Fixed effects	**β (95 % CI)**	**β (95 % CI)**
Intercept*	-0.18 (-0.69, 0.31)	-6.46 (-12.80, -1.20)
Test number[a]	0.32 (-0.15, 0.90)	0.21 (-0.27, 0.75)
Female mass[a]	0.11 (-0.39, 0.64)	-
Relative size difference[b]	-	**5.86 (0.90, 11.78)**
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Male ID	0.16 (0.00, 0.91)	0.16 (0.00, 0.87)
Female ID	0.97 (0.00, 6.22)	0.68 (0.00, 4.30)

*Reference category; estimate for mean values of fixed effects; [a]Mean centered and standardized using the standard deviation within all trials; [b]Value calculated by dividing female size by male size

Chapter II

Manuscript I: Female spiders performing maternal care reduce silk-bound chemical signalling

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Unpublished manuscript

Abstract

Chemical communication often is the base for mate assessment as animals signal their reproductive qualities to the other sex. Female reproductive qualities change during an individual's lifetime, resulting in variation in chemical signaling based on developmental or reproductive state. Yet, in spiders, research on variation in female chemical signaling often stops at mating, leaving chemical communication post-mating and especially during egg-laying and maternal care largely unexplored. We use the web-less hunting spider *Pisaura mirabilis* to test if females vary in their chemical signaling during i) developmental, ii) reproductive and iii) egg production and maternal care state by observing changes in male courtship behavior upon contact with female silk. We found differential behavioral responses of males exposed to silk of females varying in their developmental and reproductive state, including female egg-production and maternal care state, suggesting variation in female chemical signaling in web-less spiders. Males are more likely to court silk of adult females (opposed to juvenile or subadult silk), and do not differentially court silk of unmated and mated females. Notably, males significantly less often courted silk of females that laid and cared for fertilized egg-sacs. Females likely reduce chemical signaling during maternal care to minimize costs of attracting males and superfluous matings. Male discriminatory abilities might have evolved to reduce reproductive costs and consequently only target females that reproduce. Our findings highlight the importance of including post-mating states when investigating chemical signaling as well as the potential for male mate choice in this system characterized by costly male nuptial-gifts.

Keywords: Mate choice, silk, chemicals, spider, signaling, maternal care

Introduction

Chemical communication is widespread throughout the animal kingdom. Often considered the most ancient channel of information conveyance (Wyatt 2014), chemical communication in a reproductive context is indispensable for mate search, sex recognition, mate assessment and inter- as well as intra-sexual encounters in many species across taxa (Regnier and Law 1968; Burger 2005; Johansson and Jones 2007; Houck 2009; Breithaupt and Thiel 2011; Yew and Chung 2015). Chemical cues (i.e. inadvertently transmitted information) and signals (i.e. information evolved to be transmitted, such as pheromones) (Wyatt 2014) can effectively convey information about conspecific properties, such as identity, sex, immune competence, age or sexual receptivity (Rantala et al. 2002; Johansson and Jones 2007; Wyatt 2010; Thomas 2011), and consequently form the base for mate assessment. If chemical signaling that is depending on the sender's phenotypic state (e.g. developmental, reproductive) brings benefits to the sender, selection should favor strategic release of chemicals during transitory states while promoting the receiver's ability to detect such changes in chemical signals. Understanding sex-specific chemical release and detection will hence ease our understanding of mating system dynamics.

Spiders represent a particularly well-suited group for investigating chemical communication as many species use chemical means for communicating due to their often limited vision, solitary lifestyle and widespread distribution in their habitats (Foelix 2010; Uhl and Elias 2011). Together with body cuticle, spider silk, consisting of protein fibers and other substances such as pheromones (Foelix 2010), is an important carrier of chemical cues that are often essential for mate assessment and reproduction. In many web-building spiders, females add airborne pheromones to their webs, attracting males over long distances (Witt 1975; Ross et al. 1979; Jackson 1987; Kasumovic and Andrade 2004; Chinta et al. 2010; Fischer et al. 2021). Upon contact, the webs then induce male exploration and/or courtship behavior (Gaskett 2007). Web-less, cursorial spiders also produce silk in the form of silk lines deposited during movements, also known as draglines or safety lines, that stabilize the silk producer (van der Kraan and Richter 1970). These draglines, similarly to webs, function as a medium for chemical communication between the sexes, attracting mates (Anderson and Morse 2001; Nelson et al. 2012; Bell and Roberts 2016) and eliciting male courtship behavior (Jackson 1987; Papke et al. 2001; Baruffaldi et al. 2010; Chinta et al. 2010). Female silk alone generally provides mate searching males with information about the female's phenotype or state, important for male mate choice. For example, males can infer female mating state, often preferring webs (Riechert and Singer 1995; Herberstein et al. 2002; Stoltz et al. 2007; Xiao et al. 2009) or draglines (Jackson 1981;

Roberts and Uetz 2005; Baruffaldi and Costa 2010; Scott et al. 2018) of unmated females, avoiding competition over fertilizations of already mated females. Such reproductive state-dependent chemical release will ultimately benefit females from avoiding risks of remaining unmated, and potentially preventing additional males from mating if polyandry imposes fitness costs (Tuni et al. 2020). Females can also communicate their developmental state, by eliciting courtship responses in males contacting draglines (Baruffaldi and Costa 2010; Eberhard et al. 2021) or attracting partners only when sexually mature, and/or allowing males to find subadult unfertile females and guard them on their webs until females molt to adulthood to mate (Bel-Venner and Venner 2006; Uhl et al. 2015). In some species, males respond to and court silk of females depending on their age (Baruffaldi and Costa 2010, 2014; Weiss and Schneider 2022), preferring older females that are close to their egg deposition (Riechert and Singer 1995; Baruffaldi and Costa 2014; Cory and Schneider 2016; Waner et al. 2018). It is also possible for males to infer body condition from silk of webs (Weiss and Schneider 2022) or dragline silk , or hunger state from female silk, with males avoiding hungry and consequently more cannibalistic females (Johnson et al. 2011; Baruffaldi and Andrade 2015; Henneken et al. 2015a, 2017a).

Interestingly, studies on variation in spider chemical signalling, mainly focus on temporal scales up to the time when the females are mated (Gaskett 2007; Fischer 2019), disregarding the females' reproductive time beyond that time point, including egg-laying and egg-sac production and care (but see, e.g., (Baruffaldi and Costa 2010)). Females of many spiders provide maternal care for their offspring, both as eggs and spiderlings (Xiao et al. 2009; Ruhland et al. 2016a), by guarding them in a fixed location or carrying them on their body (Bristowe 1958; Eason 1964, 1969; Schneider 1996; Foelix 2010; Yip and Rayor 2014; Ruhland et al. 2016a). Brood care in form of egg carrying allows females living in an unpredictable environment to move around more freely while still caring for their eggs, hence increasing offspring fitness as females protect their eggs against predators and parasites, while providing optimal temperature and humidity conditions for egg development (Nørgaard 1956; Kullmann 1972; Lubin 1974; Li et al. 1999; Toyama 1999; Vieira and Romero 2008). However, such parental care is costly for the female as it incurs both metabolic and ecological costs, for example, in form of lost foraging opportunities, increased energy expenditure or predation risk (Colancecco et al. 2007; Ruhland et al. 2016a, b). To reduce costs associated with female maternal care, females should cease chemical signaling after egg laying to avoid sexual harassment by additional males attracted during that time. Male spiders, that on the contrary do not perform parental care, adopt strategies to ensure multiple matings and paternity share for themselves, including removal of existing egg-sacs and infanticide (Schneider and Lubin 1997; Gonzaga and Leiner 2013). For example, in the species *Stegodyphus lineatus*, males that arrive on the female's web may be successful in removing any existing egg-sac, forcing the female to re-mate and re-lay eggs, with replacement clutches being less successful (Schneider and Lubin 1997). Hence, sexual conflict may shape inter-sexual chemical communication, with egg-tending females being particularly reluctant to attract additional partners.

Females of the web-less hunting spider *Pisaura mirabilis* embed chemical cues in their draglines, known to elicit male courtship display (Beyer et al. 2018; Eberhard et al. 2021). As part of their courtship, male *P. mirabilis* offer nuptial-gifts (i.e., silk-wrapped prey donations) to females, essential for mate acceptance and mating (Drengsgaard and Toft 1999; Stålhandske 2001; Bilde et al. 2007; Tuni and Bilde 2010; Albo et al. 2011b; Tuni et al. 2013; Ghislandi et al. 2018). Nuptial-gifts entail a variety of costs for the male, including lost foraging opportunities as the prey is donated to the female (Albo et al. 2009), costly silk production for gift construction (Lang 1996; Albo et al. 2011a), and movement (Prokop and Maxwell 2012) as well as metabolic costs (Prokop and Okrouhlík 2021) from carrying the gift during mate search. *P. mirabilis* females are naturally polyandrous (i.e., they mate multiply) (Matzke et al. 2022), and females derive both direct nutritional benefits from gift consumption (Bilde et al. 2007; Toft and Albo 2015) and indirect benefits from mating with multiple partners (Tuni et al. 2013). Consequently, females are hypothesized to have evolved to signal their receptivity and to continuously attract males regardless of their previous mating experience, as supported by the finding that males court both unmated and mated females (Tuni and Bilde 2010) or their silk lines (Eberhard et al. 2021) equally, and indistinctively follow silk trails laid by both types of females (Beyer et al. 2023). Males further share an approximately equal chance of paternity of the offspring sired by multiple fathers (Matzke et al. 2022), hence lowering selective pressures for discriminating female mating state. Males instead are shown to differentiate between subadult and adult females, both by courting silk of the latter more frequently (Eberhard et al. 2021), and by preferentially following adults over subadults (Beyer et al. 2023), suggesting that female *P. mirabilis* vary their chemical signaling in relation to their developmental state, and males adjust their courtship investment accordingly.

Chemical signalling in mated females performing maternal care remains entirely unexplored. Once mated, females lay eggs inside a silken egg case (i.e., cocoon) and carry these in their mouthparts for 2-3 weeks (Austad and Thornhill 1986), a period of time in which the female does not feed (personal observations). Females then produce a nursery web with a tent-like shape, hang the cocoon on it and remain on the web to guard it (Austad and Thornhill 1986). Females guard the egg-sac until spiderlings hatch, and for 1-2 days after hatching

(Austad and Thornhill 1986). The emerged spiderlings disperse within a week. Females can produce multiple, sequential, egg-sacs through their lifetime as reported in laboratory conditions (Nitzsche 1988; Drengsgaard and Toft 1999). As reported in other spiders (Eason 1964), maternal care in form of egg carrying occurs even if the eggs are unfertilized, with *P. mirabilis* females being physiologically triggered to lay eggs and care for them for a period of time until fertilized eggs would have hatched, at which point they are abandoned. Female chemical signalling during maternal care and male responses remain unknown.

In this study, we hypothesize that females vary in their chemical signalling depending on their internal state (both developmental and reproductive) and that males respond to this variation with adjusted courtship display such as the likelihood, latency to and duration of courtship when in contact with female dragline silk. In detail, we hypothesize that females change their chemical signalling during i) their development (i.e. from juveniles to subadults and to adults), indicated by males increasing their courtship efforts as females get close to sexual maturity (i.e., courting silk of juveniles the least and silk of sexually mature adults the most (Eberhard et al. 2021)); ii) their reproductive state (i.e., from unmated to mated, and after production of a fertilized egg-sac carried by the female), indicated by a decrease in male courtship towards silk of egg-tending females as these females are expected to decrease their chemical signalling to reduce harassment from males and/or loss of the current clutch. Given the benefits of polyandry to both sexes, we predict no differences in male courtship display between silk of unmated and mated females (Eberhard et al. 2021; Beyer et al. 2023). Finally, iii) we hypothesize female chemical signalling to vary according to the female egg production state (i.e., after production of fertilized or unfertilized egg-sacs that are either carried by the female or experimentally removed). If variation in chemical signalling of females with egg-sacs is triggered by physiological changes that follow insemination and/or successful fertilization of the eggs, we expect females with fertilized eggs to reduce chemical advertisement compared to those with unfertilized eggs. Chemical signalling should recover once the egg-sac is removed (resembling loss of the cocoon or hatching) as multiple matings would no longer impose costs.

Methods

Collection and rearing

Pisaura mirabilis of both sexes were collected as juveniles or subadults in autumn 2020 (*n* = 88) and spring 2021 (*n* = 35) in grass fields near the Ludwig-Maximilians-University of Munich (Planegg-Martinsried, Germany) and brought to the laboratory, where they were housed individually in plastic vials (5 cm diameter x 10 cm height) covered with a sponge lid and filled with a layer of moss. Every 2-3 days, the moss was sprayed with water and the spiders were fed either 10 fruit flies (*Drosophila melanogaster*, *Drosophila hydei*), 2-3 houseflies (*Drosophila domestica*) or 3-5 cricket nymphs (*Gryllus bimaculatus*, *Acheta domesticus*). Prey type varied due to logistic reasons. On feeding days, all animals were fed the same type of prey to minimize possible effects of feeding status on silk production or behavior (Craig et al. 2000; Tso et al. 2005; Henneken et al. 2015b, 2017b). Additionally, spiders were not fed 24 hours prior to a trial. Animals were reared under natural photoperiod (autumn: 10 hours light: 14 hours dark, spring: 15 h light: 9 h dark). Spiders reared in autumn/winter were placed on heating mats to ensure comparable room temperature (approx. 24 °C). Putative seasonal differences between the two cohorts of spiders were statistically addressed (see below). Vials were inspected for molted exoskeletons every other day, and molting dates were noted down to determine individual spider age. Due to logistic reasons, during autumn, 27 animals were moved to a different location, but reared under equivalent conditions (see Electronic Supplementary Material Table S1-S2).

Experimental design and setup

We investigated male responses to silk lines produced by females varying in a) developmental state (juvenile, subadult, adult unmated), b) reproductive state (adult unmated, adult mated, after production of fertilized eggs: carried egg-sac), and c) egg production state (unsuccessful fertilization: unfertilized eggs removed/carried; successful fertilization: fertilized eggs removed/carried), by conducting no-choice assays using a plastic box (15 cm length x 15 cm height x 15 cm width). We included two control treatments, where we tested males i), on silk lines left by another male conspecific, to understand male reactions towards conspecific male’s silk and ii) in an empty plastic box to observe male behaviors without contact to female silk or putative chemical cues.

Silk lines were obtained by placing a spider inside the box and letting it move around for 15 min. To ensure an area-wide silk covering of the box, every 5 min, spiders were urged to move by gently shaking the box. After the 15 min timeframe, the silk laying individual was returned to its housing vial and a male was introduced to the test box, where it could move around freely for 15 minutes. During this time, we scored occurrence and duration of courtship behavior (i.e. abdominal vibrations) as well as latency to first courtship display. In order to exclude variation in gift investment between males as a potential factor affecting male courtship decisions (Ghislandi et al. 2014; Heimerl et al. 2022), used males did not carry nuptial-gifts. In case a male produced a gift in its vial after the last feeding before the trial, the gift was gently removed using forceps.

Trials were video-recorded for a total of 15 min, using a web camera (Logitech HD Pro Webcam C920), connected to a laptop and fixed at a distance of 40 cm over the plastic box. After each trial, the box was thoroughly cleaned using water and detergent, and dried using paper towels.

Variation in female individual state

To obtain females varying in a) developmental state, b) reproductive and c) egg-production state, we proceeded as follows. Female *developmental* state was ensured by observing female molting events and selecting females in their juvenile (i.e. at least second to last instar before adulthood) ($n = 12$; mean ± s.e. days before molt to adulthood: 23.92 ± 2.59), subadult (i.e., last instar before adulthood) ($n = 27$; days before molt to adulthood: 8.43 ± 1.12) and adult (i.e., after their molt to adulthood) ($n = 25$; days after molt to adulthood: 13.55 ± 0.78) age stage. Female reproductive state was manipulated by mating a randomly chosen subset of unmated females ($n = 17$) that was mated to a random male one to two days prior to the experiment, following established protocols for this species (Tuni and Bilde 2010; Tuni et al. 2013). Briefly, a male was placed in an empty plastic box (15 cm x 15 cm x 15 cm), in which an adult unmated female had been left for 15 minutes to deposit silk lines, known to stimulate male sexual behaviors (Lang 1996; Bilde et al. 2007; Ghislandi et al. 2017; Tuni et al. 2017; Magris and Tuni 2019). After one minute of acclimation, the male was offered a house fly that the male caught and started to wrap in silk to produce a nuptial gift. Once the gift construction was completed (i.e. the male picked up the silk-wrapped prey with its mouthparts), the same female that produced the silk lines used for stimulating the male's gift construction, was placed into the same box, and the pair could interact (i.e., male courtship display followed by mating). The pair was kept together until male and female separated from each other, leaving the mating position, after which both spiders were returned to their individual vials. In case the pair did not successfully mate (i.e. the male was not observed performing two pedipalp insertions during mating), the male was replaced with a new male and the trial was repeated on the same day. Mated males were not used as test subjects. Data collected on unmated females was used both for the developmental and the reproductive state analysis. We let mated females produce egg-sacs (*n* = 13) and tested them one to two days after egg laying. In a subset of females that produced egg-sacs, the egg-sac was removed using forceps and the female tested 1 day after (*n* = 9). Female *egg production* state was ensured by testing unmated adult females that produced unfertilized egg-sacs while they were still carrying it (*n* = 13), or one day after the unfertilized egg-sac was removed (*n* = 19). Not all females that were tested after egg-sac removal were tested while still carrying the egg-sac. Data collected on females with fertilized egg-sacs was used both for reproductive and egg production state analysis.

Control treatments

A subset of males (*n* = 16) was tested on male silk and in an empty box. Male silk was collected as described above. For the empty box treatment, the box was thoroughly cleaned before each trial using detergent, and dried using paper towels.

Use of individuals and sample sizes

We tested a total of 59 males and 64 females. Each male was at least tested twice (mean number of trials ± standard error: 4.85 ± 0.32), with each male having been randomly assigned to a treatment. Due to random assignment, it was possible that males were tested multiple times with the same treatment. Due to the nature of the experiment (i.e. testing the same animal when it was subadult, adult unmated, mated, carrying eggs and after the eggs were removed) and low available numbers of females in a treatment, many females (*n* = 46) were used with multiple males.

Video scoring

All videos were scored using video event-logging software BORIS (v7.10.5). For each video, we measured latency to and duration of courtship (i.e., abdominal vibrations) of the male for 15 minutes.

Statistical analyses

We investigated the likelihood of males to court, courtship duration and latency to courtship, silk of females varying in their developmental state (juvenile, subadult, unmated adult), reproductive state (unmated, mated, carrying their fertilized egg-sac), and egg-sac production state (carrying unfertilized eggs, having their unfertilized eggs removed, carrying fertilized eggs, having their fertilized eggs removed), by fitting a model with treatment and testing order (accounting for increasing male experience with the experimental setup) as fixed effects. Spider identities of each sex were added as random effects, accounting for repeated measurements and avoiding pseudo-replication. As the season of experiment conduction (autumn vs. spring) did not explain variation in the data (see Electronic Supplementary Material, Table S3-S4), we removed this variable from the model simulations. Females in the egg-production group significantly varied in their age, with fertilized females being older than unfertilized females (ANOVA: $F(3, 52) = 17.58$, $p < 0.001$; Electronic Supplementary Material Table S5). These differences were reduced, when including only those females in the analysis that were tested with their first egg-sac ($n = 43$) opposed to including first and second egg-sac ($n = 56$). This strategy resulted in decreased but still significant overall age differences between treatments (ANOVA: $F(3,39) = 2.96$, $p = 0.04$), but age differences between specific treatments were no longer significant (Electronic Supplementary Material Table S6). We consequently repeated our main model for egg production state, adding a binomial variable for number of female egg-sac.

We fitted explorative models to investigate whether male courtship varies in dependence of the closeness to female egg laying date using the data on adult unmated females, expecting that males are more likely to court and also to court sooner and longer the closer the female is to its oviposition date. Results on exploratory models are reported in the Electronic Supplementary Material.

To ease biological interpretation, we standardized numeric variables (i.e., testing order) by subtracting each value by the variable's overall mean and standardized the variable using the standard deviation of the variable. Statistical analyses were performed using R (version 4.1.1, R Core Team 2021). Generalized linear mixed-effect models (GLMMs) were applied using the "stan_glmer" function, which included the simulation of posterior distributions based on 4000 iterations (package "rstanarm" (Goodrich et al. 2020). The model family was set to binomial for the variable "courtship occurrence" and to gamma (with log-link) for "courtship duration" and "latency to courtship". Model fit was visually assessed based on the model's fitted values (goodness of fit graph) and the distribution of residuals. We inferred the statistical significance of fixed effects from the Bayesian 95 % credible intervals (CI) associated with the mean parameter estimate (β) and considered effects significant in the frequentist's sense when the 95 % CIs did not overlap zero (Nakagawa and Cuthill 2007).

Results

Female developmental state

Among females varying in their developmental state, males courted silk of adult unmated females (95 %, 36 out of 38) significantly more likely than juvenile (56 %, 9 out of 16) or subadult silk (78 %, 21 out of 27), whereas males did not differ between juvenile and subadult female silk (Table 1, Table 2, Figure 1). Courtship duration and latency to courtship were not significantly affected by female developmental state (Table 1).

Female reproductive state

Males did not differ in their likelihood to court silk of unmated (95 %, 36 out of 38) or mated females (92 %, 22 out of 24), but courted significantly less likely silk of females with their fertilized egg-sac (67 %, 10 out of 15) (Table 1, Table 3, Figure 2). Courtship duration and latency to courtship were not significantly affected by female reproductive state (Table 1).

Female egg production and maternal care state

Males courted silk of females that carried their *fertilized* eggs (67 %, 10 out of 15) significantly less often than females that carried their *unfertilized* eggs (100 %, 13 out of 13) (Table 1, Table 4 Figure 3). There was no difference in likelihood to court between females that had their fertilized (84 %, 16 out of 19) or unfertilized eggs removed (78 %, 7 out 9). Courtship duration and latency to courtship were not significantly affected by female egg production state (Table 1). Results were retained when adding number of egg sac to the model, thus controlling for age differences between treatments (Electronic Supplementary Material Table S7).

Controls

As the number of males courting in the control treatments was very low (4 out of 17 (23.5%) and 3 out of 17 (17.5 %) for male silk and an empty box, respectively), courtship duration and latency to courtship were not statistically investigated in this treatment group.

Random effects to the models reported in Table 1 can be found in the Electronic Supplementary Material (Table S8).

When investigating the likelihood of males to court females in dependence of the number of days until female egg laying, males did not court females more often that were closer to laying their eggs (Electronic Supplementary Material Table S9). Courtship duration and latency to courtship were not significantly affected by the closeness to female oviposition.

