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Sperm storage mediated by cryptic female choice for nuptial gifts

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Polyandrous females are expected to discriminate among males through postcopulatory cryptic mate choice. Yet, there is surprisingly little unequivocal evidence for female-mediated cryptic sperm choice. In species in which nuptial gifts facilitate mating, females may gain indirect benefits through preferential storage of sperm from gift-giving males if the gift signals male quality. We tested this hypothesis in the spider *Pisaura mirabilis* by quantifying the number of sperm stored in response to copulation with males with or without a nuptial gift, while experimentally controlling copulation duration. We further assessed the effect of gift presence and copulation duration on egg-hatching success in matings with uninterrupted copulations with gift-giving males. We show that females mated to gift-giving males stored more sperm and experienced 17% higher egg-hatching success, compared with those mated to no-gift males, despite matched copulation durations. Uninterrupted copulations resulted in both increased sperm storage and egg-hatching success. Our study confirms the prediction that the nuptial gift as a male signal is under positive sexual selection by females through cryptic sperm storage. In addition, the gift facilitates longer copulations and increased sperm transfer providing two different types of advantage to gift-giving in males.

1. Introduction

Cryptic mate choice refers to sexual selection during mating (syncopulatory) or afterwards (postcopulatory) that leads to differences in sperm use among competing males [1–5]. Species in which nuptial gifts are transferred during mating [6] are particularly promising for studying cryptic mate choice, because gifts that are nutritious to females may be an honest signal of male quality [7]. If nuptial gifts are honest signals of good hunting ability and this trait is heritable, females would benefit from biasing paternity towards males that offer gifts [8]. The potential for acquiring benefits of cryptic choice is particularly high when there is variance in the quality of the nuptial gift. Gift-giving may, for example, be a target for sexually antagonistic coevolution, which favours the evolution of deceit or fake gifts, reducing the benefit of precopulatory female mate choice [9–12]. However, females may counteract deceit by favouring males that present genuine nuptial gifts during or after mating [12]. In nuptial gift-giving species, female preference for genuine gifts is thus expected to favour the evolution of cryptic female mate choice. Whereas the influence of the gift-giving trait on male mating success and the potential for males to exploit female preference for nuptial gifts have attracted much interest [9,11,12], remarkably little is known about cryptic female choice for nuptial gifts.

Internal processes that result in fertilization bias can be mediated by both sexes or by interactions between male and female traits [2,3,13]. There is a wealth of studies on syn- or postcopulatory processes focusing on male traits that confer advantages in sperm competition [2,4,5]. By contrast, there are few studies showing unequivocal evidence for cryptic sperm choice [14]. This is surprising, as cryptic female mate choice should be very common precisely because it is occurring in the genital tract, where females are expected to be

more in control of internal processes after copulation than males [3]. Empirical studies have suggested postcopulatory selection for males of high quality based on traits such as body condition, attractiveness, social status or relatedness [15–23]. However, few studies conclusively show that sperm selection is driven by female cryptic sperm choice [24–26]. This may be because it is intrinsically difficult to control for strategic male sperm allocation [27], and hence to disentangle male- and female-driven processes [28]. Unequivocal evidence of female sperm choice was shown in crickets [25,26], where males form a spermatophore before they encounter the female, and therefore are unable to strategically mediate sperm allocation in response to female phenotype. Preferential storage of sperm from unrelated males [25], or conspecific males [26], could therefore be attributed to cryptic female choice.

We tested the hypothesis that the nuptial gift is a target of cryptic female choice in the polyandrous spider *Pisaura mirabilis* (Pisauridae). In this species, males often offer an insect prey wrapped in white silk as a nuptial gift, which the female feeds on during copulation [29]. While it is possible for males to copulate without a gift, gift-giving males experience dramatically higher mating success [12,30,31]. Furthermore, males that offer a nuptial gift achieve longer copulations (100 min on average) than males without a gift (10 min on average), leading to a higher number of fertilized eggs for gift-giving males [12,30,32]. Females appear to have full control over both the initiation and termination of mating [12,30]. Spiders are excellent models for testing female cryptic sperm choice, because males use external sperm transfer by modified intromittent organs, the pedipalps, which are loaded with sperm prior to mate searching [33], thus limiting the scope for strategic sperm allocation. Females store sperm from multiple males in sperm storage organs, the spermatheca, for later fertilization of eggs when she produces an egg-sac.

