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 minibilis* (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 fields 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. minibilis* [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 minibilis* 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 domestica*), 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 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 H2O 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 (b). 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 (‘emmmeans’-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.
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).

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].

### 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).

|Fixed effects| Gift acceptance| Latency to acceptance |
|-------------|----------------|-----------------------|
|Gift type    | β (95% CI)     | β (95% CI)            |
|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| | |
|Identities    | σ² (95% CI) | σ² (95% CI) |
|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)|

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

| comparison between gift types | gift acceptance | latency to acceptance |
|-------------------------------|-----------------|-----------------------|
|                               | 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, males being known to add chemicals to the silk cover of their nuptial gifts that 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 quality [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.

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