Table 1. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of male likelihood to court (model type A, binomial GLMM), male courtship duration and latency to courtship (model types B and C, Gamma GLMMs) depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized eggs), *egg production* state (carrying/removed unfertilized/fertilized eggs) and *control* treatments (male silk, empty box), including treatment and test number. Significance is shown in bold.

	A) Courtship occurrence	**B) Courtship duration**	**C) Latency to courtship**
	***β* (95 % CI)**		
Developmental state			
Intercept^{*1}	0.31 (-0.97, 1.71)	5.16 (4.10, 6.19)	5.07 (3.79, 6.59)
Treatment (subadult)^{a1}	1.14 (-0.49, 2.87)	0.12 (-0.99, 1.20)	-0.36 (-2.05, 1.17)
Treatment (adult unmated)^{a2}	**3.13 (1.18, 5.49)**	0.67 (-0.41, 1.75)	-0.96 (-2.58, 0.51)
Test number^{m}	-0.22 (-1.08, 0.68)	0.05 (-0.27, 0.38)	0.12 (-0.37, 0.67)
Reproductive state			
Intercept^{*2}	3.66 (2.02, 6.08)	5.84 (5.51, 6.19)	4.10 (3.43, 4.78)
Treatment (mated)^{b1}	-0.58 (-2.93, 1.81)	0.02 (-0.53, 0.58)	-0.13 (-1.17, 0.97)
Treatment (carrying fertilized eggs)^{b2}	**-2.74 (-5.30, -0.62)**	-0.65 (-1.39, 0.10)	0.30 (-1.13, 2.08)
Test number^{m}	-0.26 (-1.19, 0.68)	-0.05 (-0.28, 0.21)	0.02 (-0.11, 0.16)

	A) Courtship occurrence	**B) Courtship duration**	**C) Latency to courtship**
Egg production state			
Intercept[*3]	5.15 (2.02, 9.29)	5.21 (4.58, 5.92)	4.42 (3.25, 5.87)
Treatment (removed unfertilized eggs)[c1]	-2.77 (-6.90, 0.55)	0.60 (-0.24, 1.42)	0.61 (-1.15, 2.40)
Treatment (carrying fertilized eggs)[c2]	**-4.24 (-8.50, -0.82)**	-0.16 (-1.16, 0.84)	0.19 (-1.39, 1.65)
Treatment (removed fertilized eggs)[c3]	-3.23 (-7.60, 0.48)	0.85 (-0.27, 2.00)	-0.48 (-2.13, 0.97)
Test number[m]	-0.23 (-1.40, 0.89)	**-0.42 (-0.79, -0.06)**	0.40 (-0.18, 0.99)
Controls			
Intercept[*4]	-2.07 (-4.22, -0.51)	-	-
Treatment (empty)[d]	0.17 (-1.99, 2.32)	-	-
Test number[m]	**1.28 (0.17, 2.72)**	-	-

[*1] Reference category; estimate for female *developmental* state (juvenile) and normalized mean values for remaining continuous fixed effects; [a1/2] Difference between reference category and female developmental state (subadult/juvenile); [*2] Reference category; estimate for female *reproductive* state (adult unmated) and normalized mean values for remaining continuous fixed effects; [b1/2/3] Difference between reference category and female reproductive state (mated/carrying/removed fertilized eggs); [*3] Reference category; estimate for female *egg production* state (unfertilized eggs carrying) and normalized mean values for remaining continuous fixed effects; [c1/2/3] Difference between reference category and female egg production state (removed unfertilized eggs/carrying/removed fertilized eggs); [*4] Reference category; estimate for *control* treatments (male silk) and normalized mean values for remaining continuous fixed effect; [d] Difference between reference category and control treatment (empty box); [m] Mean centred within all trials and normalized over the standard deviation within all trials

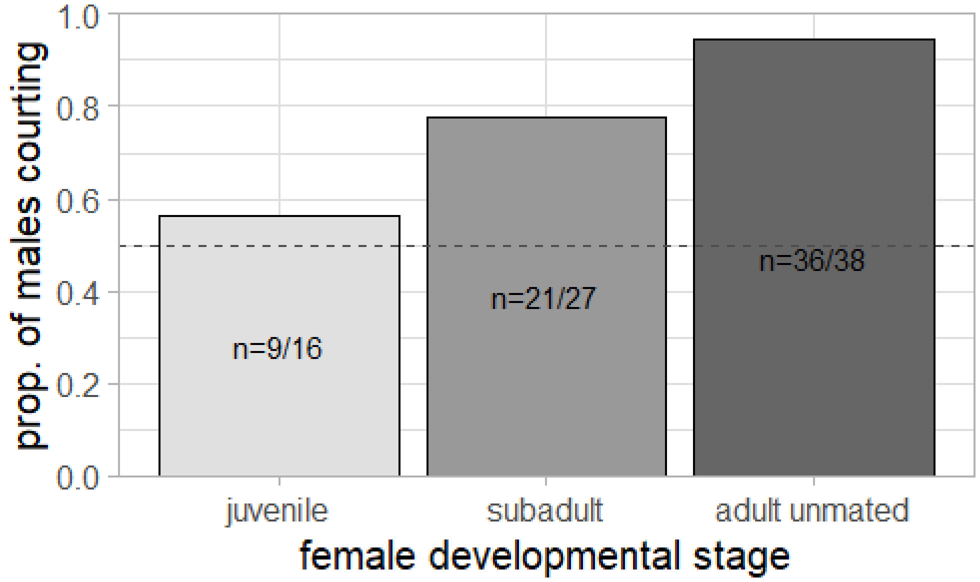

Figure 1. Male likelihood to court silk of females of varying developmental stages (juvenile, subadult, adult unmated). Numbers within bars = number of trials with courtship occurrence out of all trials within a treatment. Dashed line = random chance of courting. Juvenile females were not courted more often than subadult females. Adult unmated females were courted more often than juvenile or subadult ones.

Table 2. Post-hoc analysis of the GLMM (binomial) for influence of female developmental state on male likelihood to court, using Estimated Marginal Means (EMMs). Significance is indicated in bold.

Treatment comparison	Estimate	Lower 95 % - CI	Upper 95 % - CI
juvenile – subadult	-1.12	-2.82	0.53
juvenile – adult unmated	**-3.04**	**-5.42**	**-1.13**
subadult – adult unmated	**-1.91**	**-3.97**	**-0.10**

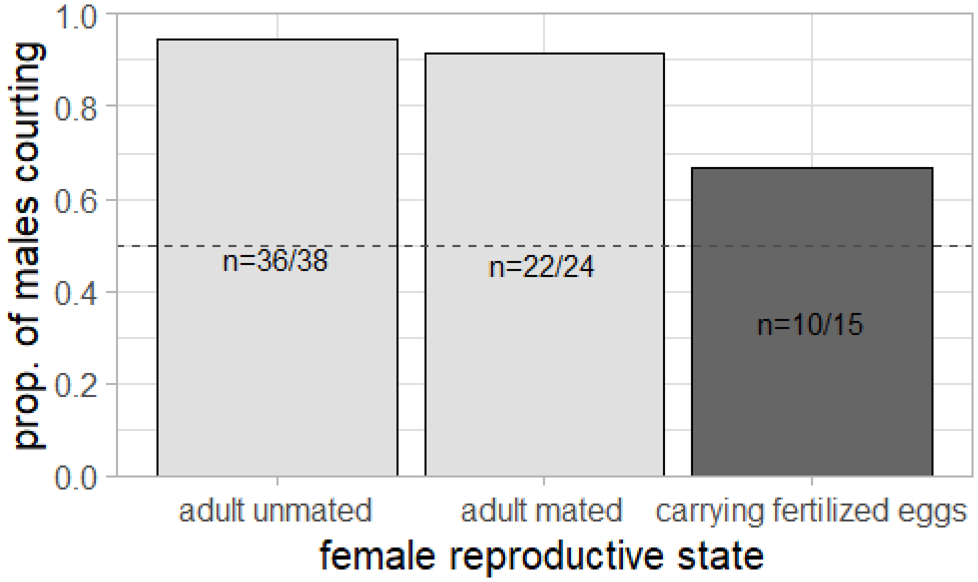

Figure 2. Male likelihood to court silk of females of varying reproductive state (adult unmated, adult mated, carrying fertilized egg-sac). Numbers within bars = number of trials with courtship occurrence out of all trials within a treatment. Dashed line = random chance of courting. Females carrying fertilized eggs are courted significantly less often than unmated or mated females.

Table 3. Post-hoc analysis of the GLMM (binomial) for influence of female reproductive state on male likelihood to court, using Estimated Marginal Means (EMMs). Significance is indicated in bold.

Treatment comparison	Estimate	Lower 95 % - CI	Upper 95 % - CI
unmated – mated	0.58	-1.79	2.94
unmated - fert. egg-sac carried	**2.66**	**0.46**	**5.08**
mated - fert. egg-sac carried	**2.10**	**0.02**	**4.55**

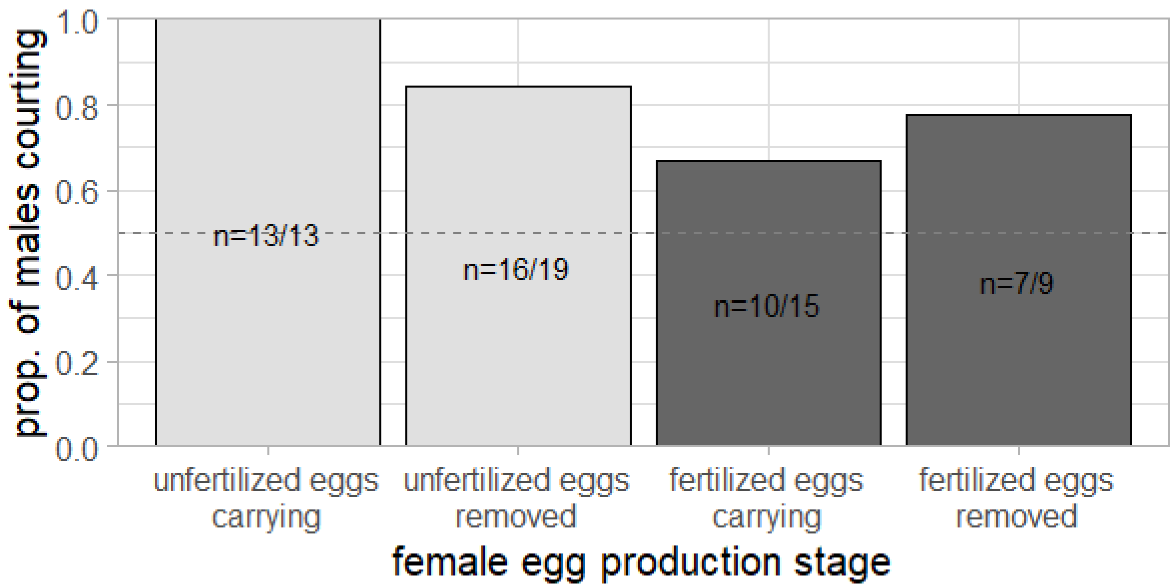

Figure 3. Male likelihood to court silk of females of varying egg production state (light grey = unsuccessful: unfertilized egg-sac removed/carrying; dark grey = successful: fertilized egg-sac removed/carrying). Numbers within bars = number of trials with courtship occurrence out of all trials within a treatment. Dashed line = random chance of courting. Silk of females carrying a fertilized egg-sac was less likely to be courted by males than silk of females carrying an unfertilized egg-sac.

Table 4. Post-hoc analysis of the GLMM (binomial) for influence of female egg production state on male likelihood to court, using Estimated Marginal Means (EMMs). Significance is indicated in bold.

Treatment comparison	Estimate	Lower 95 % - CI	Upper 95 % - CI
unf. egg-sac carried – unf. egg-sac removed	2.53	-0.88	6.50
unf. egg-sac carried – fert. egg-sac carried	**4.00**	**0.57**	**8.06**
unf. egg-sac carried – fert. egg-sac removed	3.02	-0.81	7.19
unf. egg-sac removed – fert. egg-sac carried	1.39	-0.67	3.86
unf. egg-sac removed – fert. egg-sac removed	0.43	-2.22	3.07
fert. egg-sac carried – fert. egg-sac removed	-0.99	-3.59	1.31

Discussion

In our study, we show differential behavioural responses of males exposed to silk of females varying in their developmental and reproductive state, including female egg-production and –caring state, suggesting variation in female chemical signalling in web-less spiders. Males are more likely to court silk of adult females (opposed to juvenile or subadult silk), and do not differentially court silk of unmated and mated females, but significantly decrease their courtship towards silk of females that laid and cared for fertilized egg-sacs. These findings add novel insights into chemical emission during the poorly explored post-mating phase of female maternal care, while corroborating existing evidence for variation in female chemical signalling during development.

Interestingly, we found that attractiveness of silk from females carrying their fertilized egg-sac decreased in comparison to females that were unmated or mated, with males being less likely to perform courtship behaviours. This finding, in line with results from the wolf spider species *Schizocosa malitiosa* (Baruffaldi and Costa 2010), suggests costs of continued attraction and additional matings during maternal care, and/or physiological changes of female internal chemistry following egg fertilization and laying, but not insemination (i.e., sperm storage of mated females). Insemination is known to influence female chemical production across taxa (Thomas 2011), including spiders, for example, by decreasing female attractiveness to other males after mating (Aisenberg and Costa 2005). This does not appear to be the case in *P. mirabilis*, as silk lines of mated and unmated females are courted equally by males (Eberhard et al. 2021), likely explained by direct and indirect benefits females gain from multiple matings and consumption of nuptial-gifts (Bilde et al. 2007; Tuni et al. 2013; Toft and Albo 2015), as well as males equally sharing paternity in multiple-sired clutches (Matzke et al. 2022). Once a female lays and cares for eggs, continued emission of chemical attractants may increase risks of male sexual harassment and egg loss, followed by forced matings and re-laying of eggs that may impose energetic and survival costs to females or their clutches (Schneider and Lubin 1997; Gonzaga and Leiner 2013). An alternative explanation may rely on energetic constraints of signal emission. Female attractiveness via silk lines in this species is dependent on female feeding state and body condition (Beyer et al. 2023), likely due to chemical production depending on prey-derived nutrients such as amino acids that may be used as precursors for pheromones (Tillman et al. 1999; Blomquist et al. 2018; Blomquist and Ginzel 2021). As female *P. mirabilis* do not feed during brood care (Drengsgaard and Toft 1999), their chemical production may be restricted by such nutrition-dependent energetic constraints. However, this explanation appears be unlikely, as it should also apply to females carrying and caring for egg-sacs containing unfertilized eggs. The costs of superfluous matings, together with other metabolic or ecological costs associated with maternal brood care in spiders, as increased energy expenditure or predation risk (Colancecco et al. 2007; Ruhland et al. 2016a, b), should lead to egg-caring females stopping or limiting production of chemical attractants. From the male perspective, and in line with our results, females that care for eggs should be avoided and courtship investment minimized as female spiders providing maternal care are very aggressive as they vigorously defend their eggs (Schneider and Lubin 1996, 1997; Whitehouse and Jackson 1998) and unreceptive towards additional matings (Calbacho-Rosa et al. 2017). Interestingly, these changes in chemical signaling occurred within a relatively short time frame, with the majority of females (11 out of 15) being tested within 24 hours after egg deposition. While our results need cautious interpretation due to the limited sample size for males tested on silk of egg-carrying females, our findings suggest variation in chemical signaling linked to egg fertilization and possible egg-caring, and male ability to recognize and respond behaviourally to these changes.

To understand the relative importance of egg fertilization and maternal care (egg-caring) state on female chemical signalling and male courtship investment, we allowed females to produce both fertilized (i.e,, after mating) and unfertilized egg-sacs (i.e., females remained unmated until egg production), testing males both on silk of females that carried their eggs and on females that had their egg-sacs removed by us. We found that attractiveness of silk from females that carry their fertilized eggs was lower than the silk of females that were carrying unfertilized eggs, while finding no differences for females whose eggs were removed. Our findings overall suggest that physiological changes in the female that are likely triggered by egg fertilization in conjunction with maternal care behaviour (egg carrying) lead to variation in female signalling, and that such signalling is not reversible on the short term after egg-sac loss. Female *P. mirabilis* care for their egg-sacs irrespective of them being fertilized or unfertilized, with the latter only being abandoned at the very end of the egg caring phase (Drengsgaard and Toft 1999), similar to observations in other spider species (Eason 1964). This apparent lack of discrimination and the continued provision of brood care, leading to high costs while gaining no reproductive output, may suggest female inability to recognize the fertilization state of their eggs. Contrasting, *P. mirabilis* males differentiated between females with varying egg fertilization state, courting more often females that carried unfertilized eggs.

When female egg brooding care is completed, meaning the eggs have hatched or the egg-sac is lost, polyandrous females might start chemically re-advertising their receptivity, for example, in order to replenish depleted sperm storages (Perampaladas et al. 2008). However, in our study, males did not discriminate between females that carried their egg sacs and those that had it removed, suggesting similar chemical profiles between treatments. In case of the females with unfertilized egg-sacs, the lack of difference between females carrying eggs and those that had it removed could be explained by the female being unmated and remaining attractive throughout her egg-caring time, highlighting the importance of fertilization for induction of variation in chemical signaling. For fertilized spiders, the lack of difference between egg carrying and eggs removed could be explained through the female having received enough sperm in their previous mating to fertilize eggs for multiple egg-sacs as is the case in many spider species (Schneider and Lubin 1998). However, in *P. mirabilis*, hatching success increases with multiple number of mates, indicating that in this species sperm of a single male might not be enough to optimize reproductive output (Tuni et al. 2013). Females in this species would consequently benefit from re-advertising again. We cannot exclude that re-commencing chemical signaling after it was once shut down takes time, even if the shut-down occurred within one or two days. For example, female redback spiders, *Latrodectus hasselti*, become unattractive almost immediately after mating, but start re-advertising only three months later (Perampaladas et al. 2008). While *P. mirabilis* don't share the longevity of redback spiders, it is possible that re-advertising takes multiple days to take place, and would thus evade our detection as we tested animals one day after egg removal. While our results need cautious interpretation due to the low sample size for females in the egg production group, male discrimination between females carrying fertilized and unfertilized eggs suggests innate changes in female physiology likely occurring after egg fertilization, potentially coupled with but not solely based on the female providing maternal care.

Our finding that males court adult females more often than subadult ones is in line with previous findings in which males invest more in courtship toward adult silk (Eberhard et al. 2021) and tend to prefer silk lines of adults over subadults (our study), indicating female silk-borne signaling based on developmental state. Female spiders commonly start producing chemical attractants once they moulted to sexual maturity (Uhl 2013), as most spiders become sexually receptive at this point and matings lead to successful fertilizations. Subadults instead usually do not emit chemical attractants (Chinta et al. 2010), with exceptions being, for example, spider species in which subadult matings lead to successful reproduction (Biaggio et al. 2016; Waner et al. 2018; Baruffaldi and Andrade 2020). Still, it remained unknown, how female chemical emission changes during spider growth, if it is for example gradually increasing as the young spider grows or if emission is getting “switched” on after the molt to adulthood and reaching sexual maturity. This would result in males either gradually courting more the closer the young spiders come to their final molt, or suddenly courting after the female's final molt with no courtship displayed towards subadults. We tried to fill this knowledge gap by exposing males to silk of non-adult spiders including both subadults (i.e., last instar before adulthood) and juveniles (i.e. instars at least 2 molts away from adulthood), and found evidence for both gradual and sudden chemical emission. In favor of gradual development of chemical emission, males courted both silk of juvenile and subadult females, with males courting even females that are multiple weeks away from adulthood in 50 % of the cases. However, for gradual emission, we would have expected significant differences in male courtship behaviors for juveniles and subadults. Instead, male courtship was similar between juveniles and subadults. In addition, males courted adult females significantly more often than subadult ones, indicating a significant increase and/or change in female chemical signaling after their molt to adulthood. In *P. mirabilis*, females mature later in the season than males and female chemicals are known to accelerate male development and maturation. Male reactions towards both juvenile and subadult females, coupled with the sudden increase of courtship investment as soon as females become sexually mature, suggests presence of chemicals starting from a very early age, but likely in much lower concentrations and/or varying blends than in adults.

When investigating, whether unmated female *P. mirabilis* increase their chemical signaling as they age and get closer to their first egg-sac production, we found no influence on male courtship occurrence, latency or duration: out of the 24 unmated females of which egg laying dates were known, 23 were courted. The lack of differences in male courtship behavior as proxy for female chemical signaling is surprising, as the production of and subsequent caring for unfertilized eggs appears to be very energetically demanding, with *P. mirabilis* females losing large amounts of body weight during brood care as they do not feed (personal observation). Consequently, females are expected to increase their chemical signaling as they age, also due to young adults often signaling less due to chemical production costs, and they have not encountered a mate yet (Welke and Schneider 2012; Umbers et al. 2015; Cory and Schneider 2016). While it is possible that female *P. mirabilis* do not increase their chemical signaling as they age unmated, this option seems unlikely considering the apparent high costs involved in brood care of unfertilized eggs. Rather we see it more likely that the involved chemicals might elicit other behaviors beside courtship, such as exploration or attraction. Chemical analyses (e.g., measuring concentration differences) could help with verifying differences in chemical production as females age. Alternatively, female chemicals could affect male courtship vigor, for example in form of number of vibrations emitted by the male in response to female silk. While we could not measure such detailed vibrations, laser vibrometry would allow more in depth analysis of male courtship behaviors. Male reactions towards juvenile/subadult unreceptive females, coupled with the finding that males court male silk similarly unlikely than an empty box suggests the presence of a sex-specific chemical to which males react with courtship.

In conclusion, our study shows differential behavioral responses of males exposed to silk of females varying in their developmental and reproductive state, importantly including the time after egg-production and during maternal care, suggesting state-dependent variation in female chemical signaling. Females are likely to cease chemical signaling to reduce the costs of attracting males while tending for their egg-sacs, as males appeared to be able to discriminate females based on their fertilization and/or egg-caring state. Such discriminatory abilities might be favored by selection as males have to safeguard their reproductive investment in form of nuptial-gifts by targeting sexually reproducing females. Our findings also highlight the potential for mate choice in this system.

Statements and Declarations

Author contributions: CT and MB conceived and designed the study; MB collected data, drafted the manuscript and analyzed data; CT contributed to writing and editing. All authors have read, provided comments and agreed to the final version of the manuscript.

Data accessibility: All data generated or analyzed are included in the supplementary information files.

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Supplementary Material

Female spiders performing maternal care reduce silk-bound chemical signalling

Michelle Beyer, Cristina Tuni

Location differences for autumn trials

In autumn, 27 trials were conducted in another location (opposed to 170 in location 1), consisting of 1 trial with a subadult, 8 with unmated adults, 2 with females carrying fertilized egg sacs, 4 with females with removed fertilized egg sacs, 6 with females carrying unfertilized egg-sacs and 6 with removed unfertilized egg sacs. No control trials were conducted in location 2. We verified the comparability of conditions between locations by adding location as a random effect to our models for courtship occurrence on female developmental, reproductive and egg production state.