We tested the effect of gift presence on the number of sperm stored by the female by experimentally terminating copulation duration in males with a gift (GT) to match the average duration of shorter copulations with males without a gift (NG). Following copulation, we determined the number of sperm in the female sperm storage organs. If females bias sperm storage towards males that offer a gift, females in the GT group were expected to store more sperm than females in the NG group. To examine the relationship between copulation duration and sperm storage, we also determined the number of sperm stored after uninterrupted copulations with gift-giving males (G). Finally, we examined the effect of gift presence and copulation duration on female fecundity and egg-hatching success in an additional set of females mated to NG, GT and G males to gain further insight into benefits of providing a nuptial gift.

2. Material and methods

We collected juveniles and subadults of *P. mirabilis* (Clerck, 1757) in April 2011 at the Mols Laboratory near Aarhus, Denmark. In the laboratory at Aarhus University, spiders were housed individually in vials (30 ml) containing moist moss (*Sphagnum* spp.). Water was provided regularly to maintain humidity. We raised individuals at room temperature ($23.4 \pm 0.1^\circ\text{C}$) and natural photoperiod and fed them with blowflies (*Calliphora* sp.) three times per week until maturation.

(a) Mating behaviour and experimental design

Males court females by offering the nuptial gift held in their chelicerae while waving their pedipalps (male intromittent organs). Once the female accepts and grasps the gift with her chelicerae, the male initiates sperm transfer by performing alternate insertions of the pedipalps into the female genital tract. During copulation, the male retains contact with the gift with the tarsal claws of his third pair of legs, while the female is consuming it. After each insertion, the male returns to a face-to-face position with the female, grabbing the gift with his chelicerae. No courtship in the form of pedipalp waving is performed before the second (and possibly subsequent) palp insertion. Copulation occurs in a similar way in matings with no gift, although these males experience a much reduced acceptance rate [29,34]. In staged mating trials, we registered the number of pedipalp insertions and copulation duration. Copulation duration was recorded from pedipalp insertion until pedipalp disengagement and was calculated as the sum of the duration of all insertions occurring within a trial. Total copulation duration thus represents the time of potential sperm transfer.

Our experimental design included three groups. NG males ($n = 53$) offered no gift and were allowed to copulate without interruption; copulation duration (mean \pm s.e.): 10.4 ± 1.6 min, number of insertions (mean \pm s.e.): 2.0 ± 0.2 . GT males ($n = 39$) offered a nuptial gift and had copulation experimentally terminated after 10 min to match the average copulation duration of NG males. G males ($n = 36$) offered a gift and were allowed to perform uninterrupted copulations; copulation duration (mean \pm s.e.): 86.0 ± 6.0 min; number of insertions (mean \pm s.e.): 4.2 ± 0.6 . Experimental manipulation of the GT group was based on the average copulation duration and number of insertions found in the NG group, thus allowing two pedipalp insertions. In the GT group, we terminated the first insertion after 5 min using a paintbrush. Subsequently, we allowed the male to resume copulation and perform the second insertion for another 5 min before the final separation. We analysed the number of sperm transferred in a subset of females, and fecundity and egg-hatching success in another subset of females from each of the three experimental groups (see details below).

