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7 **Males mate guard in absentia through extended effects of postcopulatory**
8 **courtship in the parasitoid wasp *Spalangia endius* (Hymenoptera: Pteromalidae)**
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22 **Abstract**
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24 The proximal mechanisms leading to monandry have been little-studied in most
25 insect orders, including Hymenoptera. In the parasitoid wasp *Spalangia endius*, mated
26 females are less attractive (less often mounted) than virgins and are unreceptive
27 (unlikely to allow copulation). Which aspects of mating are responsible was tested by
28 observing male responses toward females whose mating had been interrupted at
29 various stages. All females were allowed to receive precopulatory courtship and to
30 open their genital aperture to copulate. Then some were interrupted before copulation;
31 some after copulation but before postcopulatory courtship; and some were allowed to
32 complete postcopulatory courtship. Females that had copulated were not less attractive
33 than females that had not. In contrast, females that had received postcopulatory
34 courtship were clearly both less attractive and less receptive. Thus, postcopulatory
35 courtship functions as extended mate guarding, by making the female less attractive
36 and less receptive to subsequent males even after the original male is no longer present.
37 The effect of postcopulatory courtship on female attractiveness was persistent but
38 imperfect: when males were presented sequentially to mated females, most but not all
39 males retreated without mounting, and a female could repulse more than twenty males
40 in succession.
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42 *Key words:* Mating; Monandry; Parasitoid wasp; Receptivity; Unattractiveness

1. Introduction

Insect mating systems exhibit considerable diversity, although among males, polygyny appears to predominate (Thornhill and Alcock, 1983). Within both eusocial and parasitic Hymenoptera, among females, monandry (mating with just one male) appears to predominate (e.g., Boomsma and Ratnieks, 1996; Ridley, 1993). However, the proximal mechanisms leading to monandry have not been well-studied in Hymenoptera and many other orders, most studies being on Diptera, Lepidoptera, and Orthoptera (Eberhard, 1996; Ringo, 1996; Simmons, 2001).

Among animals generally, females can avoid remating, and thus be monandrous, through a range of mechanisms, including moving away from males, being unattractive and being unreceptive. Definitions of attractiveness and receptivity in the literature are not always consistent or clear. Here we use attractiveness to indicate whether the male mounted or courted. We use receptivity in its narrow sense, i.e., whether copulation occurred. In the literature, "receptivity" is also used in a broader sense, referring to whether the female mated, which is affected both by her attractiveness and by her receptivity in the narrow sense.

This study examines attractiveness and receptivity in the parasitoid wasp *Spalangia endius*, which is largely monandrous and polygynous (King et al., 2005). In general, mating behavior has been poorly studied in parasitoid wasps, particularly given their large numbers and ubiquity (van den Assem, 1986; Godfray, 1994).

The mechanisms for loss of attractiveness and receptivity following mating in Hymenoptera may differ from other insects. In strictly sexual species, mating may need to trigger not only loss of attractiveness and receptivity but also ovulation (reviewed in Eberhard, 1996; Ringo, 1996; Simmons, 2001) because ovulation is useless until sperm are present. However, in hymenopterans, ovulation is useful even when sperm are not available because sons are produced parthenogenetically. When mating (or the availability of sperm) needs to trigger only loss of attractiveness and receptivity and not also ovulation, does this remove constraints on the mechanism by which females become attractiveness and unreceptive? If so, loss of attractiveness and receptivity mechanisms in hymenopterans may differ from those possessed by strictly sexual species. Evaluating this prediction of a difference in mechanisms awaits more studies on hymenopterans, of which the present study is one.

S. endius is a small wasp that parasitizes the pupal stage of certain fly species that are found in manure and decaying organic matter (Rueda and Axtell, 1985). *S. endius* produces one offspring per host. The hosts are often in groups, and male *S. endius* emerge before females and wait at the cluster of hosts for females to emerge (King, personal observation). Both sexes are sexually mature at emergence.