Results were retained, location did not affect male courtship (Tables S1-S2).

Table S1. Estimated effect sizes (β) and 95 % credible intervals (CIs) for fixed effects of the binomial GLMMs testing for location effects in animals tested in autumn on predictors of male likelihood to court, depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized eggs), and *egg production* state (carrying/removed unfertilized/fertilized eggs), including treatment and test number as fixed effects, and spider identities and location as random effects. Significance is shown in bold.

	Courtship occurrence
Fixed effects	***β*** **(95 % CI)**
Developmental state (*n* = 81)	
Intercept[*1]	0.13 (-1.97, 1.96)
Treatment (subadult)[a1]	1.13 (-0.53, 2.83)
Treatment (adult unmated)[a2]	**3.20 (1.18, 5.65)**
Test number[m]	-0.21 (-1.16, 0.66)
Reproductive state (*n* = 77)	
Intercept[*2]	3.12 (0.31, 5.95)
Treatment (mated)[b1]	-1.05 (-3.82, 1.51)
Treatment (carrying fertilized eggs)[b2]	**-3.09 (-5.98, -0.92)**
Test number[m]	-0.03 (-1.03, 1.02)

Egg production state (*n* = 56)	
Intercept[*3]	5.08 (1.62, 9.42)
Treatment (removed unfertilized eggs)[c1]	-2.82 (-7.02, 0.50)
Treatment (carrying fertilized eggs)[c2]	**-4.30 (-8.66, -0.87)**
Treatment (removed fertilized eggs)[c3]	-3.25 (-7.61, 0.50)
Test number[m]	-0.20 (-1.51, 1.11)

[*1] Reference category; estimate for female *developmental* state (juvenile) and normalized mean values for remaining continuous fixed effects; [a1/2] Difference between reference category and female developmental state (subadult/juvenile); [*2] Reference category; estimate for female *reproductive* state (adult unmated) and normalized mean values for remaining continuous fixed effects; [b1/2/3] Difference between reference category and female reproductive state (mated/carrying fertilized egg sac); [*3] Reference category; estimate for female *egg production* state (carrying unfertilized egg-sac) and normalized mean values for remaining continuous fixed effects; [c1/2/3] Difference between reference category and female egg production state (removed unfertilized egg-sac/carrying fertilized egg sac/removed fertilized egg-sac)

Table S2. Variation (σ) and 95 % credible intervals (CIs) of the random effects of the binomial GLMMs testing for location effects in animals tested in autumn on predictors of male likelihood to court depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized egg-sac), and *egg production* state (carrying/removed unfertilized/fertilized egg-sac), including treatment and test number as fixed effects, and spider identities and location as random effects.

	Courtship occurrence
Random effects	**σ (95 % CI)**
Developmental state	
ID female	0.63 (0.00, 4.11)
ID male	0.66 (0.00, 4.23)
Location	1.06 (0.00, 7.27)
Reproductive state	
ID female	1.34 (0.00, 8.64)
ID male	0.89 (0.00, 5.45)
Location	2.68 (0.00, 13.87)
Egg production state	
ID female	2.13 (0.00, 10.86)
ID male	0.49 (0.00, 3.02)
Location	1.09 (0.00, 7.64)

Seasonal differences

In autumn, 178 trials were conducted, compared to 19 trials conducted in spring. Treatment groups tested in spring consisted of 5 juvenile, 1 subadult, 2 adult unmated, 6 mated and 5 females carrying fertilized egg sacs. No control treatments were conducted in spring. We investigated whether the season of experiment conduction (autumn vs. spring) influenced our model output female developmental, reproduction and egg production state by adding the variable season as random effect to the models described in the main manuscript.

Results were retained, season did not affect male courtship (Table S3-S4).

Table S3. Estimated effect sizes (β) and 95 % credible intervals (CIs) for fixed effects of the binomial GLMMs testing for seasonal effects in animals tested in autumn on predictors of male likelihood to court, depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized eggs), and *egg production* state (carrying/removed unfertilized/fertilized eggs), including treatment and test number as fixed effects, and spider identities and location as random effects. Significance is shown in bold.

	Courtship occurrence
Fixed effects	***β* (95 % CI)**
Developmental state (*n* = 81)	
Intercept^{*1}	0.10 (-2.03, 1.89)
Treatment (subadult)^{a1}	1.08 (-0.60, 2.85)
Treatment (adult unmated)^{a2}	**3.09 (1.13, 5.47)**
Test number^{m}	-0.27 (-1.21, 0.59)
Reproductive state (*n* = 77)	
Intercept^{*2}	3.51 (1.33, 6.06)
Treatment (mated)^{b1}	-0.55 (-2.97, 1.87)
Treatment (carrying fertilized eggs)^{b2}	**-2.72 (-5.28, -0.60)**
Test number^{m}	-0.28 (-1.30, 0.69)
Egg production state (*n* = 56)	
Intercept^{*3}	5.14 (1.73, 9.69)
Treatment (removed unfertilized eggs)^{c1}	-2.81 (-7.29, 0.56)
Treatment (carrying fertilized eggs)^{c2}	-4.30 (-8.82, -0.86)
Treatment (removed fertilized eggs)^{c3}	-3.29 (-7.89, 0.43)
Test number^{m}	-0.21 (-1.38, 0.96)

[*1] Reference category; estimate for female *developmental* state (juvenile) and normalized mean values for remaining continuous fixed effects; [a1/2] Difference between reference category and female developmental state (subadult/juvenile); [*2] Reference category; estimate for female *reproductive* state (adult unmated) and normalized mean values for remaining continuous fixed effects; [b1/2/3] Difference between reference category and female reproductive state (mated/carrying fertilized egg sac); [*3] Reference category; estimate for female *egg production* state (carrying unfertilized egg-sac) and normalized mean values for remaining continuous fixed effects; [c1/2/3] Difference between reference category and female egg production state (removed unfertilized egg-sac/carrying fertilized egg sac/removed fertilized egg-sac)

Table S4. Variation (σ) and 95 % credible intervals (CIs) of the random effects of the binomial GLMMs testing for seasonal effects in animals tested in autumn on predictors of male likelihood to court depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized egg-sac), and *egg production* state (carrying/removed unfertilized/fertilized egg-sac), including treatment and test number as fixed effects, and spider identities and location as random effects.

	Courtship occurrence
Random effects	**σ (95 % CI)**
Developmental state	
ID female	0.67 (0.00, 4.03)
ID male	0.74 (0.00, 4.42)
Season	1.07 (0.00, 7.29)
Reproductive state	
ID female	1.29 (0.00, 8.07)
ID male	0.97 (0.00, 5.75)
Season	0.95 (0.00, 6.55)
Egg production state	
ID female	2.11 (0.00, 11.15)
ID male	0.49 (0.00, 3.10)
Season	1.14 (0.00, 7.59)

Influence of female age in the egg production treatment

Females in the egg production treatment significantly varied in their age, with females that were fertilized being older than unfertilized ones (Table S5). To smoothen this difference, we excluded females that were tested with their second instead of first egg sac, resulting in age differences between treatments no longer being significant (Table S6). We then repeated our main model, adding female number of egg sac (binomial) as an additional fixed effect to account for female egg sac number).

Results were retained (Table S7).

Table S5. Post-hoc analysis of the ANOVA testing for differences in female age between egg production states, using Tukey multiple comparisons of means. Significance is indicated in bold.

Treatment comparison	**Estimate**	**Lower 95 % - CI**	**Upper 95 % - CI**	**p adj**
unf. egg-sac rem. – unf. egg-sac car.	1.52	-3.34	6.38	0.84
fert. egg-sac car. – unf. egg-sac car.	**8.62**	**3.50**	**13.74**	**<0.001**
fert. egg-sac rem. – unf. egg-sac car.	**13.26**	**7.41**	**19.12**	**<0.001**
fert. egg-sac car. – unf. egg-sac rem.	**7.10**	**2.43**	**11.76**	**<0.001**
fert. egg-sac rem. – unf. egg-sac rem.	**11.74**	**6.28**	**17.21**	**<0.001**
fert. egg-sac rem. – fert. egg-sac car.	4.64	-1.05	10.34	0.15

Table S6. Post-hoc analysis of the ANOVA testing for differences in female age between egg production states when using females with their first egg-sac, using Tukey multiple comparisons of means. Significance is indicated in bold.

Treatment comparison	**Estimate**	**Lower 95 % - CI**	**Upper 95 % - CI**	**p adj**
unf. egg-sac rem. – unf. egg-sac car.	1.52	-1.97	5.01	0.65
fert. egg-sac car. – unf. egg-sac car.	3.53	-0.83	7.88	0.15
fert. egg-sac rem. – unf. egg-sac car.	5.82	-0.39	12.03	0.07
fert. egg-sac car. – unf. egg-sac rem.	2.01	-2.08	6.09	0.56
fert. egg-sac rem. – unf. egg-sac rem.	4.30	-1.72	10.32	0.24
fert. egg-sac rem. – fert. egg-sac car.	2.29	-4.27	8.85	0.79

Table S7. Estimated effect sizes and 95 % credible intervals (CIs) of the binomial GLMM testing effects on predictors of male likelihood to court (*n* = 81), depending on female *egg production* state, including treatment (adult unmated, adult mated, adult female carrying fertilized egg-sac), test number and egg-sac number (binomial) as fixed effects, and spider identities and location as random effects. Significance is shown in bold.

	Female egg production state
	Courtship occurrence
Fixed effects	***β* (95 % CI)**
Intercept*	5.34 (2.15, 9.73)
Treatment (unf. removed)[a]	-2.83 (-7.21, 0.53)
Treatment (fert. carry)[b]	**-3.99 (-8.41, -0.36)**
Treatment (fert. removed)[c]	-2.83 (-7.54, 1.44)
Test number[d]	-0.23 (-1.46, 0.97)
First egg-sac (no)[e]	-0.66 (-3.74, 1.94)
Random effects	**σ^2 (95 % CI)**
Id Female	2.75 (0.00, 13.83)
Id Male	0.52 (0.00, 3.36)

* Reference category; estimate for female reproductive state (unfertilized eggs-sac carried), female's first egg sac (yes) and normalized mean values for remaining continuous fixed effects, [a] Difference between reference category and female reproductive state (unfertilized egg-sac removed), [b] Difference between reference category and female reproductive state (fertilized egg-sac carrying), [c] Difference between reference category and female reproductive state (fertilized egg-sac removed), [d] Mean centred within all trials and normalized over the standard deviation within all trials, [e] Difference between reference category and female first egg sac (no)

Random effects: Variation and 95 % credible intervals

Table S8. Variation and 95 % credible intervals (CIs) of the random effects of the GLMMs testing for predictors of male likelihood to court (model type A, binomial GLMM), male courtship duration and latency to courtship (model types B and C, Gamma GLMMs) depending on female *developmental* state (juvenile, subadult, adult unmated), *reproductive* state (adult unmated, mated, carrying fertilized eggs), *egg production* state (carrying/removed unfertilized/fertilized eggs) and *control* treatments (male silk, empty box), including treatment and test number.

	A) Courtship occurrence	**B) Courtship duration**	**C) Latency to courtship**
	σ (95 % CI)		
Developmental state			
ID female	0.63 (0.00, 3.80)	0.07 (0.00, 0.37)	1.06 (0.00, 2.95)
ID male	0.66 (0.00, 4.10)	0.32 (0.00, 1.15)	0.18 (0.00, 0.89)
Reproductive state			
ID female	1.23 (0.00, 7.31)	0.04 (0.00, 0.22)	0.50 (0.00, 1.51)
ID male	0.90 (0.00, 5.43)	0.06 (0.00, 0.29)	0.40 (0.00, 1.53)
Egg production state			
ID female	1.92 (0.00, 10.21)	0.12 (0.00, 6.64)	0.17 (0.00, 0.88)
ID male	0.47 (0.00, 3.03)	0.15 (0.00, 0.67)	0.30 (0.00, 1.53)
Controls			
ID male	1.75 (0.00, 9.41)	-	-

Days to egg sac production

We investigated male likelihood to court, latency to and duration of male courtship behavior in dependence of female closeness to oviposition by replacing the treatment variable from the main models for female developmental state with the variable containing days until oviposition. Only data on unmated adult females was used.

We found that males do not court females more often that are closer to laying their eggs (Table S9). Courtship duration and latency to courtship were not significantly affected by the closeness to female oviposition.

Table S9. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of male likelihood to court in unmated adult females (model A, *n* = 24, binomial GLMM), male courtship duration and latency to courtship (model B and C, *n* = 23, Gamma GLMMs) depending on female age state, including treatment (juvenile, subadult, adult unmated), days to production of first egg sac and test number as fixed effects, and spider identities as random effects. Significance is shown in bold.

	Female age state		
	A) Courtship occurrence	**B) Courtship duration**	**C) Latency to courtship**
Fixed effects	***β* (95 % CI)**		
Intercept*	4.19 (1.21, 8.11)	5.48 (4.78, 6.24)	3.71 (2.13, 5.27)
Test number[a]	1.48 (-2.11, 5.83)	-0.32 (-1.18, 0.58)	-0.09 (-1.57, 1.40)
Days to 1st egg sac	-0.04 (-0.43, 0.29)	-0.02 (-0.10, 0.05)	0.10 (-0.04, 0.24)
Random effects	**σ^2 (95% CI)**		
Id Female	1.26 (0.00, 8.06)	0.15 (0.00, 0.98)	1.48 (0.00, 5.11)
Id Male	1.03 (0.00, 6.33)	0.15 (0.00, 0.95)	1.69 (0.00, 6.31)

* Reference category; estimate for female age state (juvenile) and normalized mean values for remaining continuous fixed effects, [a] Mean centred within all trials and normalized over the standard deviation within all trials

Chapter III

Publication II: Female state and condition-dependent chemical signalling revealed by male choice of silk trails

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Original Article

Female state and condition-dependent chemical signaling revealed by male choice of silk trails

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Male mate choice is predicted in systems with high costs of mating, as for those with male nuptial gifts and/or sexual cannibalism. We ask whether males of the nuptial gift-giving spider *Pisaura mirabilis* exert preferences for mates varying in their reproductive potential based on chemical information during mate search. Males were presented with binary trails consisting of silk lines and substrate-borne chemicals deposited while females were walking, from females varying in 1) body condition (high vs. low), 2) developmental state (subadult vs. adult), and 3) mating state (unmated vs. mated). If female chemical signaling co-varies with individual state, we expect males to choose trails of females that are 1) in higher body condition, indicating higher fecundity, 2) adults, which can successfully reproduce, and 3) unmated, to avoid sperm competition. We show that female signaling is condition-dependent, with males being more likely to follow trails of higher body condition females, but not dependent on female mating state. Males also tended to prefer trails of adults over subadults. Choice did not depend on male individual body condition. Our findings suggest costs to chemical signaling in nutritionally deprived females, often considered negligible, and their potential as reliable indicators of individual quality. Selection may favor male preferences for more fecund partners given the energetic investment nuptial gifts entail. Nutritional and reproductive benefits of multiple mating to females and high share of paternity for males, may instead select against signaling of female mating state, and/or male discrimination and choice.

Key words: **chemicals, mate choice, signaling, silk, spider.**

INTRODUCTION

Theory predicts that female reproductive success is limited by the number of their eggs and male reproductive success by the number of their mates (Bateman 1948). This has led to postulating distinct reproductive roles for each sex, with males being highly competitive over mates and females expressing choosiness for the most suitable partners (Andersson and Simmons 2006). Yet, males can also be choosy (Bonduriansky 2001; Edward and Chapman 2011; Fitzpatrick and Servedio 2018) and intra-sexual selection can similarly act on females (Hare and Simmons 2019). Male choosiness is predicted when there is large variation in female quality (Kvarnemo and Simmons 1999) and when reproduction incurs high costs to males (Herberstein et al. 2002). Examples of costs to males include risky mate search (Berger-Tal and Lubin 2011), energetically demanding courtship displays (Woods et al. 2007), nutritive donations (Lewis and South 2012), or parental care (Sargent et al. 1986). Reducing the magnitude of such costs can shape male preferences, as, for example, shown for male spiders preferring unmated females to avoid cannibalism risks (Herberstein et al. 2002).

Not least, male choosiness also depends on male individual quality (Pollo et al. 2022). To derive fitness benefits from mate choice, males should discriminate and select between females varying in their reproductive value by using signal traits that are indicators of female quality, most likely reflecting female fecundity (e.g., body condition) (Marshall and Gittleman 1994; Roff 2002; Leather 2018). Despite involving multiple complex signaling modalities (i.e., visual, auditory, tactile, and/or chemical) (Johnstone 1997; Candolin 2003), chemical assessment is most commonly used in a reproductive context (Wyatt 2014).

Chemical signals such as pheromones (i.e., emitted to trigger behavioral or developmental responses in other individuals (Karlson and Lüscher 1959)) are involved in a wide range of communication function during reproduction (Johansson and Jones 2007; Wyatt 2009; Jacobson 2012; Steiger and Stökl 2014; Coombes et al. 2018). The chemical information encoded in pheromones may be used to select higher quality or most compatible mating partners (Penn 2002; Thom et al. 2008). Yet, while our understanding of how female mate choice shapes male chemicals traits has progressed (e.g., cuticular hydrocarbons in male insects [Thomas and Simmons 2009; Lane et al. 2016]), studies on male choice for fe-

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male chemical signals lag behind (Harari and Steinitz 2013).

Pheromones not only can be indicators of the state of an individual (e.g., developmental and/or mating state) as they vary in quantity and quality (i.e., the ratio of their components) due to the associated changes in internal chemistry (Johansson and Jones 2007; Thomas 2011), but can also be emitted strategically. Females can vary their investment in chemical signaling in response to social and/or other environmental conditions (Havrilak et al. 2015; Rehermann et al. 2016; Pham et al. 2020), suggesting fine-scale adjustment of pheromone production. From the female perspective, sexually immature females should avoid or limit chemical signaling to prevent attraction of unwanted suitors, whose mating attempts could lead to costly sexual harassment (Arnqvist and Rowe 2013). Upon reaching sexual maturity, females may start producing pheromones that function to attract males in order to secure matings and avoid reproductive failure (Rhainds 2010), as these pheromones may convey information on female fertility to potential mates (Kücklich et al. 2019). Once mated, females may switch to emitting chemicals that repel males and/or may halt the emission of male-attracting chemicals (Thomas 2011; Weiss and Schneider 2022a) to avoid the costs of extra matings (Arnqvist and Rowe 2013). Despite pheromones having been for long considered biochemically cheap to produce (Wyatt 2014), the emerging evidence for costly chemical production (Harari et al. 2011; Holman 2012; Umbers et al. 2015) suggests that chemical signaling could also function as an honest indicator of the signaler's underlying quality and/or body condition (Zahavi 1975; Grafen 1990). Female pheromones have been shown to co-vary positively with the condition of the emitter in few taxonomical groups, such as moths (Jaffe et al. 2007; Foster and Johnson 2011; Harari et al. 2011; Gonzalez-Karlsson et al. 2021), snakes (Shine et al. 2003), and spiders (Weiss and Schneider 2022a, 2022b) (but see (Assis et al. 2017)). From the male perspective, being able to discriminate female individual state may bring large reproductive benefits, as males could avoid courting and mating with infertile (e.g., subadult) females (Cook and Cook 1975; Lewis and Iannini 1995; McCartney and Heller 2008; Zahradnik et al. 2008; Tuni and Berger-Tal 2012), and/or select partners of highest quality (e.g., body condition, size, fecundity) (Jones et al. 2001; Dosen and Montgomerie 2004; Reading and Backwell 2007; Nandy et al. 2012; Baruffaldi and Andrade 2015). Assessing the female's mating status is particularly beneficial in polyandrous species (i.e., females mate with multiple males), where recognition and avoidance of mated females can reduce sperm competition and increase paternity success, or in systems with sperm priority patterns, where first or last males to mate may gain advantages in fertilizations (Simmons 2001; Thomas 2011).

Spiders are particularly suitable organisms to investigate chemical communication, as chemicals are commonly emitted from their body surface and their silk (Gaskett 2007; Schulz 2013). Spider pheromones can be airborne (volatile), transmitted over long distances as those involved in mate location and attraction, or mechanical (tactile), transmitted at contact as those eliciting courtship behaviors in males (Gaskett 2007). While only a few spider pheromones have been chemically characterized (Fischer 2019), there is vast behavioral evidence for chemical signaling (Uhl and Elias 2011). Many studies focus on web-building spiders, with evidence for males being able to differentiate between females varying in sexual maturity (Tuni and Berger-Tal 2012), age (Riechert and Singer 1995; Baruffaldi and Costa 2010), mating status (Riechert and Singer 1995; Gaskett et al. 2004; Stoltz et al. 2007; Scott et al. 2018; Weiss and Schneider 2022a), and feeding state (Baruffaldi and Andrade 2015), often based solely on silk assessment. Yet, cursorial, webless spiders also produce silk by depositing silk lines (namely, draglines) while moving. These silk lines are known to mediate chemical communication, for example, by triggering courtship behaviors (Jackson 1987; Papke et al. 2001; Ghislandi et al. 2017) or conveying information about female mating state, as reported in wolf spiders (Roberts and Uetz 2005; Rypstra et al. 2009; Baruffaldi and Costa 2010) and the running crab spider (Sentenská and Pekár 2019). Females are furthermore known to modify silk line deposition based on social context, such as male courtship behavior (Havrilak et al. 2015; Khan and Persons 2015).

Evidence for silk-borne communication also exists for draglines of the cursorial spider *Pisaura mirabilis* (Beyer et al. 2018; Eberhard et al. 2021). This species is well known for the males' courtship behavior, as males offer nuptial gifts silk-wrapped prey donations to females, which are key for mate acceptance and mating (Drengsgaard and Toft 1999; Stålhandske 2001; Bilde et al. 2006, 2007; Tuni and Bilde 2010; Albo et al. 2011b; Tuni et al. 2013; Ghislandi et al. 2018). Such a mating system is associated with high costs for males that lose foraging opportunities in order to donate prey to females (Albo et al. 2009), invest high amounts of costly silk for gift construction (Lang 1996; Albo et al. 2011a), and suffer movement (Prokop and Maxwell 2012) and metabolic costs (Prokop and Okrouhlík 2021) from carrying gifts during mate search. Females, on the other hand, derive direct nutritional benefits from gift consumption (Bilde et al. 2007; Toft and Albo 2015), and indirect benefits from mating with multiple partners (Tuni et al. 2013). Hence, selection may act on adult females to signal receptivity, regardless of their previous mating experience (Tuni and Bilde 2010), and to attract multiple males due to the gained fitness benefits associated to their nuptial feeding behavior. Previous studies show that female draglines possess male-attracting contact chemicals (Beyer et al. 2018), commonly known to trigger gift construction behavior and that are therefore included in many study designs (Bilde et al. 2007; Albo et al. 2011a; Ghislandi et al. 2017; Tuni et al. 2017; Magris and Tuni 2019). In a recent study on male vibratory courtship, using laser vibrometry, pulses generated by drumming of the male abdomen were measured in males exposed to silk draglines of females varying in their developmental and mating state (Eberhard et al. 2021). Silk lines conveyed information on female developmental state, as vibratory courtship occurred more frequently when males contacted silk of adults compared to subadult females, but seemingly not on female mating state, as no differences in vibratory performance were detected toward silk of unmated and mated females (Eberhard et al. 2021). Hence, there is evidence for variation in female chemical signaling and high mating costs for males that may select for adaptive male mate choice decisions. Yet, it remains unknown whether males use chemical information carried by female silk Hence, there is evidence for variation in female chemical signaling and high mating costs for males that may select for adaptive male mate choice decisions. Yet, it remains unknown to exert mate preference in the context of mate search.