Staged mating experiments were carried out in May 2011. A female was placed in a transparent plastic cage ($22 \times 17 \times 6$ cm) with paper-covered bottom at least an hour prior to the experiment, allowing her to deposit silk threads. We then removed the female and exposed the male to the female silk for 15 min. In the GT and G groups, a housefly (*Musca domestica*) was added to the cage which the male caught for gift construction. After 15 min, we gently reintroduced the female into the cage and mating trials started. NG males were exposed to the same procedure but with no prey available. Male spiders load their pedipalps (intromittent organs) with sperm after the final moult, and thus prior to mate search and production of nuptial gifts [34]. With this design, we aimed to minimize any effect of the presence or absence of the gift on differential loading of the pedipalps. Males were randomly allocated to the three treatment groups. Individuals used in the experiments were virgins and used only once.

(b) Sperm count

Females were frozen at -50°C between 3 and 6 h after mating (NG: $N = 39$; GT: $N = 20$; G: $N = 18$). For counting the number of sperm transferred, specimens were transferred to the University of Greifswald, where the female sperm storage organs were dissected out under a stereomicroscope (ZEISS) and treated by following a protocol established for *P. mirabilis* ([35], modified after [36]). The female spermathecae were transferred to $20 \mu\text{l}$ of saline solution (Casytone, Schärfe System). To homogenize samples, we ruptured the spermathecae with forceps and

applied ultrasonic treatment. We avoided sample loss by using indirect ultrasonic processing in a cup booster designed for small volumes (Bandelin UW 2070). Ultrasonication was done twice for 30 s at 50% power with a break of 30 s to avoid overheating of the sample. Afterwards, we centrifuged samples at 5000 g for 1 min and vortexed for 1 min. We placed 10 μ l on each counting chamber of the haematocytometer (1 mm Neubauer). The sperm were counted in 16 squares under a microscope 400 \times (Olympus).

Our main aim was to test whether the number of sperm found in the female spermatheca after NG and GT matings differ. To reduce the variance in copulation duration in NG compared with the GT group, we reduced the dataset by selecting NG data with an average copulation duration of 9.6 min (± 0.9 s.e., range: 6–16 min, $N = 14$).

A copulation could be completed with a male using one or both pedipalps, therefore we tested the effect of the number of pedipalps used on the number of sperm stored. Whether one or more than one pedipalp insertion was performed in NG matings did not significantly affect the number of sperm found in the female genital tract (mean \pm s.e. one insertion: 3020 ± 1079 ; more than one insertion: 4727 ± 946 ; $t = -1.18$, $p = 0.25$, $N_{\text{one insertion}} = 6$, $N_{\text{two or more insertions}} = 8$). Additionally, as males performing two or more insertions could have used the same or different pedipalps in the second or later insertions, we checked whether this affected the number of sperm stored by females in NG and GT matings. We found no significant differences in the number of sperm stored neither in the NG (mean \pm s.e. same pedipalp: 2625 ± 892 , different pedipalp: 5885 ± 1736 ; $U = 19.5$, $p = 0.29$, $N_{\text{changing palps}} = 12$, $N_{\text{same palp}} = 5$) nor in the GT group (same pedipalp: 7098 ± 1001 , mean \pm s.e. different pedipalp: 6197 ± 2332 ; $t = 0.92$, $p = 0.36$, $N_{\text{changing palps}} = 6$, $N_{\text{same palp}} = 14$). In two cases data points on pedipalp change are missing.

For analysing the relationship between copulation duration and sperm number, we used all data available from the NG group ($N = 39$) and pooled these with data from the G group ($N = 18$).

(c) Hatching success

To study the effect of gift presence and copulation duration on reproductive success, we determined the reproductive output of 14 females from the NG group, 19 females from the GT group and 18 females from the G group. After the mating trials, females were kept individually in 30 ml vials at room temperature ($23.4 \pm 0.1^\circ\text{C}$) and were fed one blowfly (*Calliphora* sp.) per day. After egg-sac construction, light bulbs were placed 20 cm above the vials to raise the temperature to 26.7°C (± 0.1 s.e.) during 3 h starting at noon in order to enhance hatching success of the eggs. As females carry the egg-sac in their chelicerae until the eggs hatch, feeding was stopped after oviposition. For the first egg-sac a female produced, we counted the total number of laid eggs (hatched + unhatched eggs = clutch-size). Hatching success was calculated as the proportion of hatched eggs.