In a typical interaction between the sexes, when a *S. endius* male detects a female, he very briefly fans his wings while running toward her (King et al., 2005). Whether and how quickly he wing-fans are independent of her mating status. As he contacts or nearly contacts her, he either retreats or proceeds to mount. Upon mounting, he begins precopulatory courtship, which lasts about 6 s and consists of vibrating his whole body rapidly up and down and tapping her sides with his middle legs (King unpublished data). The courtship vibration continues through copulation and postcopulatory courtship, stopping only after the male dismounts. Copulation is

brief, about 6 s, and requires that the female open her genital orifice. During the postcopulatory courtship, which lasts about 22 s, the female closes her genital orifice, and then she begins stroking her hind legs between her body and his until he dismounts.

Mating decreases an *S. endius* female's attractiveness and receptivity (King et al., 2005). Males retreat sooner from, and are less likely to mount, a mated female than a virgin. Retreats are often abrupt. Not mounting appears to be adaptive for males given that a mated female rarely opens her genital aperture for copulation even if mounted. Whether it is adaptive to females, e.g., by reducing interference with oviposition, remains to be seen. This study examines when in the mating sequence a female becomes unattractive and when she becomes unreceptive as well as how many males she can repulse (cause to retreat). If mated females are repulsing males by releasing antiaphrodisiac, as currently thought (King et al., 2005), they might deplete their supplies after repeated encounters with males.

2. Materials and methods

2.1 Wasps

The *S. endius* were from a laboratory-cultured stock of wasps arising from a 1996 collection in Zephyr Hills, Florida. They were reared on house fly pupae in a 25°C incubator. Single parasitized pupae were placed in glass test tubes in order to isolate virgins. The wasps were fed honey on the day of emergence and were less than 1 d old when used in experiments. Although both virgin and mated males avoid mounting mated females, this avoidance is less pronounced in virgin males (King et al., 2005). Thus, we used mated males in our tests because larger effects are easier to detect statistically.

2.2 Females interrupted before copulation

This experiment was designed to compare the interaction of mated males with fully mated females to that of mated males with females that had not received copulatory or postcopulatory courtship. Thus there were two treatments or female types, control females ($n = 19$) and interrupted females ($n = 19$). In the interruption treatment, courting was allowed to the point of the female opening her genital aperture; then the male was removed from the female with clean metal forceps before he inserted his aedeagus (Table 1). In the control treatment the male was removed as he dismounted to allow a complete mating while controlling for any disruption caused by removing the male with forceps in the interruption treatment. (An additional control, comparison to virgin females from another study, is in the Discussion.)

Testing was with a recently mated male: the mating had been observed, was uninterrupted, and was with a virgin female. He was left with that female until just before testing. Testing was in a small plastic dish filled about two-thirds full of sand and with a glass cover. The top of the sand was wet with water in order to reduce static

electricity build up. Clean containers and sand were used for each test. A female from one of the treatments and a test male were put in the dish and then were observed until a mating was completed or for 5 min, whichever came first.

Presence or absence of mounting, precopulatory courtship, opening of the female genital aperture and copulation were recorded. Number of retreats from the female by the male prior to mounting was also recorded. Males that mounted almost always courted and when the female opened her genital aperture, copulation always followed (Fig. 1). Thus, the statistical analyses focused on whether the occurrence of mounting, the occurrence of copulation, and the frequency of retreats depend on treatment.

Because control females received more of the mating sequence than interrupted females, the two groups differed not only in the mating stimuli that they received but also in the duration between each aspect of mating and testing. However, testing was at least 10 min after treatment. This is more than enough time for the stimuli to act in all treatments. Mated females become unattractive and unreceptive immediately after mating, and the entire mating sequence takes less than a minute. Thus, since the stimuli are part of the mating sequence, the stimuli cannot be taking more than a minute to act.