We conducted binary choice assays to test male preference for trails consisting of silk lines and substrate-borne chemicals deposited upon movements, of females varying in 1) body condition (high vs. low), 2) developmental state (subadult vs. adult), and 3) mating state (unmated vs. mated). In order to understand whether information from silk trails is derived chemically, we also allowed males to choose between silk trails washed of any putative chemical and empty no-silk trails, as a lack of preference would point to chemicals and not exclusive tactile contact to silk being the main information channel. If female chemical signaling varies with female individual state and such variation is indicative of the female's reproductive potential, it would be advantageous for *P. mirabilis* males to discriminate between trails and preferentially choose one over the other due to the costs of nuptial gifts. Specifically, we predict males to prefer trails of females in higher body condition (i.e., females experimentally provided with a high feeding regime), a trait closely associated to higher fecundity in spiders (Marshall and Gittleman 1994), over females of low body condition. The latter may pose additional costs (e.g., injury and/ or mortality) to males due to increased incidence of sexual cannibalism in starved females (Toft and Albo 2016). Males should prefer silk trails of adults over subadults, in order to avoid costly sexual interactions with immature females that would not yet be reproductive. Finally, although *Pisaura mirabilis* males do not differ in their courtship behavior when paired with unmated or mated females (Tuni and Bilde 2010; Tuni et al. 2013) or when exposed to silk of females of different mating status (Eberhard et al. 2021), if given a choice between unmated and mated females, we may expect males to prefer unmated females due to reduced sperm com- petition and the possibility to monopolize the female through long copulations via nuptial gifts. Yet, the gain in paternity obtained by mating with previously mated females (Matzke et al. 2022) may weaken the strength of selection for unmated partners. Given evidence that the strength of male choice is higher in males of higher individual quality (Pollo et al. 2022), we additionally explored the effect of male body condition on choice.

MATERIALS AND METHODS

Animal raising

Spiders (~150) were collected by hand from grass fields in the Großhadern area of Munich (Germany) in May 2021. Captured spiders consisted mainly of juveniles and subadults (in four cases as adults, see details below). They were brought to the laboratory at the Ludwig Maximilian University and individually placed into transparent plastic vials (10 cm height and 5 cm diameter) containing moss and closed by sponge lids. Spiders were fed three times a week with either 15 fruit flies (*Drosophila spp)*, two cricket nymphs (*Gryllus bimaculatus)*, or two houseflies (*Musca domestica)*, and moss was sprayed with water on the feeding days. Animals were kept at room temperature (~23 °C) and natural photoperiod (15 light:9 dark hours). Vials were inspected daily for presence of molted exoskeletons, and date of final molts to adulthood was noted to determine post-maturation age. Animals were used in experiments two weeks after reaching adulthood, or two weeks after their capture in the case of the four male spiders captured as adults. These four spiders were assumed to be unmated and relatively young as, at the time of capture, only subadults were found in the field and the captured males were all lacking nuptial gifts, suggesting limited mating opportunities. Individual body mass was measured to the nearest 0.01 g using a digital scale (Kern PKT, Kern & Sohn GmbH, Germany) immediately before testing, and prosoma width was measured to the nearest 0.01 mm using electronic calipers (Aerospace, China) as a proxy for spider body size, once the experiment was completed.

Experimental design and setup

To test whether males show preference for silk trails produced by females varying in 1) body condition (high vs. low), 2) developmental state (subadult vs. adult), and 3) mating state (unmated vs. mated), we conducted binary choice assays using a V-shaped walking corridor (40 cm length × 12 cm height × 7 cm width, with a 90° angle) made of polystyrene foam and with all internal surfaces covered with brown polypropylene packing tape (Tesapack 64014) for easy cleaning. Spiders could enter the device at the intersection of both arms where their housing vial was inserted through a pre-made hole (7 cm diameter) in the wall, and could exit the device through holes present at the end of each arm. Specifically, female silk trails (i.e., silk lines and substrate-borne chemicals) were obtained by allowing two females varying in their individual state (body condition, developmental state, and mating state) to sequentially walk, each, through one of the arms of the device (i.e., starting from the opening at the intersection to the end of the arm) while the other arm was blocked with a polystyrene foam sheet. Since the intersection area was crossed by both females, it contains silk trails and putative body chemicals of both individuals. In case the female did not walk through the entire arm of the experimental device (stopped or reversed direction), the spider was removed, the device cleaned with 70% ethanol (EtOH, purest, Carl Roth, Germany), and the procedure repeated. The state of the female depositing silk trails in each arm of the device, left or right, was randomized to avoid biases. At the end of the procedure, females were returned to their housing vials and the device was inspected visually to ensure silk presence. This procedure does not allow distinguishing silk types, but only confirms that female silk deposition occurred.

A male was allowed to enter the device from the intersection area and could freely move, and the trial was considered completed when the male reached one of the holes at the end of the device's arms. A time limit of 15 min was set within which the male had to reach one of the ends of the device, otherwise the trial was stopped. However, all males reached an end of the device within this timeframe. In cases where males had produced a gift after feeding on the previous day, these were gently removed from their mouthparts using forceps before testing. By using gift-less individuals, which are also known to wander in search of mates in the field (Ghislandi et al. 2018), we excluded variation in gift investment between males (Ghislandi et al. 2014; Heimerl et al. 2022) as a potential factor affecting male mate choice decisions. We also scored occurrence and duration of male courtship behavior (i.e., abdominal vibrations) to investigate further differences in male responses toward silk of females varying in their condition and reproductive state.

Variation in female individual state

To obtain females varying in 1) body condition, 2) developmental state, and 3 mating state, we proceeded as follows. Female *body condition* was manipulated by applying a differential feeding regime to a randomly chosen subset of females as soon as they reached adulthood, for a total of 2 weeks: low-fed regimes were implemented by providing spiders once a week with two house flies (n = 10 females), and high-fed regimes by providing two house flies three times a week (n = 15 females). Female *mating state* was manipulated by mating a randomly chosen subgroup of adult females (n = 10 females), kept on high-fed feeding regimes, 1–2 days prior to the experiment, following well established procedures for this species (Tuni and Bilde 2010; Tuni et al. 2013). In brief, a male was placed in an empty plastic transparent box (10 × 10 × 7 cm) where a female had been previously left for approximately 30 min to leave dragline silk, known to stimulate male sexual behaviors (Lang 1996; Bilde et al. 2007; Albo et al. 2011a; Ghislandi et al. 2017; Tuni et al. 2017; Magris and Tuni 2019). After 1 min of acclimation, the male was offered a house fly for gift construction. The spider caught and killed the prey, and started silk-wrapping it. Once the male picked up the silk-wrapped prey with its mouthparts, an indication that gift construction was completed, the same adult female used for stimulating gift construction was placed into the same arena, and the pair could interact (i.e., male courtship and then mating). In two cases, the pair did not successfully mate, hence the male was replaced with a new male and the trial repeated on the same day. Individuals were kept together until they separated from each other by leaving the mating position. Then, both male and female spiders were returned to their individual vials. Mated males were not used as test subjects. All females al- located to this treatment eventually mated. Unmated females were randomly chosen from the adult females of our laboratory population. Variation in female *developmental state* was ensured by monitoring female molting events and selecting females—kept on high-fed feeding regimes—in their subadult (i.e., last instar before adulthood) (n = 13 females), and adult (2 weeks post-maturation, unmated) (n = 20 females), stages. We verified that females tested were subadults by monitoring their subsequent molt, and adults by inspection of the opening of their reproductive tract (epigynum) that darkens upon maturity.

Silk trail washing treatment

We used the same procedure and device outlined above for an additional test to understand whether males respond to chemical in- formation present in the silk trails or to silk presence per se. In such a test, a single adult female on a standard feeding regime (n = 21 females) provided a trail by walking through one of the device's arms (in randomized order) that was washed of putative (polar) chemicals as it was sprayed with 96% ethanol (EtOH, purest, Carl Roth, Germany) (as done in Beyer et al. 2021), yet retaining physical cues from silk presence. Ethanol was chosen at it is able to dissolve both polar and (weakly) non-polar substances due to its polar (hydroxyl (OH)) and non-polar (ethyl (C_2H_5)) groups, and consequently covers a range of putative chemicals for removal. The other device arm was left empty, hence it contained no structural and/or chemical cues derived from female silk and/or body surface. The device was then left to dry (~5 min) before introducing the male at the intersection, and proceeding with the test (i.e., no female silk or cues). A male was introduced at the intersection, and we proceeded with the test.

Use of individuals and sample sizes

A total of 31 males were tested. Each male was used in all four tests (female body condition, developmental state, mating state, and silk trail washing treatment) in a randomized order and on subsequent days. In total, 60 females were used, with 51 of them having been used multiple times (specifically, 17 out of 25 in body condition, 14 out of 33 in developmental state, 17 out of 28 in mating state, and 9 out of 21 washing treatments) in order to optimize use of individuals and keep the age at testing constant.

Statistical analyses

We investigated male preference for silk trails produced by females varying in 1) body condition, 2) developmental state, and 3) mating state, and for washed silk trails, by fitting three distinct binomial generalized mixed-effects models (GLMMs) (one for each female state) and one generalized linear model (GLM) for the silk washing treatment. We specifically included male choice (yes/no) for the arm containing silk trails of 1) high-fed females (when testing high- fed vs. low-fed trails), 2) adult females (when testing adult vs. sub- adults trails), 3) unmated females (when testing unmated vs. mated trails), and 4) washed silk (when testing washed vs. no trails), as response variable. In other words, a positive male choice was assigned when the male first reached the hole at the end of the device arm with silk of, for example, high-fed females when running choice tests of high-fed versus low-fed silk trails. Male testing order (1–4) was included to control for increasing male experience, as fixed effect. Female identity was added as random effect to account for pseudo-replication due to multiple usage of females (unlike males that were tested only once per treatment) in the models involving female states, but not in the model analyzing washed silk trails. As female body condition is known to influence male reproductive behavior in arthropods, for example by males preferring high condition and consequently more fecund females (Leather 2018), we calculated a residual body condition index as the residuals of a linear regression of body mass and prosoma width (Jakob et al. 1996), and included the residual body condition index as an additional covariate in our model when testing for the effect of female mating state on male choice. Female body condition was directly linked to the feeding regimes (high-fed females are of higher condition than low-fed) and developmental state (adult females are of higher condition than subadult), and consequently was not added for these models. We verified that body condition indices differed between low-fed and high-fed females using a two-sample *t*-test. Since male individual quality is known to influence the expression of male choice (Pollo et al. 2022), we also included male body condition in our models.

Due to low sample sizes, male occurrence of courtship toward silk of females varying in their condition, developmental and mating state, as well as toward washed silk and an empty device was not analyzed statistically. Descriptive statistics can be found in the Supplementary Material.

All numeric variables were grand-mean-centered by subtracting each value from the variable's overall mean, and standardized using the standard deviation of the variable. Statistical analysis was per- formed using R (version 4.1.1) (R Core Team 2021). Binomial GLMMs were applied using the "stan_glmer," and the binomial GLM using the "stan_glm" function of the package "rstanarm" (Goodrich et al. 2020) and included the simulation of posterior dis- tributions based on 4000 iterations. Model fit was assessed using the models' fitted values (goodness of fit graphs) and residual distributions. For all fixed effects, Bayesian 95% credible intervals (CIs)— describing with 95 % probability that the true estimate lies within the given intervals—and associated mean parameter estimates (β) were calculated using the posterior distributions of the models. Fixed effects were considered significant, when the 95% CIs did not include zero (Nakagawa and Cuthill 2007).

RESULTS

Our experimental feeding regimes yielded significant differences in female body condition, as the estimated residual body condition index varied significantly between high-fed and low-fed females (two-sample *t*-test: $t = -12.88$, df = 45.86, $P < 0.001$; mean residual body condition index ± SE; low-fed: -23.28 ± 2.14, $n = 20$; high-fed: 15.02 ± 2.07, $n = 31$). High-fed females were also heavier than low-fed females (two-sample *t*-test: $t = -10.09$, df = 58.33, $P < 0.001$; mean female mass ± SE in mg: low-fed: 88.87 ± 2.54, $n = 31$; high-fed: 128.71 ± 3.02, $n = 31$), whereas there was no significant difference in body size (two-sample *t*-test: $t = 0.52$, df = 47.18, $P = 0.61$; mean female prosoma width ± SE in mm: low-fed: 3.75 ± 0.02, $n = 20$; high-fed: 3.72 ± 0.04, $n = 31$).

Males were three times more likely to choose silk trails of high- fed females than those of low-fed ones (Table 1, Figure 1A). Males did not exert preferential choice between silk trails of adult and subadult females, although this effect is marginally non-significant with males being twice as likely to choose silk trails of adults (Table 1, Figure 1B). Males did not prefer silk trails of unmated over mated females (Table 1, Figure 1C), or of washed silk trails over no silk trails (i.e. an empty arm of the device) (Table 1, Figure 2). These results were not explained by male individual body condition (Table 1). In the experiment on female mating state, with increasing testing experience males were less likely to choose the unmated females' silk, whereas female body condition did not affect male preferences (Table 1).

DISCUSSION

When encountering trails, consisting of silk lines and substrate-borne chemicals, of females varying in their state and condition, males of the spider *Pisaura mirabilis* exert strong preference for those of females that are of high body condition (opposed to low condition females) and tend to prefer those of sexually mature females (opposed to immature subadult females), but exert no preference for trails of females varying in their mating state (unmated vs. mated). Males also responded similarly to the presence of silk trails washed of any putative chemicals and absence of any trail, suggesting that chemicals bonded to the silk and/or derived from the female's body cuticle, and not silk presence per se, may be responsible for transferring information about female quality. We hence highlight two key findings: 1) female signaling is state and condition-dependent, and 2) males can discriminate between female phenotypes and exert preferences based solely on chemical signals that are likely to be indicators of the female's underlying reproductive potential.

Variation in female chemical signaling

By manipulating female feeding regimes, we found that the attractiveness of female trails to males varied, as trails of females in better body condition (high-fed) were more likely to be followed by males. These findings uncover potential costs to chemical signaling, suggesting that, by negatively affecting the individual's body condition, nutritional stress may impair the production of chemicals, in the form of reduced release of silk and its associated silk-borne chemicals, and/or reduced release of body chemicals, or drive female strategic allocation to chemical production. Body condition is considered the available pool of resources from which the individual can draw to allocate toward trait production and maintenance (Rowe and Houle 1996; Hill 2011). Pheromone production may make use of such energetic resources (Blomquist and Bagnères 2010) while also potentially directly depending on nutrition, as dietary amino acids (South et al. 2011) and/or other specific diet compounds can be used as precursors for pheromo-

Table 1

Estimated effect sizes (β-value) and 95% credible intervals (CIs) of the binomial GLMMs (models A–C) and GLM (model D) testing for the likelihood of males to follow silk trails of females that are (A) of higher body condition (high-fed vs. low-fed, *n* = 31), (B) adults (adult vs. subadult, *n* = 26), (C) unmated (mated vs. unmated, *n* = 31), and (D) to follow silk trails that were washed from putative chemicals (washed vs. empty arm of the device, *n* = 28)

	(A) Body condition (high-fed, low-fed)	(B) Developmental state (adult, subadult)	(C) Mating state (mated, unmated)	(D) Silk treatment (empty, washed)
Fixed effects	β (95% CI)			
Intercept[a]	**1.31 (0.33, 2.54)**	0.95 (−0.05, 2.07)	0.10 (−0.89, 1.14)	−0.17 (−0.96, 0.61)
Test order[b]	−0.38 (−1.39, 0.56)	−0.29 (−1.32, 0.72)	**−1.27 (−2.51, −0.27)**	0.32 (−0.45, 1.10)
Male condition[b]	0.08 (−0.94, 1.12)	−0.22 (−1.27, 0.77)	−0.15 (−1.17, 0.79)	0.26 (−0.57, 1.10)
Female condition[b]	-	-	0.25 (−0.80, 1.39)	-
Random effects	σ^2 (95% CI)			
Female ID	0.83 (0.00, 4.80)	0.80 (0.00, 5.22)	1.25 (0.00, 7.76)	-

We accounted for testing order (1–4) and male body condition in all models, and female body condition in model C, including those variables as fixed effects. Effect of female individual condition and state (A–C), and silk treatment (D) is given in the respective intercept. Significance (i.e., when 95% CIs do not include zero) is shown in bold.

[a] Reference category; estimate for respective female state and silk treatment chosen (silk trail of high-fed in model A, adult in model B, unmated in model C, washed in model D) and mean values of remaining fixed effect.

[b] Mean-centered and standardized using the standard deviation within all trials

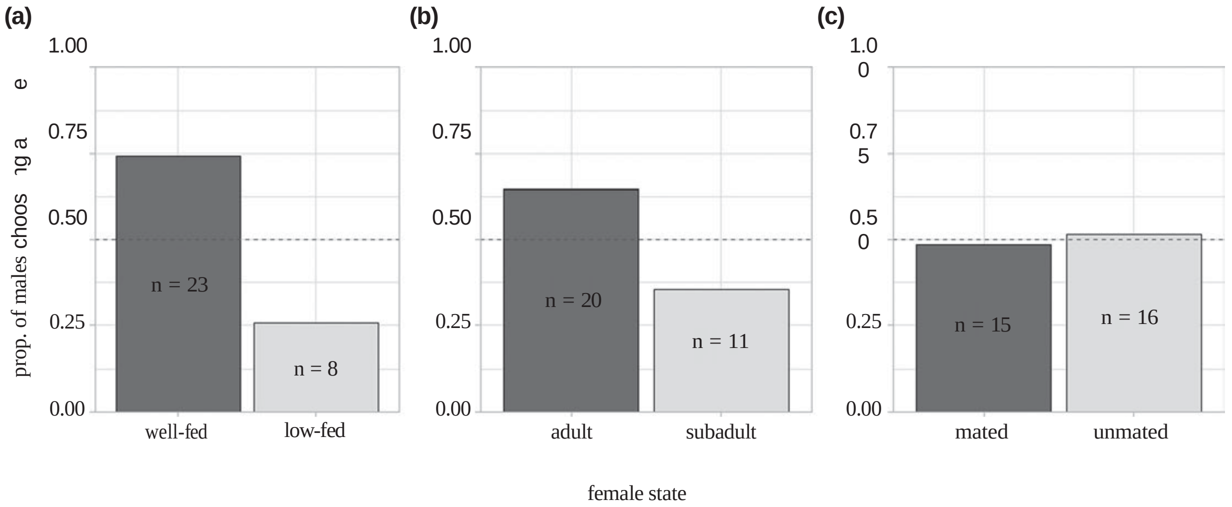

Figure 1
Proportion of males choosing silk trails of females varying in their (A) body condition (high-fed vs. low-fed), (B) developmental state (adult *vs.* subadult), and (C) mating state (mated *vs.* unmated). Dashed lines indicate random choice. Numbers within bars = number of males choosing a trail of a certain female state out of all males within the female state- treatment.

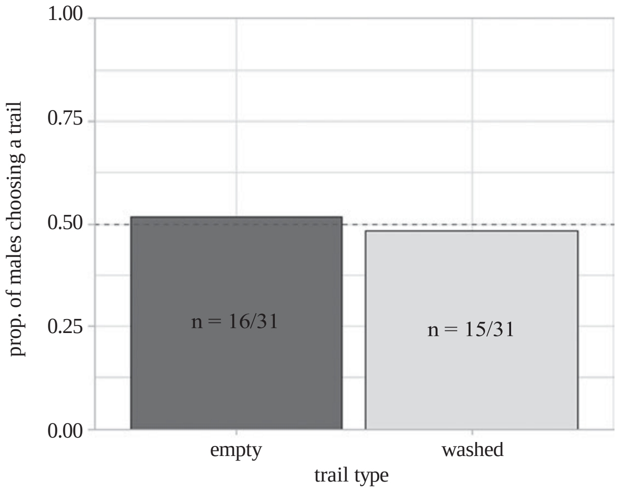

Figure 2
Proportion of males choosing a side of the testing device that contains either a female's silk trail washed with solvents (light gray) or is empty (dark gray). The dashed line indicates random choice.

nes, or even act directly as sex pheromones themselves (Landolt and Phillips 1997; Eisner and Meinwald 2003). Evidence for variation of chemical signaling linked to female nutritional state and/or body condition remains limited to a handful of studies. Pheromone production (quantitative, not qualitative) in the moth *Heliothis virescens* is reduced in sugar-stressed females and allows males to assess variation in female phenotypes (Foster and Johnson 2011). In the fruit borer moth *Neoleucinodes elegantails*, females that produce the blend of pheromones preferred by males are heavier and have larger wings (Jaffe et al. 2007). Starvation significantly reduced the amount of body pheromones—absolute amounts and relative amounts per unit body mass—released in the wasp spider *Argiope bruennichi* (Weiss and Schneider 2022b). Apart from diet-mediated changes in chemical production, costs of chemical signaling have been uncovered through trade-offs in resource allocation (Stearns 1992), where energy allocated to one function (e.g., pheromone production) comes at the expense of investment in others (e.g., soma maintenance). Female moths are, for example, shown to suffer reduced survival from chemical advertising for males (Harari et al. 2011). Strategic pheromone release from females, where signaling is tailored to the reproductive needs, as described in moths (Umbers et al. 2015) and in the wasp spider (Weiss and Schneider 2022a), are further suggestive of costs of pheromone production. All the above examples refer to females, yet condition-dependent chemical signaling is also described in males; glands of poor-fed males contain less pheromone precursor in the cockroach *Nauphoeta cinerea* (Clark et al. 1997), female mealworm beetles *Tenebrio molitor* prefer pheromones from high-fed males, where enhanced pheromone attractiveness is coupled to weakened male immunity (Rantala et al. 2003), and males of the lekking fruit fly *Drosophila grimshawi* that emit higher pheromone amounts have shorter lifespans (Johansson et al. 2005).