(d) Statistical analyses

Statistical analyses were performed using JMP 7.0 software (SAS institute). Assumptions of parametric tests were examined using Shapiro–Wilk tests for normal distribution of residuals and Levene’s test for homogeneity of variances. Generalized linear models (GLMs) were used for analysing sperm number (Poisson) and hatching success (binomial) among groups, and to examine the effect of copulation duration on hatching success (binomial). We used linear regression to analyse the effect of copulation duration on the number of sperm stored (log transformed).

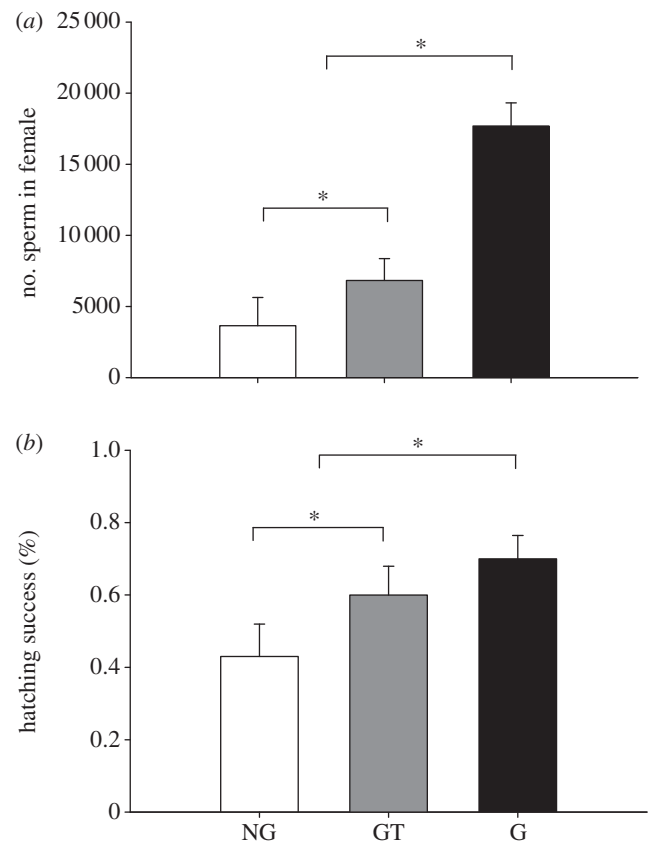


Figure 1. (a) Number of sperm in the female sperm storage organs and (b) hatching success, in NG (short copulations without gift), GT (short copulations with gift) and G matings (long copulations with gift). Asterisk (*) indicates significant differences ($p < 0.05$).

3. Results

(a) Sperm count

The number of sperm in the female spermatheca differed significantly among the three experimental groups (GLM, $\chi^2 = 174442.1$, $p < 0.0001$, d.f. = 2, $N = 52$; figure 1a), and was significantly lower in the NG group compared with the GT group ($\chi^2 = 12165.4$, $p < 0.0001$, d.f. = 1, $N_{\text{NG}} = 14$, $N_{\text{GT}} = 20$; figure 1a). Females in uninterrupted matings with gift-giving males G stored significantly higher numbers of sperm compared with females from the NG and GT groups ($\chi^2 = 162276.8$, $p < 0.0001$, d.f. = 1, $p < 0.0001$, $N_{\text{G}} = 18$, $N_{\text{NG+GT}} = 34$; figure 1a). In the G group, copulations resulted in significantly more sperm stored in the female genital tract (Linear regression, effect of copulation duration on log sperm number: $F = 11.6$, $p = 0.004$, $N_{\text{G}} = 18$; figure 2a). No parametric linear relationship was found in the group of males with no gift ($N = 39$, figure 2a).