2.3 Females interrupted before postcopulatory courtship

This experiment was designed to compare the interactions of mated males with fully mated females to that of mated males with females that had not received postcopulatory courtship. Thus again there were two treatments or female types, control females ($n = 20$) and interrupted females ($n = 19$). In the interruption treatment, the male was removed immediately after copulation, thereby preventing postcopulatory courtship (Table 1). The control and testing were the same as in the previous experiment.

2.4 Persistence of unattractiveness

The purpose of this experiment was to determine whether having repulsed males decreases a mated female's ability to repulse subsequent males. Matings were observed. A mated female ($n = 13$) or virgin female ($n = 20$) was presented with mated males sequentially. Any given male was only used once. Whether a male first retreated or fully mounted was recorded prior to presenting a new male.

For virgin females, males were presented sequentially until one fully mounted (Fig. 2). For mated females, males were presented sequentially, up to 25 males or until we ran out of males, and regardless of whether a full-mounting occurred (Fig. 3). (In only one case, involving a mated female, did any of the males fail to interact with the female within 10 min; those two males, the third and twentieth, were excluded and replaced with different males.)

2.5 Statistical analysis

Analysis of the presence versus absence of response (i.e., of mounting and copulation) in the first two experiments was by likelihood ratio tests, as described below. Comparison of number of retreats between treatments was by Mann-Whitney U tests because assumptions of parametric tests were strongly violated. Because the controls in the first two experiments were not statistically different, they were combined prior to the analyses presented below. In the *Persistence of unattractiveness* experiment, the test of the relationship between the proportion of males that fully mounted and the order of presentation to the female was by Spearman rank correlation; however, a regression leads to the same conclusion.

3. Results

3.1 Females interrupted

Female attractiveness, defined by whether the male mounted, did not differ between females that had previously copulated and females that had not: in both interruption treatments 18 of 19 females were mounted (Fig. 1). The two interruption treatments were then combined for comparison to the control. Whether a male mounted differed significantly between the interruption treatments and the control (likelihood ratio test $X^2 = 16.95$, $df = 1$, $P < 0.001$): a much greater proportion of males mounted interrupted females, i.e., females that had not received postcopulatory courtship (95% versus 56%, Fig. 1).

Comparison of the two interruption treatments suggested reduced receptivity for females that had previously copulated relative to those that had not (63% versus 89%) (likelihood ratio test $X^2 = 3.81$, $df = 1$, $P = 0.05$; Fig. 1). Each interrupted treatment also differed from the control: a much greater proportion of males copulated with females that had not previously copulated (likelihood ratio test $X^2 = 40.79$, $df = 1$, $P < 0.001$) and with females that had not previously received postcopulatory courtship (likelihood ratio test $X^2 = 20.15$, $df = 1$, $P < 0.001$) than with control females, where only 8% copulated.

The number of male retreats did not differ between the interrupted treatments (mean: 1.1, range: 0 – 9, $n = 19$ versus mean: 0.63, range: 0 – 3, $n = 19$; Mann-Whitney $U = 161.0$, $P = 0.45$). In other words, whether a female failed to receive both postcopulatory courtship and copulation or just postcopulatory courtship had no statistically detectable effect on the number of retreats a subsequent male made from her. In contrast, males made significantly fewer retreats from such interrupted females (the two interruption treatments combined) than from control females (mean: 0.84, range: 0 – 9, $n = 38$ versus mean: 3.45, range: 0 – 19, $n = 38$; $U = 396.0$, $P < 0.001$). This pattern persisted when the analysis was restricted to pairs in which the male subsequently mounted (mean: 0.64, range: 0 – 7, $n = 36$ versus mean: 1.14, range: 0 – 5, $n = 22$; $U = 307.5$, $P = 0.04$).