The finding that males tend to prefer silk of adults over subadults points to variation in *Pisaura mirabilis* female silk-borne signaling

The finding that males tend to prefer silk of adults over subadults points to variation in *Pisaura mirabilis* female silk-borne signaling-based on their developmental stage, as previously suggested (Eberhard et al. 2021). Signaling receptivity is crucial for adults, and female spiders are commonly known to start production of sex pheromones to attract males once they molted to sexual maturity (Uhl 2013). On the contrary, subadults are thought to not emit sex pheromones (e.g., the wasp spider *Argiope bruennichi* (Chinta et al. 2010). Nevertheless, empirical studies show contrasting male behavioral responses toward silk of subadults, with males of some species displaying courtship only on webs or silk extracts from webs of adult females (i.e., *Steatoda grossa* (Fischer et al. 2018) or preferring silk of adults over silk of subadults (i.e., *Stegodyphus lineatus* (Tuni and Berger-Tal 2012)), and males of other species expressing courtship behaviors toward silk of subadults (i.e., *Schizocosa ocreata* (Roberts and Uetz 2005)). Attracting a mating partner at a subadult stage would only be beneficial to females when the benefit of securing a mate exceeds the costs of male cohabitation, for example due to male cleptoparasitic foraging behavior on subadult female webs (Erez et al. 2005) and/or subadult matings lead to successful re- production (Biaggio et al. 2016; Waner et al. 2018; Baruffaldi and Andrade 2020). None of these scenarios are likely to apply to our study system.

Overall, these findings strongly suggest that spider trails (i.e., silk lines and substrate-borne chemicals) of females varying in feeding and likely developmental state differ in their pheromone quantities or composition, with females in lower body condition and subadults releasing fewer chemicals or different blends (Baruffaldi et al. 2010; Chinta et al. 2010; Henneken et al. 2015, 2017; Weiss and Schneider 2022b). We cannot distinguish between the sources of such chemicals being either silk or body cuticle, and suggest that both are likely to play a synergistic function. Pheromone production from body cuticle is, for example, shown to strongly correlate with silk pheromones in the sexually cannibalistic spider *Argiope bruennichi* (Weiss and Schneider 2022a). While our experimental test with washed silk trails is highly suggestive of such chemically-mediated form of male choice, we cannot fully exclude that differences in silk characteristics, silk amount and/or microstructure, between females of different state explain our results. Our study did not account for the spatial arrangement of silk strands or of attachment discs (i.e., silk structures that anchor silk lines to the substrate). Silk line diameter, for example, is known to co-vary positively with the spider's body mass (Vollrath 1999). Hence, whether differences in silk diameter (~1–2 µm) are detected by males remains an open question.

Male choice for chemical trails

Adult male and female *P. mirabilis* occur in naturally patchy distributions (Ghislandi et al. 2018), hence, it is likely that mate-searching males may encounter silk lines of multiple females, highlighting the importance of adopting binary choice assays in investigating male mate choice decisions through trail following behavior. These silk lines can be considered mechanical and chemical trails that, if followed, can increase the chances of finding a mate compared to random search (Dijkstra 1976; Tietjen 1977; Anderson and Morse 2001; Bell and Roberts 2016). Given the high costs of male mate search (Fromhage et al. 2016), selection may favor male discrimination of chemical information embedded in such trails, and safeguard males' heavy investment in nuptial gifts by following females with high reproductive potential (namely, adults and females in higher body condition). Males clearly benefit from discriminating information on female body condition, as male mate choice in arthropods, including spiders (Marshall and Gittleman 1994), is often based on female fecundity (Bonduriansky 2001; Roff 2002; Edward and Chapman 2011), a trait ultimately positively correlated with female body mass, or condition (Leather 2018). Individual mass or condition may also reflect recent food intake (Stoltz et al. 2010). Hence, in cannibalistic animals such as spiders, such information may also importantly be used for assessing female satiation and consequent risk of cannibalism (Elgar and Schneider 2004). This is the case of the cannibalistic widow spider, *Latrodectus hesperus*, where males are able to discriminate female feeding state by pheromone extracts from silk and are most responsive toward those of well-fed females (Baruffaldi and Andrade 2015). In *P. mirabilis*, cannibalism rates reported in laboratory studies are relatively low, ranging between 0% and 18% (Drengsgaard and Toft 1999; Stålhandske 2001; Bilde et al. 2007; Andersen et al. 2008; Tuni and Bilde 2010). Nevertheless, hungry females are more frequently cannibalistic, especially toward males not carrying gifts (Toft and Albo 2016). In our experimental set up, for logistics, we chose to remove gifts from males before testing, hence males lack protection against potential females they may encounter by following their trails, which may further reinforce male preference in following chemical trails of high-fed (less-aggressive) females.

Males also benefit from choosing adult females over subadults, as only the former are reproductive. Unlike described for few spider species restricted to the genus *Latrodectus* (Biaggio et al. 2016; Waner et al. 2018; Baruffaldi and Andrade 2020), subadult matings were never observed in *P. mirabilis*. Production of viable offspring following subadult mating appears generally improbable, as female spiders, even if mated immaturely, may suffer from unsuccessful sperm transfer from males and/or from not retaining stored sperm upon molting to adulthood (Foelix 2010). There is also lack of pre-copulatory mate guarding in this system (i.e., guarding subadult females until maturity), commonly described in web-building species in which males are attracted and cohabit with subadults to prevent rivals from mating and reduce sperm competition (Jackson 1986; Fahey and Elgar 1997; Bel-Venner and Venner 2006). Hence, *P. mirabilis* subadults appear of little reproductive value to males. Our findings are supported by evidence for males adjusting their vibratory courtship depending on whether they are exposed to silk of adults or subadults: in particular, when contacting the lines of subadult females, a lower proportion of males performed vibratory courtship and those that did court started vibrations later in time and emitted less vibratory pulses (Eberhard et al. 2021).

So far, studies on this species have suggested poor male discriminatory abilities of females, or little use of such information in a mating context. For example, studies failed to report differences in male courtship behaviors toward unmated and mated females and/ or naive and previously experienced females (same vs. different fe- males) (Tuni and Bilde 2010; Tuni et al. 2013), regardless of males having limited or high mating opportunities (Magris and Tuni 2019). Similarly, male vibratory performance does not co-vary with female age, body mass, size, or condition (Eberhard et al. 2021). These studies have primarily investigated male mating investment and commonly use sequential mate choice designs, leaving the use of direct (binary) choice assays entirely unexplored. Yet, by investigating male preferences in the context of mate search, which entails costs associated to predation risk or energetics (Berger-Tal and Lubin 2011), our study importantly uncovers male discriminatory abilities and suggests that trail-following based on male mate-choice decisions may lead to fitness gain by allowing males to find partners of higher reproductive value.

Despite a recent meta-analysis investigating variation in the expression of male mate choice in relation to male quality in 52 species showing that males of higher quality are choosier than their counterparts, and this result also applying to males of greater body condition (Pollo et al. 2022), our findings point to lack of effects of male body condition (used in our study as a proxy for male individual quality) on choice expression. These findings indicate that females of higher reproductive value are generally preferred by males of all phenotypes, or alternatively that given our rearing conditions there may have been overall little variation in male body condition.

Female mating state

The ability of males to discriminate female mating state is pervasive across taxa (reviewed in Thomas 2011), including silk-based discrimination in spiders (Roberts and Uetz 2005; Stoltz et al. 2007; Baruffaldi and Costa 2010; Sentenská and Pekár 2019), yet, we failed to find a preference for unmated females over mated ones. This finding is consistent with a previous study where *P. mirabilis* males did not vary their vibratory courtship display when contacting silk of unmated and mated females (Eberhard et al. 2021), and may indicate that females do not vary signaling with mating experience, or that males do not make use of such information. Such lack of variation in signaling between unmated and mated females has been shown in *Pholcus beijingensis* using chemical analyses of silk (Xiao et al. 2009). Despite not being chemically verified, there is behavioral evidence for lack of variation between mated and unmated females also in the jumping spider *Evarcha culicivora* (Cross and Jackson 2009). Nutritional and reproductive benefits of mating multiply for *P. mirabilis* females (Tuni et al. 2013) may select for continuous advertisement, also after having secured insemination. Feeding from the nuptial gift allows females to achieve a meal, which is particularly valuable if females are food deprived, and such benefits persist with multiple matings (Toft and Albo 2015), by providing indirect fitness benefits from multiple partners in the form of higher hatching success of the offspring (Tuni et al. 2013). While it may generally be beneficial to advertise, we cannot exclude that signaling is time-dependent. Females in our study mated a few days prior silk collection and testing, and longer intervals may trigger different responses. Our understanding of changes in mating state-related chemical signaling is limited to black widow spiders (*Latrodectus hasselti*), where signaling is interrupted immediately after mating (Stoltz et al. 2007) and resumed only after months (Perampaladas et al. 2008). Yet, in other species such as wolf spiders females are often arbitrarily tested 2–3 days after mating (Riechert and Singer 1995) and attraction to- ward silk cues declines with time (after 11 days) (Baruffaldi and Costa 2010).

From the male perspective, a relatively secure and high share of paternity in mixed fathered broods, as revealed from a recent study investigating paternity outcomes under intense sperm competition in *P. mirabilis* (Matzke et al. 2022), may instead select against discrimination of mating state. This would also be supported by the lack of differential investment in matings, in the form of investment in nuptial gifts and/or courtship performance, toward unmated and mated females (Tuni and Bilde 2010; Tuni et al. 2013).

CONCLUSIONS

Our study shows that female signaling through silk trails is state and condition-dependent, and males can discriminate between female phenotypes and exert preferences, most likely based on chemicals reflecting the female's underlying reproductive potential. These findings overall contribute important empirical evidence supporting costs associated to chemical signaling, often considered negligible, and the potential role such chemicals play as reliable indicators of female quality. Results also highlight the potential for male mate choice in this system. In the context of mate search, selection may favor discriminatory abilities in males which have to safeguard their valuable reproductive investment in nuptial gifts and, therefore, target more fecund and sexually reproducing females. Nutritional and reproductive benefits of mating multiply for females and high share of paternity for males may instead select against variation in signaling and/or male discrimination of mating state.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco. oxfordjournals.org/

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AUTHOR CONTRIBUTIONS

Michelle Beyer (Data curation [Equal], Formal analysis [Lead], Methodology [Equal], Visualization [Lead], Writing – original draft [Equal]), Kardelen Özgün Uludag (Data curation [Equal], Formal analysis [Supporting], Investigation [Lead], Methodology [Equal], Writing – original draft [Equal]), and Cristina Tuni (Conceptualization [Lead], Funding acquisition [Lead], Project administration [Lead], Resources [Lead], Supervision [Lead], Writing – review & editing [Lead])

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

Analyses reported in this article can be reproduced using the data provided by Beyer et al (2023).

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Chapter IV

Publication III: Silk-borne chemicals of spider nuptial gifts elicit female gift acceptance

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Animal behaviour

Silk-borne chemicals of spider nuptial gifts elicit female gift acceptance

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Chemical communication is important in a reproductive context for conveying information used for mate recognition and/or assessment during courtship and mating. Spider silk is a common vehicle for chemical communication between the sexes. However, despite being well described in females, male silk-borne chemicals remain largely unexplored. Males of the spider *Pisaura mirabilis* silk-wrap prey (i.e. nuptial gifts) that is offered to females during courtship and eaten by the female during mating. Interestingly, rejected males often add more silk to their gift which leads to successful mating, suggesting the presence of silk-borne chemicals that facilitate female gift acceptance. To test this hypothesis, we offered females standardized gifts covered with male silk that was either washed in solvents or unwashed, respectively, to remove or not any chemically active components. We scored female gift acceptance, and as expected in the case chemicals that mediate female mating behaviour are present in male silk, females were more likely to accept gifts covered with unwashed silk. Our findings suggest that silk-borne chemicals of nuptial gifts prime female responses, potentially signalling male quality or manipulating females into mating beyond their interests given the occurrence of male cheating behaviour via nutritionally worthless gifts in this system.

1. Introduction

Chemical communication is widespread among arthropods [1] and considered one of the most ancient modes of information transfer. In a mating context, communicating individual qualities (e.g. genetic quality, immunocompetence or body condition) to a prospective partner is key to successful reproduction [2]. Male chemicals (i.e. pheromones) are critical for courtship and mating; they have the potential to be shaped by sexual selection through female choice and appear to reliably reflect an individual's underlying condition [3]. Alternatively, they can be shaped by sexual conflict, if males manipulate females into matings beyond their reproductive interests [4].

Spiders represent a particularly well-suited taxonomical group for investigating chemical communication, as they are often solitary wandering species with marked variation in their visual capacities [5]. Furthermore, spiders produce silk, a protein fibre that is known to transmit contact or airborne chemicals [6] important for courtship and mating. Silk varies greatly in its structure and properties—with different silk types used to build webs, wrap prey or deposit safety lines during movement—and potentially in its chemicals [5,7]. However, while much is known about the chemical composition of female silk functioning to attract mate-searching males [8], chemicals in male silk remain poorly explored [9]. Male silk pheromones are, to our knowledge, undescribed [10]. Only a handful of studies address female behavioural responses to chemical properties of male silk, indicating that male silk eases female orientation [11], mate recognition and/or attraction [12–14], or decreases female aggression [15].

Males of the spider species *Pisaura mirabilis* (Pisauridae) silk-wrap prey and offer it to females during courtship [16], with such nuptial gifts being an important prerequisite for male courtship and mating success [17–19]. Once accepted, females feed on the gift while copulating. The silk around the gift facilitates keeping the mating position and prolongs copulation [20,21]. Silk-wrapping generally occurs prior to female encounters as mate-searching males are found in the field carrying gifts in their mouthparts [22,23]. Regardless of the gift being silk-wrapped, males that are rejected by the female during courtship are known to add silk to their gift before re-offering, a behaviour eventually leading to successful mating [20,24]. This observation suggests that silk-wrapping elicits female gift acceptance, as also shown for another spider [25]. Given that gift appearance plays a minor role in female attraction in *P. mirabilis* [20,21,26] and pisaurids are primarily acute in visually detecting motion [27], enhanced female responses to silk-wrapping of gifts may likely occur in response to the male embedding of silk-borne chemicals. To test this hypothesis, we conducted behavioural assays offering females standardized gifts covered with male silk that was either washed in solvents to remove any chemically active components, or unwashed and hence hypothesized to contain chemically active components. We scored female gift acceptance and expected that, if chemicals that mediate female mating behaviour are present in male gift silk, females should be more likely to accept gifts with unwashed silk than those covered in washed silk.

2. Material and methods

Approximately 120 juvenile *Pisaura mirabilis* were collected in fields near the Ludwig-Maximilians-University of Munich (Germany) during autumn 2020. They were housed individually in vials (5 × 10 cm) covered with a sponge lid and filled with a layer of moss and reared in the laboratory on heating mats (temperature, mean ± s.e.: 24 ± 0.16°C) and natural photoperiod (8 h light : 16 h dark). Every 2–3 days, moss was sprayed with water and spiders were fed with either 10 fruit flies (*Drosophila* spp.), 2–3 houseflies (*Musca domestica*) or 3–5 cricket nymphs (*Gryllus bimaculatus*, *Acheta domesticus*), and vials were inspected for moulted exoskeletons to determine spider maturation to adulthood. All animals were tested 2–3 weeks after maturation, remained unmated through the course of the experiment and were not fed prior to testing. Due to logistic reasons, approximately 30 animals were moved to a different location, but reared in equivalent conditions (see electronic supplementary material).

(a) Gift production and silk treatments

Males were provided with a newly euthanized housefly (placed at −22°C for 20 min) inside a 15 × 15 × 10 cm arena for gift production. Previously, an adult female had been kept for 15–30 min inside the arena to deposit silk lines known to stimulate male prey wrapping [22]. In the unwashed silk treatment, the male was allowed to silk-wrap the prey for up to 30 min, after which the gift was collected with forceps and offered immediately to the female (see below) to minimize the potential fading of chemicals. This procedure, however, did not yield sufficient silk amounts for the washed silk treatment; hence in the latter, the male was allowed to silk-wrap for 4 or 16 h (overnight) (see electronic supplementary material). In the washed treatment, silk was removed from the gift under a stereomicroscope (Zeiss, Germany) using forceps, being careful to avoid contamination from fly body parts or fluids by discarding these silk portions. It was weighed to the nearest of 0.01 mg using a semi-micro scale (Mettler Toledo GmbH, Switzerland) to account for potential effects of silk amounts between and within treatments. Silk was then placed around a small piece of polyurethane foam (3 × 3 mm) to preserve its shape and immersed in approximately 3 ml of EtOH 90%, and then H_2O_{bidest}, for 30 min each, to remove known silk-born and pheromone-related substances such as acids, esters, alcohols or ketones (e.g. [28]). Silk was dried overnight at room temperature inside a glass desiccator jar. Prior to offering it to the female (see below), the foam was removed and replaced with a newly euthanized fly.

We established two control groups in which gifts consisted of a newly euthanized housefly and a ball of black polyurethane foam (0.5 cm diameter), to assess the influence of prey alone and of the offering methodology on gift acceptance, respectively.

(b) Gift-offering assays

We conducted gift-offering assays instead of exposing females to silk or extracts in olfactometers to test whether silk primes biologically relevant behaviours such as female acceptance, as residency time or movements toward silk appear irrelevant for this species [29]. A female was placed in a 15 × 15 × 10 cm arena and left to acclimatize for 15 min. A gift was offered to the female at a fixed distance (2 cm) from the spider's mouthparts using long (15 cm) forceps in a standardized manner: the gift was offered for 3 s, and if not accepted it was removed and re-offered after a 3 s break, for a total of three times. This sequence was repeated for a maximum of three times, with a 20 s interval between each, resulting in a maximum number of nine offerings. The gift was considered accepted if the female grasped it in her mouthparts, and the number of offering (1–9) noted as a measure for latency to gift acceptance. Once accepted, the gift was promptly removed from the female to prevent her from feeding and silk was removed and weighed as described for the washed silk.

Each female ($n = 35$) was tested four times with all gift types and offered in a randomized order on 4 consecutive days. Each female was matched with one male that produced the silk-wrapped gifts (unwashed and washed) to control for silk source.

(c) Statistical analyses

Data were analysed using RStudio (version 1.3.959, RStudio, Inc.; R v. 3.6.0, https://www.r-project.org/), and the 'lme4' package [30] was used to run generalized linear mixed-effect models (GLMMs). To test for the effects of gift treatments on female gift acceptance and latency to acceptance (i.e. number of offerings until acceptance), we included gift type (unwashed silk, washed silk, silk-less fly, silk-less foam) as a fixed effect and spider identities as random effects as spiders were used repeatedly (once per gift type), using a binomial (GLMM-b) and a Poisson distribution, respectively.

For both response variables, the model formula was

$$\text{ResponseVariable} \sim 1 + \text{giftType} + (1|\text{IdFemale}) + (1|\text{IdMale}).$$

We used the 'sim' function ('arm' package) to simulate the posterior distribution of the model parameters based on 2000 simulations [31]. The statistical significance of fixed effects was inferred from the 95% credible intervals (CI) associated with the mean parameter estimate (β). We considered an effect as 'significant', when the 95% CI did not overlap zero [32]. Estimates of the GLMMs for gift acceptance and latency to acceptance were pairwise-compared for each gift type ('emmeans'-package) [33]. We further investigated the effect of silk quantity, location, male age and testing order on gift acceptance and latency to accept, to control for methodological-driven sources of variation. A detailed description of the statistics can be found in the electronic supplementary material.

Table 1. Estimates and 95% CI for predictors of female gift acceptance (GLMM-b) and number of trials until gift acceptance (= latency to acceptance) with number of data points each model used for its calculations (*n*).

	gift acceptance binomial (Y/N) *n* = 140	latency to acceptance count (no. of gift offerings) *n* = 64
fixed effects	β (95% CI)	β (95% CI)
gift type		
unwashed (intercept)	1.47 (0.50, 2.42)	0.93 (0.66, 1.17)
washed	−2.35 (−3.54, −1.19)	0.32 (−0.07, 0.73)
control fly	−1.60 (−2.73, −0.50)	−0.03 (−0.43, 0.37)
control foam	−3.04 (−4.27, −1.77)	0.26 (−0.22, 0.74)
random effects	σ^2 (95% CI)	σ^2 (95% CI)
identities		
female	0.47 (0.27, 0.71)	0.00 (0.00, 0.00)
male	0.75 (0.40, 1.23)	0.05 (0.03, 0.09)

3. Results

Gifts covered with unwashed silk were accepted significantly more often than all other gift types (table 1 and figure 1), while latency to acceptance did not differ significantly between gift types (table 1 and figure 2). Between-group comparisons for each gift type (unwashed, washed, control fly, control foam) show that, besides the unwashed gifts for gift acceptance, gift types did not differ significantly from each other (table 2).

Due to methodological reasons (i.e. longer time available for gift wrapping), gifts for the washing treatment were covered in significantly more silk (electronic supplementary material, table S1 and figure S1). Silk quantity did not significantly affect gift acceptance and having less silk around the gift led to shorter latency to acceptance (electronic supplementary material, table S2). Testing order, male age and change of location did not significantly affect female gift acceptance and latency to acceptance (electronic supplementary material, table S3).

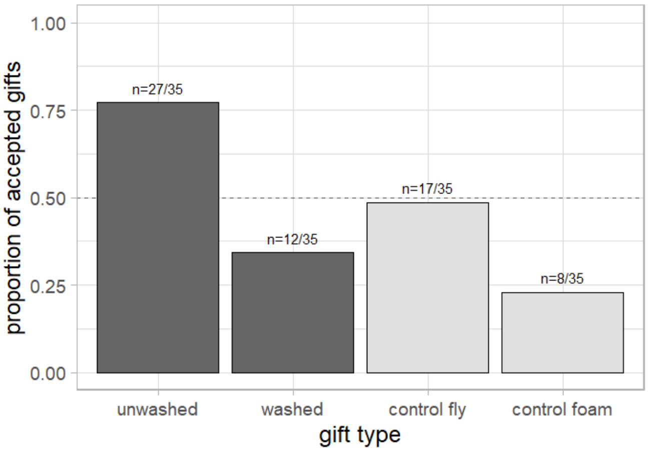

Figure 1. The proportion of accepted gifts per gift type belonging to treatment group (dark grey) or controls (light grey). The dashed line indicates a proportion of acceptance of 0.5. Numbers above bars = number of accepted gifts/total number of gift offerings for each gift type.