(b) Hatching success

Generalized linear models showed that the hatching success differed significantly among groups ($\chi^2 = 235.9$, $p < 0.0001$, d.f. = 2; $N = 51$; figure 1b) and was lower in the NG compared with the GT group ($\chi^2 = 92.7$, $p < 0.0001$, d.f. = 1, $N_{\text{NG}} = 14$, $N_{\text{GT}} = 19$). Females in the G group experienced the highest hatching success compared with NG and GT females ($\chi^2 = 143.2$, $p < 0.0001$, d.f. = 1, $N_{\text{G}} = 18$, $N_{\text{NG+GT}} = 33$). We found a positive relationship between copulation duration

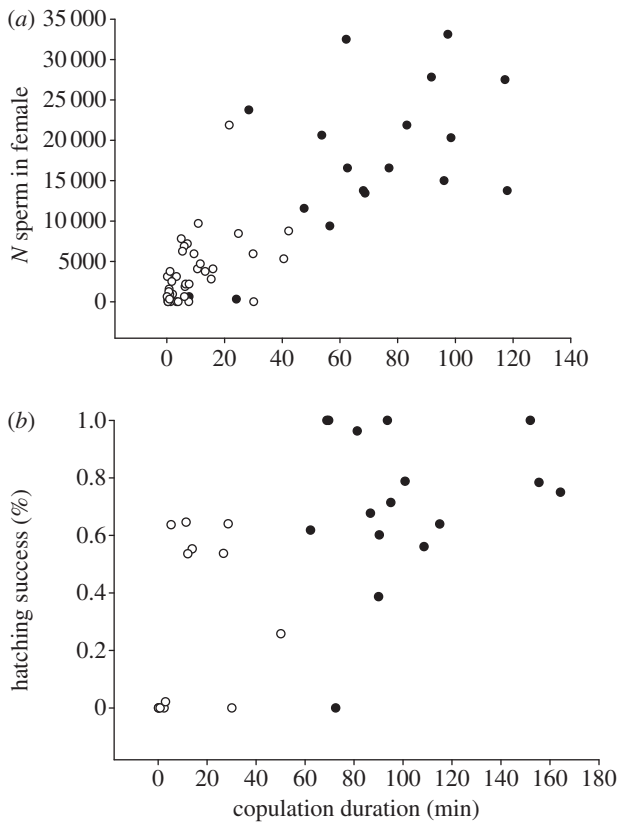


Figure 2. (a) The number of sperm stored in female sperm storage organs as a function of copulation duration in NG (short copulations without gift) and G (long copulations with gift); and (b) proportion of hatched eggs as a function of copulation duration in the NG and G groups. Statistics were performed using GLM with Poisson (sperm number) and binominal function (hatching success) and log link (see text). Filled circles, G group; open circles, NG group.

and hatching success (NG and G pooled, $N_{\text{NG+G}} = 32$, GLM binominal: $\chi^2 = 139.9$, $p < 0.0001$; figure 2b).

4. Discussion

Our data show that females stored significantly more sperm from gift-giving males than from males without a gift when we kept copulation duration constant. By allowing gift donors to store more sperm females can gain indirect benefits, either through Fisherian processes if females produce ‘sexy sons’ that are more likely to provide nuptial gifts, or by elevated fitness of offspring if gift-giving males pass on genes that are superior for survivorship traits [7,8,13,19]. *Pisaura mirabilis* males that offer nuptial gifts may signal good hunting abilities that are inherited by their offspring and through which male offspring would further gain an advantage in attracting females. Indeed, males that were satiated, and therefore in good condition were shown to achieve higher mating and paternity success compared with starved males in poor condition, suggesting that females can select males based on their quality [37]. Our data suggest that sperm storage is at least partially under female control through cryptic sperm choice. Differential sperm storage may result from preferential sperm uptake during mating (syncopulatory sexual selection) [25,26], or through differential sperm selection or ejection immediately after mating (postcopulatory sexual selection) [24], processes that are proposed to occur widely [3].