3.2 Persistence of unattractiveness

The unattractiveness of mated females persisted even with repeated exposure to males. Males usually retreated from mated females; and as mated females were presented with mated males in succession, there was no significant change in the proportion of males that fully mounted (Fig. 3; Spearman's $r = -0.094$, $n = 25$, $P = 0.66$). Of the 246 males presented to the 13 mated females, 83% fanned then retreated, 11% fanned and only mounted less than half way onto the female, 1% fanned, fully mounted and then dismounted, 5% fanned, fully mounted, courted and then dismounted without copulation and less than 1% fanned, fully mounted, courted and copulated. A given female was fully mounted by 0 – 27% of the males that she encountered. Females repulsed up to 24 males in succession.

In contrast to mated females, virgin females were usually fully mounted at their first male encounter ($n = 17$ of 20 pairs), and in all but one of those cases they then copulated. The 4 of 20 females that did not copulate with the first male did so with the second male (Fig. 2).

4. Discussion

4.1 Source of unattractiveness and loss of receptivity

Both the mounting data and the male retreat data indicate that the postcopulatory courtship that mated females receive is responsible for their subsequent unattractiveness, decreasing mounting from 95% to 56%. Aspects of courtship prior to postcopulatory courtship do not appear to contribute. Copulation did not contribute because without postcopulatory courtship 95% of females were mounted, regardless of whether or not they had previously copulated (the two interruption treatments). Precopulatory courtship and genital orifice opening did not contribute because females that had experienced both did not differ from females that had experienced neither: 95% were mounted in the two interruption treatments versus 96% of virgin females that had been tested in similar circumstances (King et al., 2005) (likelihood ratio test $X^2 = 0.068$, $df = 1$, $P = 0.80$). (Note: the trial duration was 6 min for the virgin treatment versus 5 min in the present study; however, nothing relevant happened in the additional minute: all the mountings had occurred by 2.55 min.)

Postcopulatory courtship also appears to be the major stimulus for loss of receptivity: only 8% of females that received postcopulatory courtship subsequently copulated versus 63% of females that had not received it (Fig. 1). Copulation made a smaller contribution to loss of receptivity, decreasing it from 89% to 63%. Precopulatory courtship and genital orifice opening did not contribute because females that had experienced both did not differ from females that had experienced neither: 89% copulated among females that were interrupted after precopulatory courtship and genital orifice opening versus 86% of virgin females that had been tested in circumstances similar to this study (King et al., 2005) (likelihood ratio test $X^2 = 0.093$, $df = 1$, $P = 0.76$). (Again, although the trial duration was 1 min longer in the virgin treatment, nothing relevant happened in the additional minute: all the copulations had occurred by 2.57 min.)

Females that received pre- but not post-copulatory courtship were not as unattractive and as unreceptive as females that received both, yet qualitatively pre- and post-copulatory courtship are not obviously different. Perhaps loss of attractiveness and receptivity is related to the total duration of all courtship. Postcopulatory courtship is much longer than precopulatory courtship (see Introduction). Alternatively, pre- and post-copulatory courtship may be different in some subtle way, e.g., perhaps the male releases a chemical only during postcopulatory courtship, or perhaps courtship has to follow copulation. Which aspect of postcopulatory courtship is responsible for loss of receptivity and unattractiveness remains to be determined; physical or chemical stimuli are possibilities.

4.2 Source of unattractiveness and receptivity in other species

Which aspects of mating induce loss of receptivity appears to vary considerably among species. *S. cameroni* has a very similar lifestyle to *S. endius*; but mated females are only unreceptive, not unattractive (King, 2000). In contrast to *S. endius*, in *S. cameroni* loss of receptivity seems to commence with the closing of the female's genital aperture, even though males provide postcopulatory courtship (King, 2000).

Whereas postcopulatory courtship affected both receptivity and attractiveness in *S. endius*, in the parasitoid wasp *Aphytis melinus*, postcopulatory courtship appears to affect only receptivity. Postcopulatory courtship of a female does not reduce the chances that a second male will mount her; and in fact, it increases the second male's number of mounts prior to copulating, as well as the duration of his precopulatory courtship (Allen et al., 1994). However, postcopulatory courtship decreases her chances of copulating with the second male from 97 to 70%, due to her not opening her genital orifice even when mounted. In *A. lingnanensis* postcopulatory courtship decreases a female's chances of copulating with a second male from 40% to 8% (Gordh and DeBach, 1978).