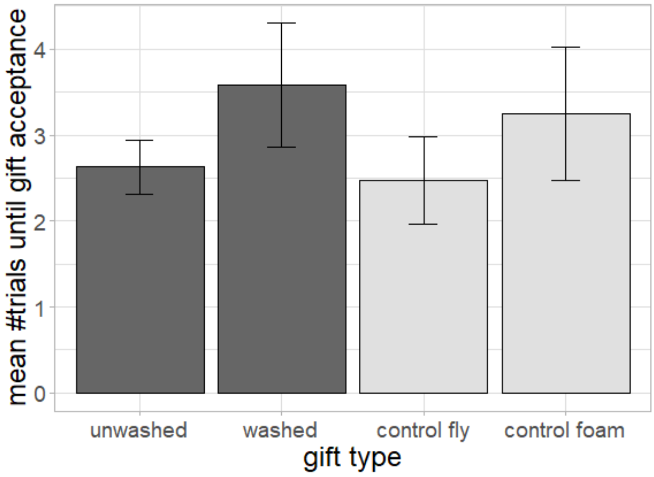

Figure 2. Mean number of trials until acceptance per gift type belonging to treatment group (dark grey) or controls (light grey). Error bars indicate standard errors of the means.

4. Discussion

In our study, we investigated the potential for silk of male nuptial gifts to carry chemicals used for communicating in a reproductive context. By applying a washing treatment to silk to remove putative chemicals, we show that nuptial gifts covered in unwashed silk were accepted by females in higher proportions than those covered in washed silk. These findings strongly suggest the presence of silk-borne chemicals that prime female behavioural responses at mating. Interestingly, despite chemicals in spider silk being common [8,34], they are seldom described in males [9]. The handful of studies investigating male silk-borne chemicals (namely reported in seven species across the families Agelenidae, Lycosidae, Salticidae, Scytodidae and Theridiidae, reviewed in [9]) show that these are used by females for orientation [11], sexual stimulation and/or overcoming female reluctance to mating [15,35] and courtship [15]. In the context of nuptial feeding, silk-borne chemicals are suggested to be present in the silk cover of nuptial gifts of the Neotropical spider *Paratrechalea ornata* and similarly induced higher female gift acceptance [25].

Using silk to chemically elicit a positive response in reluctant females may occur, if, for example silk-borne chemicals transmit information about the male’s individual characteristics (e.g. age, body condition) important in mate choice [2].

Table 2. Post hoc analysis (Tukey's test) of the estimates for differences in gift acceptance and latency to acceptance between gift types.

	gift acceptance		latency to acceptance	
comparison between gift types	*z*-value	*p*-value	*z*-value	*p*-value
unwashed – washed	3.58	<0.01	−1.56	0.40
unwashed – control fly	2.60	<0.05	0.16	1.00
unwashed – control foam	4.24	<0.001	−1.06	0.71
washed – control fly	−1.35	0.53	1.51	0.43
washed – control foam	1.16	0.65	0.22	1.00
control fly – control foam	2.38	0.08	−1.12	0.68

P. mirabilis males in higher body condition are indeed known to cover gifts in more silk [36], potentially varying the silk's quantitative or qualitative chemical composition, and conveying honest information of their superior nutritional state to females. Yet, females appear to ignore condition-dependent information carried by male gifts during mate choice [26], suggesting a marginal role for silk quantity *per se*. Silk-borne chemicals may, however, also function to manipulate females into mating beyond their reproductive interests. Despite the fitness advantage derived by multiple matings [37,38], *P. mirabilis* females become increasingly reluctant to undergo additional matings [37,39], a common feature among spiders [40]. Males may instead benefit from mating with mated females due to their gained paternity share [41]. Enticing reluctant females into mating may be particularly relevant as males often offer females nutritionally worthless gifts consisting of silk-wrapped prey leftovers or plant parts [23]. Silk-borne chemicals may potentially ease such cheating behaviour, with males being known to add higher amounts of silk to worthless gifts [22]. Apart from potentially masking gift contents, increasing gift size and compensating for the lower nutritional value of the gift, silk may allow males to chemically attract females into disadvantageous matings [19].

Interestingly, as shown here and in other studies, *P. mirabilis* females do not require gifts to be silk-wrapped as silk-less prey may be promptly recognized as a meal and trigger female foraging responses. Additionally, females accept unwrapped prey quicker than wrapped prey when offered by male spiders [20,21]. In our study, latency to gift acceptance did not differ across gift types, although a tendency for quicker acceptance of unwashed compared to washed gifts is visible. The similar results for latency to acceptance of unwashed and silk-less prey gifts may be explained by our methodology, whereas the presence of the male during gift offering could have influenced female acceptance behaviour, for example through male courtship movements, vibrations [42] or male quality [19].

Overall, our findings point to gifts covered in washed silk to be the most undesired by females, comparable to the piece of foam used as control. Although our experimental procedure aimed at minimizing differences between washed and unwashed gifts, we cannot entirely exclude that differences in gift appearance influenced female acceptance behaviour. The washing treatment caused stiffness of the silk [43], making it difficult to cover the flies to fully resemble unmanipulated gifts. Despite vision potentially playing a role, it seems to be of little importance in *P. mirabilis* in the context of discriminating gifts based on silk quantity [26,44]. Furthermore, female gift-grasping always occurred without the female touching the gift beforehand, indicating that gift acceptance was not influenced by tactile cues such as altered silk structure.

To conclude, our findings suggest that male *P. mirabilis* add chemicals to the silk cover of their nuptial gifts that elicit female gift acceptance and consequently mating. Whether silk-borne chemicals signal male underlying quality or manipulate females into mating beyond their interests remains an interesting venue for future research, especially given the occurrence of male cheating behaviour via nutritionally worthless gifts in this system. Finally, our study also highlights that spiders represent a promising taxonomical group for studying the evolutionary function of male chemical traits in the context of reproduction and their potential to be shaped by sexual selection or conflict.

Data accessibility. Data, code and a description of the data associated with this manuscript are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.0cfxpnw2x [45].

The data are provided in the electronic supplementary material [46].

Authors' contributions. C.T. conceived, designed and coordinated the experiment, and revised the manuscript; J.M. conducted the experiment, participated in data analysis and drafted the manuscript; M.B. carried out the data analysis, participated in the study design, drafted and revised the manuscript. All authors gave approval for publication and agree to be accountable for all aspects of the work.

Competing interests. We declare we have no competing interests.

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Supplementary material

Silk-borne chemicals of spider nuptial gifts elicit female gift acceptance

Michelle Beyer, Julia Mangliers, Cristina Tuni

1. Supplementary methods

Change of location

Towards the end of the study a number of animals (*n* = 30) were moved to a different location, where they were raised under similar conditions as in the laboratory (two-sample t-test: *t* = 0.9, d.f. = 51.48, *p* = 0.37, mean temperature ± standard error (s.e.): laboratory: 24.11 ± 0.22 °C for *n* = 37 days; new location : 23.82 ± 0.23 for *n* = 22 days); they were kept individually in their housing vials (5 x 10 cm), placed on top (but not in direct contact) of 20W thermos mats (Lucky reptile, Germany), sprayed with water and fed with the same regime and diet as those kept in the laboratory.

Gift production and silk treatments occurred as described in the manuscript's methods section, with the only difference that the silk removed from gifts of the silk-washed treatment was dried overnight on top of a heater since a desiccator as not available.

As shown below, location was accounted for during statistical analyses.

Statistical analyses

Silk quantity

A Kruskal-Wallis test was used test to inspect differences in mean silk weight between wrapping durations (30min, 4h, 16h). Pairwise comparisons using a Dunn's test were performed to determine specific differences between the means of groups.

Differences in mean silk weight between unwashed and washed silk were analysed using two-sample Welsh's t-test, and influence of silk weight on the response variables was accounted for by fitting generalized linear models (GLMs) with the respective response variable, and treatment and silk weight as fixed effects that were added as an interaction as silk weight differed significantly between treatments. No random effects were added, as male and female identity showed a variance of almost zero.

Effects of location, male age and testing order

To test for the effect of possible additional factors to female gift acceptance and latency to acceptance, GLMMs were conducted with gift type (unwashed, washed, control fly, control foam), male age (days from maturation moult until day of respective experiment), testing order and/or location (laboratory, new location) added as fixed effects, and spider identities as random effect.

2. Supplementary results

Silk quantity

From 35 sets of complete experiments (i.e. female tested with four gift types), silk was weighed from 27 unwashed and 14 washed gifts. In the remaining cases, the silk amount around the gift was either too low to be weighed accurately or the silk was heavily contaminated by fly remains, rendering any silk weight measurements unreliable. In order to obtain enough silk for removal, males producing gifts for the silk-washing treatment were given more time to silk wrap their prey. The amount of silk deposited around the gift differs significantly according to available time a male has for gift construction durations (Kruskal-Wallis test: $\chi^2 = 15.43$, d.f. = 2, $p < 0.001$) (Table S1) with longer available time for wrapping resulting in higher silk weight (Figure S1). Consequently, gifts for the silk washing treatment were covered in significantly more silk (mean silk weight ± s.e.: 0.27 ± 0.05 mg) than unwashed gifts (0.09 ± 0.02 mg) (two-sample t-test: $t = -3.28$, d.f. = 16.30, $p < 0.01$).

Table S1: Post-hoc analysis (pairwise comparisons) of the estimates for differences in silk weight between different available times for wrapping using Dunn's test. Significance is indicated through *italics*.

Comparison between available times for wrapping in min	z-value	p-value adjusted
30-240	1.97	0.15
30-960	-3.67	< 0.001
240-960	-0.23	1.0

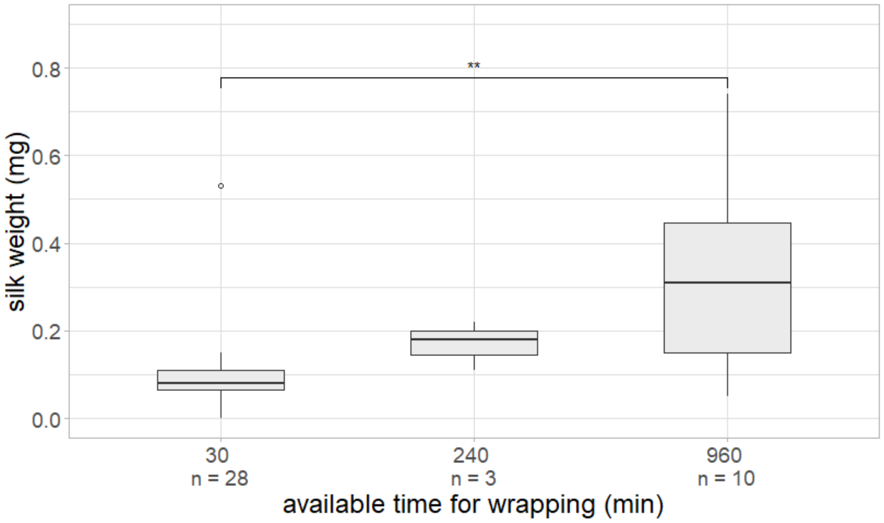

Figure S1: Differences in silk weight (mg) between different available times for wrapping.

Effects of location, male age and testing order

Silk quantity did not affect female gift acceptance, but having less silk around the gift led to shorter latency to acceptance (Table S2). Location, male age and order did not significantly affect gift acceptance or latency to gift acceptance (Table S3).

Table S2: Model estimates of the GLMs testing for the effects of gift type (unwashed silk, washed silk), silk weight and their interaction on the likelihood of gift acceptance (GLM-b) and number of trials until gift acceptance (= latency to acceptance). Presented are fixed (β) parameters with their 95% credible intervals (CI), and sample sizes for the respective effects (*n*).

	Gift acceptance		**Latency to acceptance**	
	Binomial (Y/N)		**Count (No. of gift offerings)**	
Fixed effects	***n***	**β (95% CI)**	***n***	**β (95% CI)**
Gift type				
unwashed (intercept)	27	1.52 (0.16, 2.93)	19	0.98 (0.42, 1.59)
washed	14	-2.53 (-4.98, -0.14)	6	1.01 (0.13, 1.87)
Silk weight	41	-6.94 (-19.24, 5.07)	25	-0.40 (-7.42, 6.61)
Gift type*Silk weight	41	9.52 (-4.24, 23.18)	25	-1.83 (-8.83, 5.72)

Table S3: Model estimates of the GLMMs testing for the effects of gift type (unwashed silk, washed silk, control fly and foam), location, male age and trial order on the likelihood of gift acceptance (GLMM-b) and number of trials until gift acceptance (= latency to acceptance). Presented are fixed (β) and random (σ²) parameters with their 95% credible intervals (CI), as well as respective sample sizes (*n*).

	Gift acceptance **Binomial (Y/N)**		**Latency to acceptance** **Count (No. of gift offerings)**	
Fixed effects	***n***	**β (95% CI)**	***n***	**β (95% CI)**
Gift type				
unwashed (intercept)	35	3.16 (0.86, 5.55)	27	1.04 (0.32, 1.76)
washed	35	-2.30 (-3.50, -1.13)	12	0.36 (-0.03, 0.76)
control fly	35	-1.62 (-2.75, -0.49)	17	-0.02 (-0.40, 0.35)
control foam	35	-3.13 (-4.36, -1.88)	8	0.25 (-0.22, 0.71)
Location	140	-0.98 (-2.23, 0.32)	64	-0.05 (-0.43, 0.35)
Male age	140	-0.03 (-0.14, 0.07)	64	0.00 (-0.03, 0.04)
Trial order	140	-0.17 (-0.55, 0.20)	64	-0.06 (-0.20, 0.09)
Random effects	***n***	**σ² (95% CI)**	***n***	**σ² (95% CI)**
Identities				
Female	35	0.63 (0.39, 0.95)	31	0.00 (0.00, 0.00)
Male	35	0.41 (0.20, 0.67)	21	0.06 (0.03, 0.10)

Chapter V

Manuscript II: Chemical signalling bound to silk of male nuptial-gifts does not depend on male feeding condition

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Unpublished manuscript

Abstract

Inter-sexual interactions shape the evolution and diversity of chemicals, namely pheromones. Despite accumulating evidence for costs of chemical signalling, pheromone production is traditionally considered biochemically cheap. Moreover, in many taxonomical groups, such as spiders, there is a persisting bias in assigning sex-specific roles in chemical communication, leading to major knowledge gaps in male chemical signalling and female detection mechanisms, as well as female choice. Here, we ask whether male silk-bound chemical signalling in the spider *Pisaura mirabilis* depends on male nutritional condition, and whether such information alone elicits a differential response in female mate acceptance. Males of this species silk-wrap prey, thus producing nuptial-gifts that are donated to the female during courtship. During experimental laboratory trials we tested i) silk investment in the gift (i.e., duration of silk-wrapping) of males given differential feeding regimes (low-fed vs. high-fed), and ii) female likelihood of and latency to acceptance of such gifts, presented experimentally without interference of male attributes (i.e., behaviour, chemical cuticular profile). If males transmit silk-borne information about their individual characteristics, such as body mass (and/or nutritional state), used by females during mate choice, we expected higher and quicker female acceptance of gifts of high-fed males. Instead, females did not respond differentially towards gifts of males of different nutritional conditions, despite low-fed males investing less in silk. Findings suggest lack of male condition-dependent signalling, regardless of silk quantity. Alternatively, females may ignore male silk-borne information as countermeasure to deceptive males in this system, known to offer well-wrapped, nutritionally worthless gifts.

Keywords: Mate choice, silk, chemicals, spider, signalling

Introduction

Chemical communication is one of the most widely distributed and most ancient forms of communication used throughout the animal kingdom (Bradbury and Vehrencamp 2009) and is essentially ubiquitous among insects and other arthropods (Wyatt 2014). Chemical signalling is key to a number of biological contexts, including mating. Pheromones (i.e., chemicals emitted to trigger a behavioural or physiological response in a conspecific individual, also known as chemosignals (Karlson and Lüscher 1959) are, in particular, key molecules to reproduction (Jacobson 1972; Johansson and Jones 2007; Wyatt 2009; Steiger and Stökl 2014; Coombes et al. 2018). The information that pheromones encode may be used by opposite-sex individuals for individual recognition, to find and/or select higher quality or most compatible mating partners (Penn 2002; Thom et al. 2008). Pheromones can indicate the phenotypic state (i.e., developmental and/or mating state) of the signaller as they reflect changes in internal physiology that lead to variation in the composition and/or in the quantity of chemical release (Johansson and Jones 2007; Thomas 2011). Pheromone release can also be plastic, with chemicals emitted strategically in response to the prevailing environmental conditions (e.g., social factors) (Havrilak et al. 2015; Rehermann et al. 2016; Pham et al. 2020). For example, females that have already mated, may switch from emitting male-attracting chemicals that secure fertilization to emitting chemicals that repel males to avoid the costs of additional matings (Thomas 2011; Weiss and Schneider 2022a).

Interestingly, pheromones are traditionally considered biochemically cheap to produce (Wyatt 2014). Yet, there are a growing number of studies providing evidence for associated costs to chemical production (Harari et al. 2011; Holman 2012; Umbers et al. 2015). Costs may be revealed by constraints on energetic resources linked to the individual's body condition, as pheromone production may make use of such resources (Blomquist and Bagnères 2010). Nutrition can also play a key role as some amino and fatty acids (South et al. 2011) from the diet can be used as precursors for pheromones, or even as pheromones themselves (Landolt and Phillips 1997; Eisner and Meinwald 2003). Examples for variation of chemical signalling linked to the individual's nutritional state and/or body condition can be found both in females (e.g., in the moths *Heliothis virescens* (Foster and Johnson 2011) and *Neoleucinodes elegantails* (Jaffe et al. 2007), in the nursery web spider *Pisaura mirabilis* (Beyer et al. 2023) or the wasp spider *Argiope bruennichi* (Weiss and Schneider 2022b)) and males (e.g., in the cockroach *Nauphoeta cinerea* (Clark et al. 1997), the mealworm beetle *Tenebrio molitor* (Rantala et al. 2003) and the rock lizard *Iberolacerta cyreni* (Martín and López 2010)). Costs of chemical signalling are also uncovered by trade-offs in resource allocation between current and future reproduction (Stearns

1992), with individuals that chemically advertise for partners suffering from reduced survival (e.g., males of the lekking fruit fly *Drosophila grimshawi* (Johansson et al. 2005) and female moths (Harari et al. 2011). Strategic pheromone release, in which signalling is tailored to the individuals' reproductive needs, are further suggestive of costs of pheromone production. For example, as described in moths (Umbers et al. 2015) and in the wasp spider (Weiss and Schneider 2022a), individuals increase signal release while ageing if still unmated.

Insights from studies on insects have notably advanced our understanding of how inter-sexual interactions have shaped chemicals traits (Stökl and Steiger 2017). Yet, the general view on males investing more in mate search and courtship than females (Fromhage et al. 2016) has led to considering females as the chemically advertising sex and males as the sex with corresponding sensory detection mechanisms (Kittredge and Takahashi 1972; Johansson and Jones 2007; Keller-Costa et al. 2015; Scott et al. 2018), leaving male chemical communication and female detection mechanisms and mate choice much less explored. Male pheromones can indeed function as attractants (Zhang et al. 2002), as aphrodisiacs that increase female receptivity (Baker and Cardé 1979; Grillet et al. 2006; Ejima 2015), or as anti-aphrodisiacs that reduce female attractiveness to other males after mating (Seidelmann and Ferenz 2002; Schulz et al. 2008; Estrada et al. 2011). Male chemicals can also be subjected to female choice (Thomas and Simmons 2009; Lane et al. 2016) as they convey information on the individual's suitability as a mating partner (Johansson and Jones 2007; Steiger and Stökl 2014) by conveying information about their underlying quality, such as immunocompetence (Rantala et al. 2002) or condition (Rantala et al. 2003; Ming and Lewis 2010; South et al. 2011). On the contrary, research on male chemosignals in other arthropods, such as spiders, lags behind. Spiders, are considered excellent models for studying chemical communication in a reproductive context for several reasons. The majority of spiders have poor vision, leading them to rely largely on chemo-sensory channels and they are generally solitary and occur in low densities, meaning that the long-distance characteristics of chemical communication are of great importance (Huber 2005; Gaskett 2007; Uhl and Elias 2011; Schulz 2013). Spiders also produce silk, which provides an additional means for the transmission of chemical signals together with cuticular compounds (Huber 2005; Gaskett 2007; Uhl and Elias 2011; Schulz 2013). Yet, while there is a larger body of research on chemical signalling bound to female silk (Baruffaldi and Costa 2010; Uhl and Elias 2011; Tuni and Berger-Tal 2012; Cory and Schneider 2016; Scott et al. 2018; Waner et al. 2018; Eberhard et al. 2021), less attention has been devoted to male silk.

Here, we investigate condition-dependent chemical signalling bound to silk of males of the hunting spider *Pisaura mirabilis*. This web-less species is well-studied for its nuptial-gifts: males capture, kill and wrap prey in silk (i.e., generally insects and other arthropods) and offer it to females at mating (Bristowe 1958). Copulation occurs while females feed on the food offer, with the gift allowing higher mating success (Stålhandske 2001; Albo et al. 2011b) and advantages in fertilizations tied to prolonged matings in males (Matzke et al. 2022). Gifts are costly sexual traits as they are likely to entail energetic and time costs of hunting, a missed meal, and documented transportation and metabolic costs (Prokop and Maxwell 2012; Prokop and Okrouhlík 2021). The costs of gifts may also be tied to silk production. Early investigations on the effects of male nutritional state on gift construction show that males that are given a poor feeding regime required more time to produce a given amount of silk compared to well-fed males (Lang 1996). Males in low feeding regimes are also shown to wrap gifts less frequently and thoroughly as those in high feeding regimes (Albo et al. 2011a), with resulting differences in silk amounts (Ghislandi et al. 2017), further suggesting condition-dependence of gift construction in relation to silk production. Yet, very little is known on the chemosignals of the silk cover and their putative variation tied to male body condition and/or nutritional state. A recent study by Beyer et al. (2021) suggests that chemicals present in the silk cover of gifts elicit *P.mirabilis* female's gift acceptance. By washing the silk of male gifts with solvents to remove any putative chemicals and comparing female acceptance behaviour of the gift to unwashed gifts (offered experimentally and not by the male itself) the study was able to show higher acceptance of nuptial-gifts covered with unwashed silk, pointing to silk-borne chemicals mediating female behaviour (Beyer et al. 2021). This effect was also assumed from observations during mating trials, where courting males that are rejected by females (regardless of their gift being silk-wrapped or not) have a higher chance of being accepted if they add silk layers to their gift immediately before re-offering it to the female (Stålhandske 2002; Bilde et al. 2007).