Under the risk of sperm competition, males can increase fertilization success by increasing sperm transfer through longer copulations, by a higher rate of ejaculate transfer per unit time, by increasing sperm size, by producing different types of sperm within one ejaculate, thereby directly competing with the sperm of other males inside the female reproductive tract, or by a combination of these traits [5,38–42]. Strategic ejaculate transfer is unlikely to explain the pattern of differential sperm storage observed here. We used an experimental design where males were randomly allocated to treatment groups when their intromittent organs were already loaded with sperm. Loading takes place shortly after the maturation moult, precluding that males tailor the number of sperm in their pedipalps in response to the probability of being able to acquire a gift. Furthermore, as no-gift males experience shorter copulations ([12,30] and this study), males copulating without gifts are likely to be under selection pressure to increase sperm transfer rate if possible [5].

It is possible that the difference in number of sperm stored between GT and NG males may result from having the top performing males with the longest copulations excluded from the NG group. However, as males were randomly assigned to the experimental groups, we expect each group to contain males with similar variability in performance. Further, we selected a subset of NG males close to the mean copulation duration of 10 min for comparison with the GT males in order to minimize variance in copulation duration between groups. Consequently, it seems unlikely that the observed difference in number of stored sperm between NG and GT males can be explained by other factors than cryptic female choice.

From a male perspective, nuptial feeding functions to facilitate copulations and increases copulation duration and sperm transfer [5,43–46]. This is advantageous because *P. mirabilis* females are polyandrous and control copulation duration, which is positively correlated with nuptial feeding and gift quality [12,47,48]. Our data showed that both sperm storage and fertilization success increased linearly with copulation duration, suggesting that males that are able to offer gifts that take longer to consume would gain an advantage in sperm competition. Females do not only discriminate males on gift presence, they also accept males in good feeding condition with higher probability [37]. This supports a scenario in which male hunting ability, feeding condition and the gift-giving trait are associated. Males therefore gain two different types of advantage from the nuptial gift by preferential female storage of sperm from gift-giving males, and by prolonged copulation duration that correlates positively with sperm transfer.

Cryptic female choice may be expected if males extend copulation duration by offering non-nutritive items, ‘worthless gifts’ [12]. Females can only assess gift content and quality after having fed on the gift for some time during which males transfer significant numbers of sperm (C. Tuni & M. J. Albo 2011, personal observation). Whether cryptic female choice for genuine nuptial gifts evolves to counteract male deception should depend on the direct costs of accepting worthless gifts to females and indirect Fisherian benefits of son’s mating success when offering worthless gifts [32,48,49]. The combination of cryptic female sperm storage favouring gift-giving males, and advantages in sperm competition to males offering genuine gifts, probably acts to maintain selection for high-quality

gifts and may explain the prevalence of genuine nuptial gifts in natural populations [12]. Genuine gifts may also be favoured by direct nutritional benefits to females in the form of earlier oviposition [48].

We found that egg-hatching success was significantly higher in matings with gift-giving compared with gift-less males and correlated positively with the number of sperm stored. This effect can hardly be owing to sperm limitation, because gift-less males transferred several thousands of sperm that should be more than enough to fertilize a clutch of approximately 100 eggs. Nevertheless, hatching rates are in the range of 43–70%, suggesting that sperm storage, sperm activation and processes that lead to fertilization require large sperm numbers even in the absence of rival sperm. In spiders, sperm are transferred in an encapsulated and coiled state into the female sperm storage organs, and thus require being decapsulated and uncoiled latest when oviposition is due [50]. Whether internal processes that

mediate sperm activation and fertilization are targets for postcopulatory female choice requires further examination.

In conclusion, there seem to be two different types of advantage of nuptial gifts to *P. mirabilis* males. Firstly, nuptial gifts are favoured by cryptic female choice as females store more sperm from gift-giving males. Secondly, gift-giving males increase the number of sperm transferred by prolonging copulation. These processes in concert lead to a higher paternity success for gift-giving males and may confer indirect benefits to females.

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