Interestingly, when body condition of the offering male and quality of the gift (i.e., well- and poorly-wrapped gifts, produced by males varying in their nutritional state) were experimentally mismatched, females were shown to positively respond to male body condition, but not to gift quality (Albo et al. 2012). The latter finding suggests that, despite gift wrapping being an honest indicator of the male's underlying body condition and/or nutritional state (Lang 1996; Albo et al. 2011a), females might ignore such information in mate choice decisions, and rather assess male phenotypic quality through other means. For example, multiple male sexual signals at play during mating, such as male behavioural displays during courtship, vibrational signals (Eberhard et al. 2020) and/or potential body odour derived by cuticle chemicals, may override any potential chemical signal bond to the silk cover during mate assessment. Research focusing solely on female choice for chemical signals that could reveal variation in their chemical composition and/or quantity are missing. Hence, to understand whether chemical information bound to silk of nuptial-gifts is condition-dependent, and whether such information alone can elicit a differential response in the female we i) tested gift construction abilities in terms of investment in silk wrapping (i.e., duration of silk wrapping) in males varying in their nutritional state (experimentally low-fed and well-fed), and ii) offered such gifts to the female in a standardized manner that excludes any effect of male presence and/or phenotype (Beyer et al. 2021), and measured female likelihood and latency of gift acceptance. If males use silk to transmit information about their individual characteristics, such as body mass (and/or their nutritional state), used from females during mate choice, we'd expect a higher and quicker female acceptance of gifts silk-wrapped by well-fed males.

Methods

Juvenile *Pisaura mirabilis* spiders were collected from the fields surrounding the Biocenter of the Ludwig-Maximilians-University Munich (Munich, Germany). Spiders were collected in September and October 2021 and April and May 2022. They were individually housed in transparent (5 × 10 cm) plastic vials with foam tops to allow for airflow, and lined with a layer of moss collected from fields that was regularly sprayed with water to maintain humidity. Spiders were kept in the laboratory at room temperature, whereas those housed in winter were placed on heating mats to maintain a temperature of approximately 21^{o}C. Spiders were checked for moults, provided with water, and fed three times a week with either 5-10 fruitflies (*Drosophila melanogaster),* one housefly (*Musca domestica, Lucilia sericata),* or a crickt nymph (*Gryllus bimaculatus).*

Upon reaching maturity, adult male spiders were randomly assigned to one of the two feeding regimes, aimed at generating variation in male body condition: i) high-fed males continued to be fed three times a week (n = 31), while ii) low-fed males were fed only once a week (n = 24). This feeding schedule, applied for two weeks, has been previously shown to yield differences in spider body mass and body condition (Albo et al. 2011a; Ghislandi et al. 2017). Adult females were kept on a high-fed diet (n = 52).

Silk investment in nuptial-gifts

Male body mass was measured to the closest 0.001 g using a digital scale (KERN & SOHN GmbH, Balingen, Germany) immediately before testing. To elicit male gift construction, an adult female was released into a transparent (15 × 15 × 20 cm) plastic tank for 30 minutes to release silk lines known to trigger male mating behaviours. The female was then confined under a tent-like mesh, and a male introduced. Once the male started to exhibit vibratory courtship behaviour, a fly (*Lucilia sericata* or *Musca domestica*, depending on availability) was presented with forceps for the male to grasp, kill and silk-wrap into a nuptial-gift. If males did not accept the fly, or accepted the fly but did not start wrapping it within 10 minutes, the male was removed from the test arena and returned to his housing vial. For males that accepted the fly and performed gift construction, we scored the duration of the silk-wrapping defined as the cumulative duration of all wrapping bouts (or sequences) performed consecutively. Silk wrapping was considered completed when males picked up the silk-wrapped fly in their mouthparts and did not resume wrapping for five consecutive minutes, and used as a proxy for silk release (Ghislandi et al. 2017). The gift was then collected from males using forceps. Males from the winter (8 low-fed and 9 well-fed) were tested for silk investment during gift-construction only once, males from the summer (15 low-fed and 22 high-fed) were tested multiple times (1-9) on consecutive days, with one test per day.

Female gift acceptance

During the male gift construction processes described above, a randomly chosen focal female (i.e., to be tested) was released into a clean transparent (15 x 15 x 10 cm) plastic box to acclimatize. Once the male completed silk-wrapping, the gift was collected from the male's mouthparts using forceps, it was assigned a random number to allow testing to be conducted blind in regards to feeding treatment, and was immediately offered to the focal female using existing protocols (Beyer et al. 2021). In brief, the wrapped gift was offered to the female using forceps at a 2 cm distance from the female's mouthparts (i.e., chelicerae). The gift was offered for 3 seconds, taken away for 3 seconds, and re-offered for a total of three times. This sequence was repeated three times, with 20 seconds break in between each set of offerings. We scored whether the female accepted the gift (yes/no) by grasping it in its chelicerae, and in which of the offering bouts (1-9) this occurred as the proxy for latency to gift acceptance. As soon as the female grasped the gift, it was removed from the female's mouthparts with forceps to prevent consumption of the gift. Each female was tested twice (n = 49), with exception of three females that were tested 3 (n = 2) or 5 times (n = 1), with a gift from a high-fed male and a gift from a low-fed male with randomized treatment order, and was tested once per day. Female latency to acceptance of male gifts originated from low-fed and high-fed males was scored only using spiders from the summer cohort.

Statistical analysis

We used a two-sample t-test to test for differences in body mass between males of the two feeding treatments (high-fed and low-fed) measured before the first gift construction trial. To test for the effect of treatment on wrapping duration between high-fed and low-fed males, we conducted a generalized mixed-effects model (GLMM, Gamma distribution with log-link) with treatment and number of produced male gift (1-9) as fixed effects. To investigate whether female likelihood to accept the gift (i.e., yes/no) and latency to gift acceptance (i.e., number of offerings, 1-9) is affected by the males' feeding treatment, we fitted a binomial and Poisson-distribution model, respectively, with male feeding treatment as a fixed effect. We also included female trial number (1, 2) to account for increased female experience during testing as a fixed effect. We added spider identities of each sex as random effects to account for repeated measurements and pseudo-replication.

Given the two cohorts of spiders (collected and tested in autumn and spring) used to test for male silk investment and female likelihood of and latency to gift acceptance, we accounted for the effect of season on our model output by adding the variable season as random effect to the models described above. As the season of experiment conduction (spring vs. autumn) did not explain variation in our data (as shown in the ESM), we removed the variable from the model simulations.

We further fitted explorative models, presented in the ESM, to investigate the effect of gift-wrapping duration on the likelihood and latency of female gift acceptance by substituting treatment (high fed and low fed) with wrapping duration given the interdependency of the two variables (see below). We also tested for the effect of first gift type (sourced from a low-fed or high-fed male) on female gift acceptance of subsequent gifts., expecting females to be choosier on their second gift encounter if they have experienced gifts produced by high-fed males first.

We standardized the numeric variables (i.e. testing order, wrapping duration) by subtracting each value by the variable's overall mean and standardized the variable using the standard deviation of it in order to ease biological interpretation. Data was analysed using R (version 4.1.1) (R Core Team 2021). To fit generalized mixed-effects models (GLMMs), we applied the function "stan_glmer" from the package "rstanarm", which included the simulation of posterior distributions based on 4000 iterations (Goodrich et al. 2023). Model fit was visually assessed based on the goodness of fit graph (i.e. model's fitted values) and residual distributions. Statistical significance of fixed effects was inferred from the Bayesian 95 % credible intervals (CI) associated with the mean parameter estimate (β), and considered significant in the frequentist's sense, when the 95 % CIs did not overlap zero (Nakagawa and Cuthill 2007).

Results

Silk investment in nuptial-gifts

Male body mass significantly differed between males of the two feeding regimes, with high-fed males being heavier than low-fed males (two-sample t-test: t = 3.52, d.f. = 52.96, p < 0.001; mean ± s.e. mass in mg; high-fed 95.23 ± 3.68 for n = 31; low-fed 78.21 ± 3.14 for n = 24).

Wrapping duration of the prey in silk was significantly longer for high-fed males (mean ± s.e. wrapping duration in sec: 278.06 ± 22.05 sec, *n* = 31) than low-fed males (193.75 ± 13.03, *n* = 24) (Table 1; Figure 1).

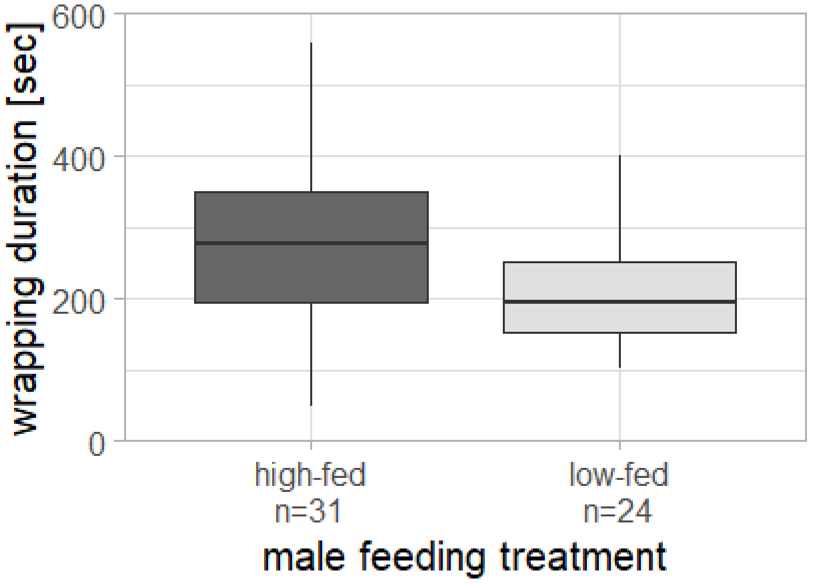

Figure 1. Male silk-wrapping duration of gifts depending on male feeding treatment (dark-grey: high-fed, light-grey: low-fed). High-fed males wrapped longer than low-fed ones.

Table 1. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of male gift wrapping duration (Gamma (log-link) GLMM, *n* = 107), including male

feeding treatment (high-fed, low-fed) and number of produced gift (1-9) as fixed effects, and male identity as random effect. Significance is shown in bold.

	Wrapping duration
Fixed effects	**β (95 % CI)**
Intercept*	5.63 (5.50, 5.75)
Treatment (low-fed)[a]	**-0.29 (-0.47, -0.10)**
Gift number[b]	-0.00 (-0.09, 0.09)
Random effects	**σ² (95 % CI)**
Id Male	0.02 (0.00, 0.07)

* Reference category; estimate for respective male feeding treatment (high-fed) and normalized mean values of remaining fixed effects; [a] Difference between reference category and male feeding treatment (low-fed); [b] Mean-centered and standardized using the standard deviation within all trials

Female gift acceptance

Male feeding treatment did not significantly affect the likelihood of female gift acceptance with females accepting 64 % of gifts produced by high-fed and 66 % from low-fed males (Figure 2a, Table 1) or latency to gift acceptance (Figure 2b; Table 2). Trial number also did not significantly affect the likelihood of female gift acceptance or latency to gift acceptance (Table 2). Female likelihood of gift acceptance or latency to acceptance was not significantly affected by the duration of silk wrapping in males (see ESM Table S2). Female acceptance of gifts during their second trial was not affected by the type of gift (produced by a high-fed or low-fed) experienced during the first trial (Electronic Supplementary Material Table S3).

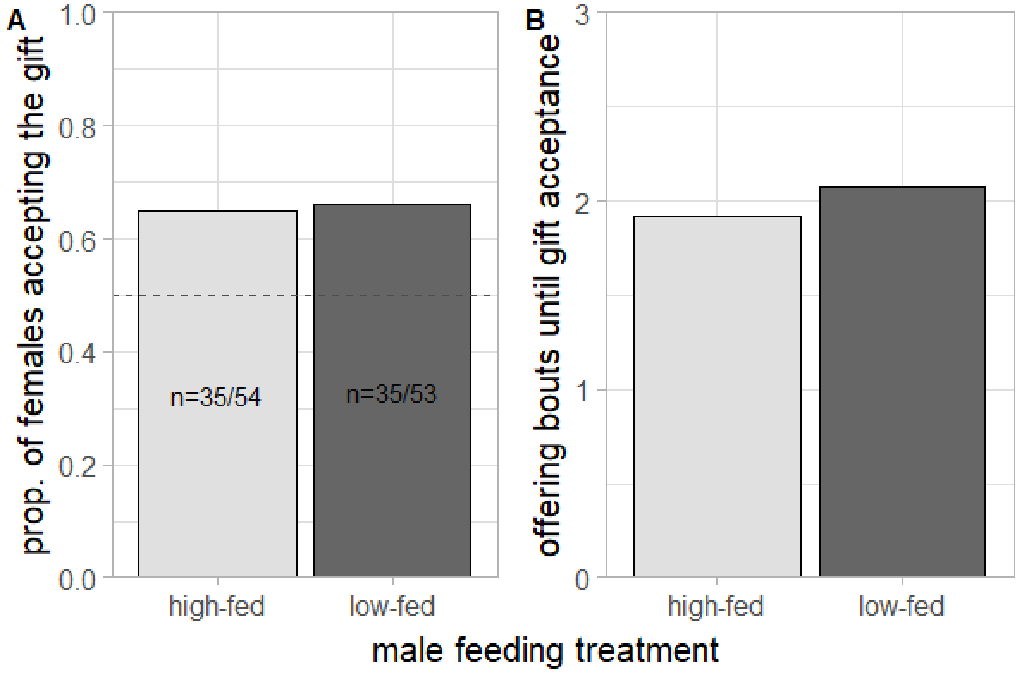

Figure 2. Female likelihood (A) and latency (B) to accept gifts produced by males varying in their body condition (high-fed, low-fed). Numbers within bars = number of trials with gift acceptance out of all trials within a treatment. Dashed line = 50 % chance of acceptance.

Table 2. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of female gift acceptance (model A, binomial GLMM, *n* = 107) and latency to gift acceptance (model B, Poisson GLMM, *n* = 70), including male feeding treatment (high-fed, low-fed) and trial number as fixed effects, and spider identities as random effects. Significance is shown in bold.

	Gift acceptance	**Latency to gift acceptance**
Fixed effects	**β (95 % CI)**	
Intercept*	0.88 (-0.11, 2.03)	0.62 (0.26, 0.94)
Treatment (low-fed)[a]	0.28 (-0.93, 1.69)	0.11 (-0.33, 0.57)
Trial number[b]	-0.23 (-1.00, 0.40)	0.20 (-0.08, 0.49)
Random effects	**σ^2 (95 % CI)**	
Id Female	4.27 (0.18, 13.63)	0.08 (0.00, 0.31)
Id Male	1.22 (0.00, 5.97)	0.06 (0.00, 0.28)

* Reference category; estimate for respective male feeding treatment (high-fed) and normalized mean values of remaining fixed effect; [a] Difference between reference category and male feeding treatment (low-fed); [b] Mean-centered and standardized using the standard deviation within all trials

Discussion

We investigated the potential for the silk cover of male nuptial-gifts to carry chemicals informing females about the male's underlying feeding condition, and hence body mass, during courtship by testing female likelihood of and latency to acceptance of gifts sourced from high-fed and low-fed males. Our findings show that, despite gift construction being constrained by male nutritional state, females do not respond differentially towards gifts of high-fed and low-fed males. This suggests that any chemical signalling bound to silk does not vary with the male's underlying feeding condition. Alternatively, if such variation is present, females do not make use of it in mate choice decisions.

Consistent with previous reports of male condition-dependent gift construction (Lang 1996; Albo et al. 2011a; Ghislandi et al. 2017), we show that males varying in feeding conditions, and therefore body mass, invested differentially in silk wrapping of the nuptial-gift, with high-fed males wrapping longer than low-fed ones. Although we did not quantify silk amount, the time a male spends wrapping the prey is known to positively correlate with the quantity of silk laid to cover prey during nuptial-gift construction (Ghislandi et al. 2017). We hypothesized that variation in silk abundance, on the one side, and in available nutrients resulting from different feeding histories, on the other side, would lead to quantitative and/or qualitative differences in pheromone release, with high-fed males releasing more or different blends of chemicals than low-fed ones (Baruffaldi and Costa 2010; Chinta et al. 2010; Henneken et al. 2015, 2017; Weiss and Schneider 2022b), consequently triggering higher or faster gift acceptance in females. Yet, contrasting to the variation in male silk investment triggered by our feeding treatments, we found lack of differential female responses towards gifts sourced from high-fed opposed to low-fed males. These results suggest that female mate choice is not influenced by the amount of silk covering a nuptial-gift, despite wrapping duration, and consequently silk amount, being indicative of male condition and/or quality. The marginal role of silk quantity on female mate choice has already been suggested in this species, as silk quantity (an indicator of variation in body condition of the gift producer) did not affect female gift acceptance during male courtship (Albo et al. 2012). Such result was retained also in the study from Beyer et al. (2021), in which females were exposed to silk-wrapped gifts without the interacting effects of male phenotype and/or behaviour (i.e. all males of that study were of similar and high-fed condition). Other studies on this species also suggest low importance of silk quantity or presence *per se* on female mating decisions. In fact, females have been reported to accept gifts sooner when they were unwrapped compared to silk-wrapped ones (Bilde et al. 2007), as gifts without silk are likely promptly chemically and/or visually recognized as prey and stimulate female foraging responses. However, this quick female latency to gift acceptance appeared to be influenced by male presence as unwrapped and silk-wrapped gifts that were artificially offered using forceps (i.e. without male presence) were accepted equally quickly (Beyer et al. 2021). Taken altogether, the similar response of females towards gifts of both types can be explained by lack of qualitative and/or quantitative difference of gift silk chemicals, regardless of the documented variation in silk investment. This finding is yet puzzling given the associated differences in silk wrapping between the two groups of males.

An alternative explanation for our findings is that, if underlying chemical differences do exist, females do not make use of such information during mate choice. Ignoring the information carried by the gift is hypothesized to represent a female countermeasure against cheating males (Albo et al. 2012). Males of this species are indeed known to cheat females into mating by offering non-nutritious gifts (i.e., prey remains) (Albo et al. 2011b; Ghislandi et al. 2018), which are covered in higher amounts of silk, potentially to disguise their low quality contents (Ghislandi et al. 2017). Hence, females may base their mate choice exclusively on traits that can provide direct reproductive benefits, such as gift presence *per se* (Stålhandske 2001; Albo et al. 2011b; Tuni et al. 2013) and male - not gift - attributes, such as body condition that was shown to lead to higher siring success (Albo et al. 2012). Chemical analyses (e.g., GC-MS) are needed to verify presence of chemicals in silk of males varying in their condition, in order to understand whether females ignore present information.

The experimental feeding regimes applied to males in our study were successful in generating a difference in male body mass, as males from the high-feeding treatment were approximately 20 % heavier than low-fed males. A similar feeding regime applied to *P. mirabilis females* resulted in condition-dependent chemical signaling via draglines revealed by male trail-following behavior: when given a choice, males preferred silk of high-fed females with higher body condition rather than low-fed/low body condition females (Beyer et al. 2023). When comparing our current results with this study on females, findings point to sex-differences in chemical signaling, indicating female (but not male) condition-dependent variation in silk-bound chemical signaling and male (but not female) mate choice based on silk-borne condition-dependent information. One general assumption on sex differences in chemical release is that when search costs are high, females are most likely the sex to produce signals (Johansson and Jones 2007). This may be the case for cursorial spiders like *P. mirabilis* that need to roam in the environment to find each other (Foelix 2010). Moreover, females in our study system may be under selection to signal their underlying body condition and/or mass, a trait positively correlated with fecundity in arthropods (Leather 2018), to ensure both mating and nutrition from gift-giving males (Tuni et al. 2013; Toft and Albo 2015). Correspondingly, given the costly nuptial-gift provision, males may benefit from discriminating females based on their reproductive quality (Bonduriansky 2001; Roff 2001; Edward and Chapman 2011), and hence exert mate choice during mate search (Beyer et al. 2023). While it may be likely that males are not under the same selective pressures as females, we do note that the sex differences highlighted above stem from differences in the type of silk studied, with female spider trails consisting of dragline silk (also known as safety lines) and substrate-borne chemicals deposited during movements (Beyer et al. 2023), and male gift-silk used to wrap prey. These silk types are produced in different silk glands (i.e. the major ampullate silk glands for draglines and aciniform silk glands for gift-silk), with spiders possessing up to seven different silk gland types that produce silks varying structurally and functionally (Vollrath 1992; Schulz 2004; Garb 2013), and that are suggested to be responsible for pheromone production (Fischer et al. 2022). Further research addressing variation in silk types would be required to fully uncover potential for condition-dependent variation of chemical signalling via silk.

To conclude, our study shows that chemosignals on the silk cover of male nuptial-gifts in *Pisaura mirabilis* spiders are not dependent on male feeding condition, and do not affect female behavioural responses during courtship. While the mere presence of silk-bound chemicals on male nuptial-gifts is beneficial in triggering female gift acceptance (Beyer et al. 2021), these do not appear to be used to signal the potential partner’s quality. Female spiders might ignore condition-dependent information on male silk as cheating males adopt similar strategies in order to cover the non-nutritive contents of nuptial-gifts. Instead, females may rely on direct information on male condition, such as cuticular chemicals, visual or vibrational information, to maximize their reproductive output.

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Statements and Declarations

Author contributions: MB and CT conceived and designed the study; MSM collected data and drafted the manuscript; MB performed statistical analysis and edited the manuscript; CT wrote the manuscript. All authors have read, provided comments and agreed to the final version of the manuscript.

Data accessibility: All data generated or analyzed are included in the supplementary information files.

Competing interests: The authors declare no competing interests.

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Supplementary Material

Lack of condition-dependent chemical signalling bound to silk of male nuptial gifts

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Supplementary statistics

Seasonal differences

In spring, 90 trials were conducted, compared to 17 trials conducted in autumn. We investigated whether the season of experiment conduction (autumn vs. spring) influenced our model output female gift acceptance by adding the variable season as random effect to the model described in the main manuscript. As in autumn latency to gift acceptance was not measured, no analysis on latency was conducted.

Results were retained, season did not affect female gift acceptance (Table S1).

Table S1. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for seasonal effects on predictors of female likelihood to accept gifts (*n* = 107, binomial GLMM)) of males varying in their feeding treatment, including treatment (high-fed, low-fed) and test number as fixed effects, and spider identities and season as random effects. Significance is shown in bold.

	Gift acceptance
Fixed effects	**β (95 % CI)**
Intercept*	1.67 (-1.24, 4.59)
Treatment (low-fed)[a]	0.18 (-0.95, 1.42)
Test number[b]	-0.36 (-1.09, 0.27)
Random effects	**σ² (95 % CI)**
Id Female	1.97 (0.01, 7.67)
Id Male	0.74 (0.00, 3.79)
Location	6.61 (0.27, 26.08)

* Reference category; estimate for male feeding treatment (high-fed) and normalized mean values for remaining continuous fixed effects, [a] Difference between reference category and male feeding state: low-fed, [b] Mean centred within all trials and normalized over the standard deviation within all trials

Explorative models including silk-wrapping duration

We investigated the influence of wrapping duration on likelihood and latency to female gift acceptance by adding the variable to the models described in the main model. The treatment variable was removed due to the interdependency between treatment and wrapping duration, with high-fed males wrapping longer.

Wrapping duration did not affect female gift acceptance or latency to acceptance (Table S2).

Table S2. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of female gift acceptance (model A, binomial GLMM, *n* = 107) and latency to gift acceptance (model B, Poisson GLMM, *n* = 70), including trial number and wrapping duration as fixed effects, and spider identities as random effects. Significance is shown in bold.

	Gift acceptance	**Latency to gift acceptance**
Fixed effects	**β (95 % CI)**	
Intercept*	1.03 (0.24, 2.10)	0.66 (0.40, 0.90)
Trial number[a]	-0.17 (-0.90, 0.46)	0.19 (-0.08, 0.46)
Wrapping duration[a]	-0.57 (-1.32, 0.09)	-0.06 (-0.30, 0.17)
Random effects	**σ² (95 % CI)**	
Id Female	3.36 (0.05, 11.30)	0.08 (0.00, 0.31)
Id Male	1.15 (0.00, 5.70	0.05 (0.00, 0.24)

* Reference category; estimate for respective male feeding treatment (high-fed) and normalized mean values of remaining fixed effect; [a] Mean-centered and standardized using the standard deviation within all trials

Explorative models: testing the effect of previous gift type

We investigated influence of female experience on female acceptance, by including gift type experienced in trial number 1 (i.e., treatment of the male that produced the first gift), treatment (gift type offered in trial number 2) and their interaction to the model. Due to low sample sizes, for latency to gift acceptance no interaction calculation was possible.

In total, 69 % (36 out of 52) of gifts were accepted in the first trial, with 69 % (18 out of 26) accepted each from low-fed and high-fed. In the second trial, a total of 54 % (26 out of 48) of gifts were accepted, with 52 % (12 out of 23) of gifts from high-fed and 58 % (14 out of 24) from low-fed males accepted. Neither treatment of the male that produced the first gift nor of the male producing the second gift did significantly affect female gift acceptance or latency to gift acceptance (Table S3).

Table S3. Estimated effect sizes and 95 % credible intervals (CIs) of the GLMMs testing for predictors of female gift acceptance (model A, binomial GLMM, *n* = 107) and latency to gift acceptance (model B, Poisson GLMM, *n* = 70), including treatment of the male producing the first gift and trial number as fixed effects, and spider identities as random effects. Significance is shown in bold.

	Gift acceptance	**Latency to gift acceptance**
Fixed effects	**β (95 % CI)**	
Intercept*	2.83 (-1.72, 8.12)	-0.30 (-2.89, 1.63)
Treatment trial 1 (low-fed)[a]	-2.67 (-7.96, 2.12)	1.00 (-1.04, 3.66)
Treatment trial 2 (low-fed)[b]	-2.18 (-7.54, 2.76)	1.10 (-0.91, 3.77)
Interaction (low-fed:low-fed)[c]	14.81 (-0.85, 40.00)	-
Random effects	**σ^2 (95 % CI)**	
Id Female	5.96 (0.00, 33.80)	0.13 (0.00, 0.59)
Id Male	0.98 (0.00, 5.87)	0.12 (0.00, 0.57)

* Reference category; estimate for male feeding treatment in trial 1 (high-fed) and trial 2 (high-fed); [a] Difference between reference category and male feeding state in trial 1: low-fed, [b] Difference between reference category and male feeding state in trial 2: low-fed, [c] Difference between treatments in trial 1 (high-fed, low-fed) in the difference between treatments in trial 2 (high-fed, low-fed)

General Discussion

This dissertation contributes to filling the knowledge gaps on chemical communication by focussing on silk-borne chemicals used in mate search and mate assessment of both male and female spiders and, thus, investigating reproduction without traditional assumptions on sex roles. The importance of chemical communication in animal reproduction and its almost ubiquitous use throughout the animal kingdom has been acknowledged and studied for over a century (Darwin 1871; Wyatt 2003; Bradbury and Vehrencamp 2009). Decades of research on chemical cues and signals established vast knowledge on the functions of chemicals in reproduction, including mate search and assessment (Johansson and Jones 2007; Wyatt 2010). Yet, based on the observation that males often invest more in mate search and courtship than females (Fromhage et al. 2016), a bias in the research community persists that leads to fixed assigned sex roles in reproduction, with females considered the sex chemically advertising and males the sex with corresponding sensory detection mechanisms. This sex-bias is mirrored in the literature, with most research focussing on female chemical signalling and male chemical detection (Kittredge and Takahashi 1972; Johansson and Jones 2007; Keller-Costa et al. 2015; Coombes et al. 2018; Scott et al. 2018a), leaving male chemical communication and female detection mechanisms as well as mate choice much less explored. Given that female mate choice has the potential to shape male chemical traits (Thomas and Simmons 2009; Lane et al. 2016), I have designated my research efforts on signalling in both sexes in the study of chemical communication. In particular, I focused on spiders, a taxonomical group of arthropods for which our understanding of chemical sensing, unlike insects, lags behind.

Through a series of carefully designed experiments that address the role of both male and female silk-borne chemicals in the mating process – from mate search to mate assessment and choice – of the hunting spider *Pisaura mirabilis*, I found that: i) in the context of male mate search, general directionality information appears to be absent in female spiders' silk trails, but chemical information embedded in the trails may convey information on female phenotypic characteristics, with male directional trail-following possibly reflecting male mate choice decisions (Aim 1, Chapter 1); ii) female silk trails convey information on female phenotypic characteristics such as developmental state, reproductive state and body condition, and are subjected to male choice (Aim 2, Chapter 2 and 3); and iii) male silk covering of nuptial gifts appears to carry chemical information that increase female gift acceptance, but this information is not depending on male body-condition (Aim 3, Chapter 4 and 5).

An overview of the main results of each chapter relating to the thesis' aims is shown in Box 1.

Box 1. Overview of the chapters' main results

Aim 1. Presence of directionality information in female spider silk trails in the context of male mate search

Chapter 1: Lack of male directional trail-following (i.e., following trails in the same direction as laid by the female), but silk trails that are unwashed or sourced from relatively larger females are followed more often in the female direction than those that are washed of putative chemicals with solvents or sourced from smaller females. Chemical trail attributes assessed through probing of silk lines may convey information on female quality, with directional trail-following reflecting male mate choice.

Aim 2. Variation in female chemical signalling and its role in male mate choice

Chapter 2: Females signal their developmental and reproductive state, but not mating state. When contacting female silk males decreased courtship towards silk of juveniles and subadults compared to adults, and towards silk of females carrying fertilized egg-sacs compared to unmated or mated females. Female silk conveys information on female reproductive quality and adjusted courtship investment indicates male mate choice.

Chapter 3: Females signal their developmental state and body condition, but not mating state through silk trails. When given a choice males prefer silk trails of females in high condition over low-condition, and adult over subadult, but do not prefer trails of mated over unmated females. Female chemical signalling is state and condition-dependent, and males exert mate choice through trail-following choices.

Aim 3. The presence and variation of male chemical signalling and its role in female mate choice

Chapter 4: Presence of chemicals in silk covering male nuptial gifts shown by higher female acceptance of gifts with unwashed opposed to silk washed with solvents to remove putative chemicals. Male silk-borne chemicals prime female responses, possibly by signalling quality or manipulating females.

Chapter 5: Lack of variation in male mass-dependent silk-borne chemical signalling. Females accept equally likely gifts produced by males in high and low feeding condition. Females may base mate choice on directly accessible male traits instead of silk-borne ones.

Male mate search and the complexity of directional information in female silk trails

Finding a mating partner is key for animal reproduction and a challenging task particularly for solitarily living species that occur in low densities within their population. Trails increase the chances of finding mates in comparison to random search, especially if these trails convey directionality information indicating the movements of the trail producer. Yet, studies on trail directionality are rare and the process of information embedding poorly understood (Gehlbach et al. 1971; Ford and Low 1984; Jackson et al. 2004; Nieh et al. 2004). Chapter 1, fills this existing shortage in literature by contributing knowledge on a spider species. However, in my study, I did not get conclusive evidence for directional information in female silk trails in this species but rather uncovered a complex scenario.

I investigated the presence of directional information in female silk trails by exposing *Pisaura mirabilis* males to female silk trails that were deposited unidirectionally, and scored male trail-following direction with reference to the female. If directionality information, that males recognize and use, is present in female trails, males should follow the trails in the direction they were deposited by the female. However, I did not find evidence for general directional trail-following as only little more than half of the males followed in female direction. Lack of directional trail-following could be attributed to potential costs (e.g., chemical production, predation) outweighing benefits of eased mate finding (e.g., Fei et al. 2023), or to directional information being unnecessary in *P. mirabilis* as animals occur in clusters during mating season, drastically increasing the chances of finding mates during this time (personal observation). Alternatively, males are unable to decode present directional information. Similarly to our results, lack of directionality in female silk trails was reported in two other web-less spider species, the wolf spider *Schizocosa ocreata* (Bell and Roberts 2016) and the crab spider *Misumena vatia* (Anderson and Morse 2001), with the latter also being tested on the mechanism behind trail-following (chemical vs. mechanical). While trail-following in the crab spider was purely mechanical with solvents not affecting it, when washing female silk trails to remove putative chemicals, *P. mirabilis* males followed washed silk trails significantly less often in female direction than when tested on unwashed trails. This effect on male directional trail-following behaviour suggests an important role of chemical information for directional trail-following, either regarding the encoding of directionality itself (e.g., in form of a chemical gradient) or additional information influencing the initiation of directional trail-following.

Possibly, males choose when to directionally follow a female silk trail, likely after having assessed chemical attributes of the trail that convey information about female characteristics (e.g., body mass, size or condition) reflecting reproductive qualities (e.g., fecundity). Nevertheless, when testing this hypothesis, I found no evidence that males directionally follow females of higher reproductive values (higher body mass or condition, larger size). I expected that males preferentially follow trails sourced from females that had high body mass, size and/or condition since these characteristics are known to affect male mate choice in arthropods (Bonduriansky 2001; Leather 2018) including spiders (Danielson-Francois et al. 2002; Johnson et al. 2014). Males could use both structural silk line properties such as density and size (Vollrath 1999) and chemicals bound to silk (Weiss and Schneider 2022a, b) to assess female phenotypic differences. Surprisingly, female phenotypic traits did not affect male directional trail-following, despite heavy and large *P. mirabilis* females being more fecund, and low-mass females being more likely to cannibalize males (Toft and Albo 2016). Coupled with the high costs for males associated with their nuptial-gifts, males *should* differentiate between varying female phenotypes as early as during mate search and directionally follow trails accordingly. My results seem even more puzzling considering that in Chapter 2 I show that males are indeed able to recognize female body mass and condition, as they preferentially follow silk of high-condition females in a binomial choice assay when compared to low-condition females. Interestingly, *P. mirabilis* males followed trails in female direction when the female was relatively larger than they were – and avoided the direction when the female was relatively smaller. This result is striking as the perception of relative phenotypic differences requires individuals to self-assess their own size in relation to the size of another. This ability is widespread in intra- and intersexual encounters in arthropods, including spiders, and has already been hypothesized for female *P. mirabilis* as relatively smaller males are allowed longer copulations (Prokop 2006) and gain relatively higher paternity shares (Matzke et al. 2022). Mate discrimination based on relative size differences could be explained by compatibility and incompatibility regarding each sex’ genitalia, with large males being unable to insert their pedipalps into a smaller female’s genital opening (i.e., the epigyne), resulting in reduced success of copulation and/or insemination (Schick 1965), and larger females being preferred due to eased access to the female’s genitalia.

Overall, my work shows no overwhelming evidence for general directionality in male movements during trail-following. Nevertheless, male spiders use female silk trails as guidelines for movements, gathering chemical and possibly tactile information through probing and pulling of silk lines that might encode information on female quality. Indeed, directionality appears to be present or used under certain conditions, with chemical trail attributes providing males with relative size-dependent information on female movements. Mechanisms of embedding and/or detecting trail directionality may be weakened by the natural history of the study species (clustered populations with high densities) and possibly require a costly sensory machinery in the face of increased predation or parasitism risks. The findings reported here contribute to the lack of empirical studies on directionality, and are key for advancing our understanding on mechanisms involved in animal communication and mate search in free-living and solitary arthropods.

Variation in female chemical signalling is based on their developmental and reproductive state, and affects male mate choice decisions

Chemical communication influences and shapes mate choice as animals convey information on their developmental, reproductive and/or other physiological states that in turn allow the restriction of the set of potential mates. Traditional views on female and male reproductive roles, with choosy females selecting among vigorously courting males, led to a major sex-bias in research and literature that only slowly is beginning to be conquered by adopting sex-independent scientific approaches that include female advertisement and male choosiness (Bonduriansky 2001; Edward and Chapman 2011; Fitzpatrick and Servedio 2018). As female reproductive interests vary through their lifetime, selection should favour variation in chemical signalling reflecting these varying interests. Similarly, males should respond to this variation by selecting the most suitable females, as they strive to minimize costs associated with male reproduction. In Chapter 2 and 3, I contributed to the reduction of sex-bias in research, by investigating variation in female chemical signalling and corresponding male mate choice in a spider species. In my studies, females varied in their chemical signalling, depending on their body condition, development and reproductive state, but not mating state, and males exerted mate choice based on these female traits.

I investigated female variation in chemical signalling and corresponding male mate choice by exposing males in no-choice and binary-choice assays to silk of females varying in their body condition (binary-choice only), development and reproductive state, including egg-production and –caring (no-choice only), and scored male courtship towards a single (Chapter 2) and preference between two simultaneously offered female silk trails (Chapter 3). If female chemical signalling varies with female individual state and this information is reflecting female reproductive potential, males should respond to this variation by adjusting their courtship investment and by preferentially choosing trails of females with perceived higher reproductive potential over those of lower potential. Indeed, as expected, when given the choice, *P. mirabilis* males chose trails of females that were of high body condition over those of low condition, uncovering potential costs to chemical signalling, such as nutritional stress, limiting chemical production (Blomquist et al. 2010; South et al. 2011). I verified previous findings that males can recognize female developmental state (Eberhard et al. 2021), adding to this knowledge that males not only adjust their courtship investment, with males courting adult females more often than subadults, but also exert choice when simultaneously encountering chemical information of both subadult and adult females, by choosing to follow trails of the latter. Female spiders commonly start to chemically attract mates as soon as they reach sexual receptivity and matings would lead to successful fertilization (Uhl 2013). Subadult females instead are usually not expected to emit chemicals in order to avoid attraction of unwanted suitors and possibly sexual harassment as females are not able to reproduce at this age. Consequently, it might come as a surprise that in 50 % and more of the cases, I found that males courted both juveniles that were still multiple weeks away from their moult to sexual maturity, and subadults that were one moult away from maturity. Male courtship reactions toward both juvenile and subadult females, coupled with the sudden increase of courtship investment as soon as females become sexually mature, suggest presence of chemicals starting from a very early age, but likely in low concentrations and/or varying blends than in adults.

Given the benefits of multiple matings for both females (nutritional and genetic benefits) and males (equally shared paternity) in *P. mirabilis*, I expected male courtship and trail choice to not vary with female mating state, as previously shown (Eberhard et al. 2021). I verified these results, with males not differing in courtship or in trail choice when encountering trails of unmated or mated females. While most studies in spiders that investigate variation in female chemical signalling stop at this point of females having mated (e.g., Riechert and Singer 1995; Roberts and Uetz 2005; Stoltz et al. 2007; Eberhard et al. 2021), as part of Chapter 2, I was interested in female signalling and male courtship responses in the post-mating phase, with females having produced fertilized egg-sacs and providing maternal care by carrying them in their mouthparts. I expected females that produced fertilized egg-sacs to reduce their chemical signalling in order to minimize male harassment and/or loss of the current clutch. Indeed, I found that males court females significantly less often that carried their fertilized egg-sac compared to females that were unmated or mated, and this change occurred within the short time-frame of 24 hours post egg-sac-production. The finding of reduced attractiveness of egg-carrying females, in line with results from another web-less spider, the wolf spider *Schizocosa malitiosa* (Baruffaldi and Costa 2010), suggests metabolic or ecological costs (e.g., energy expenditure, predation risk) together with costs of superfluous matings for females during maternal care, and males reducing their courtship investment by not courting aggressive and unreceptive females that guard egg-sacs.

Taken together, my work in these two chapters shows differential behavioural responses of males exposed to silk of females varying in their physiological, developmental and reproductive state, importantly including the post-mating phase with egg-production and maternal care, and male ability to discriminate between and exert preferences towards simultaneously encountered female phenotypes. These results suggest variation in female chemical signalling, and male mate choice reflected by both adjusted courtship display and choice in trail-following. These chapters contribute important empirical evidence supporting costs associated with chemical signalling and the potential role of such chemicals as reliable indicators of female quality that males use for mate choice.

Male silk-borne chemicals affect female mate choice, but are independent of male mass

For females, it is essential to select a suitable mating partner, as this choice majorly influences female reproductive success in form of number and quality of her offspring. With females often seen as the sex investing more in reproduction (Bateson 1983), for example in form of costly games and maternal care, females are expected to be choosy in order to reduce some of the costs associated with female reproduction. However, the general view on males investing more in mate search and courtship than females (Fromhage et al. 2016) has led to considering females as the chemically advertising sex and males as the sex with corresponding sensory detection mechanisms (Kittredge and Takahashi 1972; Johansson and Jones 2007; Keller-Costa et al. 2015; Coombes et al. 2018; Scott et al. 2018a), leaving male chemical communication and female detection mechanisms and mate choice much less explored. Yet, male chemicals are also critical for reproduction as they can affect female mate choice through both signalling of male underlying condition and manipulation of the female. Similarly to males, sexual selection should favour females that are able to discriminate between males varying in their underlying quality. In Chapter 4 and 5, I tackled the pronounced lack in research on male chemical signalling and corresponding female mate choice in spiders, by investigating presence as well as condition-dependency of chemicals in male silk reflected by female mate acceptance. In my studies, I uncover presence of male silk-borne chemicals, with male underlying feeding condition and body mass nevertheless not affecting female mate choice.

In Chapter 4, I investigated the presence of chemicals in male silk and corresponding female mate choice by offering female *P. mirabilis* nuptial-gifts covered by male silk that was either washed in solvents to remove putative chemicals, or unwashed, and scored female gift acceptance. If chemicals are present in male silk that mediate female gift acceptance, females should accept gifts that are covered with unwashed more often than those covered with washed silk. As expected, I found females to accept gifts in significantly higher proportions when they were covered in unwashed compared to washed silk, strongly suggesting the presence of silk-borne chemicals that elicit female gift acceptance. However, considering that *P. mirabilis* males are known to cheat females into mating by producing worthless gifts consisting of insect exoskeletons or plant parts (Albo et al. 2011b; Ghislandi et al. 2014; Albo and Peretti 2015), that they even cover in additional silk layers to hide their non-nutritious contents (Ghislandi et al.

2017), I also wanted to know whether these silk-borne chemicals honestly signal male underlying quality or if they rather manipulate females into mating beyond their reproductive interests. For this purpose, in Chapter 5, I let males of varying feeding state and consequently body mass (derived from variation in nutritional state) produce nuptial-gifts that were offered to females, of which I again scored gift acceptance. If males use their gift silk to transmit information about their individual characteristics, and if females use this information for mate choice, I expected a higher female gift acceptance of gifts silk-wrapped by males of high mass. Despite gift construction being constrained by male nutritional state, with low-condition males investing less in gift construction than high-condition males, in my study, females equally likely accepted gifts of both high- and low-condition males. This lack of female discrimination between gifts produced by high- and low-condition males could be explained by a lack of qualitative and/or quantitative differences of gift silk chemicals, for example caused by low costs of chemical production that can be provided by males of both conditions. Alternatively, females do not use present chemical differences and ignore condition-dependent information in male silk as a countermeasure against cheating males (Albo et al. 2012), solely basing their mate choice decisions on traits that can provide direct reproductive benefits, such as gift presence per se (Stålhandske 2001; Albo et al. 2011a; Tuni et al. 2013) and male (not gift-related) attributes, with male body condition being known to lead to higher siring success (Albo et al. 2012).

My work in these two chapters suggests that males add silk-borne chemicals to their gift silk that elicit female gift acceptance and consequently mating. While female choice and/or these male chemicals are not influenced by underlying male feeding condition, these two chapters importantly highlight the potential of investigating the evolutionary function of male chemical traits and their potential to be shaped by sexual selection or conflict through female mate choice.

General Conclusion

My dissertation contributes to our understanding on the use of chemicals, specifically silk-borne chemicals, in mate search and mate assessment between the sexes of a free-roaming arthropod species, overcoming century-old views on fixed sex roles in reproduction, and consequently allowing for an unobstructed view on chemical communication. My obtained results show an impressive variety of conditions and states influencing chemical signalling on the one side, and many-faceted mate choice on the other side. Notably, both males and females produced and assessed chemicals, and consequently influenced mate choice decisions in the other sex. With this, my work sets the baseline for future studies investigating chemical signalling and mate choice in both sexes, offering the opportunity to study mechanisms of sexual selection and sexual conflict alike.

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Co-Author Statements

All co-authors have stated that they have not and will not use one of the publications or the manuscript included in this dissertation for the purpose of their own dissertation.

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Publication II (Beyer et al. 2023a) and Publication III (Beyer et al. 2021).

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I hereby declare the correctness of the abovementioned details.

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Academic Education

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List of Publications

Beyer, M., Uludag, K.Ö., Lailler, M., Wolff, J. O., Eberhard, M. J. B., Czaczkes, T., Tuni, C**.** (2023). Testing presence of directionality information in female spider silk trails through male trail-following behavior. Behavioral Ecology and Sociobiology

Beyer, M., Uludağ, K. O., & Tuni, C. (2023). Female state and condition-dependent chemical signaling revealed by male choice of silk trails. *Behavioral Ecology,* arad068.

Beyer, M., Mangliers, J., & Tuni, C. (2021). Silk-borne chemicals of spider nuptial gifts elicit female gift acceptance. *Biology Letters, 17*(11), 20210386.

Beyer, M., Czaczkes, T. J., & Tuni, C**.** (2018). Does silk mediate chemical communication between the sexes in a nuptial feeding spider? *Behavioral ecology and sociobiology, 72,* 1-9.

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2021	**32nd European Congress of Arachnology** (ECA, Online Conference), 23-26 August, Oral presentation
2020, 2022	**Open day**, Biocentre, LMU, Presentation of the Behavioural Ecology group towards citizens, and schools
10 – 11. 2021	Written **Interview** about one of my research projects for the science and technology journal New Scientist
2021	**16th Ethologische Gesellschaft e. V. (Online Conference)**, 26th

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2021	**Travel stipend** of the Ethologische Gesellschaft e. V. for a research visit in Greifswald (Germany)
2021	**Eberhard-Gwinner-Preis** for the outstanding scientific presentation of a young scientist in terms of slide design and verbal communication, Virtual Conference of the Ethological Society e. V.

11 March 2024
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