Among nonparasitic Hymenoptera, in the bee *Centris pallida* postcopulatory courtship appears to make females unreceptive but not unattractive (Alcock and Buchmann, 1985). In contrast, in Dawson's burrowing bees, females become unreceptive after less than 15 s of intromission, probably corresponding to completion of sperm ejaculation, whereas normal intromission is longer than 1 min (Simmons et al., 2000). In contrast to *S. endius*, Dawson's burrowing bees are attractive to males if they have just mated, although no longer so by the time they begin nesting, which is hours or days later (J. Alcock, personal communication).

Copulation has also been shown to reduce receptivity in some other insects, with loss of receptivity being most studied in various fly species (reviewed in Ringo, 1996; Eberhard, 1996; reviewed for *Drosophila melanogaster* in Wolfner, 2002; Chapman et al., 2003). The aspects of copulation that have been shown to be responsible include seminal components from male accessory glands and/or the presence of sperm in a female's spermatheca, as well as stretching of the female's copulatory pouch by spermatophores (Obara et al., 1975). In sagebrush crickets, mated females' reduced receptivity to subsequent matings may result simply from satiation from nuptial feeding (Weddle and Sakaluk, 2003).

Which aspects of mating contribute to unattractiveness has been less well studied than which aspects contribute to loss of receptivity, except perhaps in *D. melanogaster* (reviewed in Tram and Wolfner, 1998). In *D. melanogaster* the unattractiveness (reduced courtship index) in the few hours immediately after mating results from different stimuli than the subsequent unattractiveness that persists for days. The stimulus for the initial short-term unattractiveness comes from males during the early part of copulation. The stimulus may partly be an antiaphrodisiac that is transferred from the male's to the female's cuticle and that she herself produces later. However, for the unattractiveness to persist, the female must have received sperm (Tram and Wolfner, 1998). In the solitary bee *Osmia rufa* unattractiveness results from the male rubbing his antiaphrodisiac from his sternites across the female's wings during postcopulatory courtship (Ayasse et al., 2000).

4.3 Persistence of unattractiveness

Having mated did not protect *S. endius* females from all attempts by males to mate. About 7% of fully mated females were fully mounted by males. However, this proportion did not change significantly as females encountered additional males. Thus, females do not appear to deplete their antiaphrodisiac supply by repulsing males. These results also demonstrate that females do not readily fatigue or habituate in repulsing males.

The number of males that a female can repulse appears not to have been studied in other species. In species where the unattractiveness is due to a pheromone on the female, loss of unattractiveness with successive male retreats is not expected. However, in *S. endius*, unattractiveness appears to require action on the part of the female, e.g., release of an antiaphrodisiac by the female when the male approaches, rather than just being on her cuticle (Bratzke, 2001; Fischer, 2003).

4.4 Conclusion

The present study suggests that in *S. endius* postcopulatory courtship functions not only as immediate mate guarding but also as extended mate guarding, by making the female both less attractive and less receptive to subsequent males even after the original male leaves. Extended mate guarding should be to that male's fitness advantage but it remains to be seen whether it also benefits the female. It might benefit her by reducing harassment from other males and/or it might cost her in decreased number or quality of sperm.

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Table 1

Description of the two treatments of interrupted females and of the control females; treatments were generated by exposing a female to part of the normal mating sequence with a virgin male.

Females Interrupted Before Copulation	Females Interrupted Before Postcopulatory Courtship	Control Females
male mounts	male mounts	male mounts
precopulatory courtship	precopulatory courtship	precopulatory courtship
female opens her genital aperture	female opens her genital aperture	female opens her genital aperture
male removed	copulation	copulation
	male removed	postcopulatory courtship
		male removed

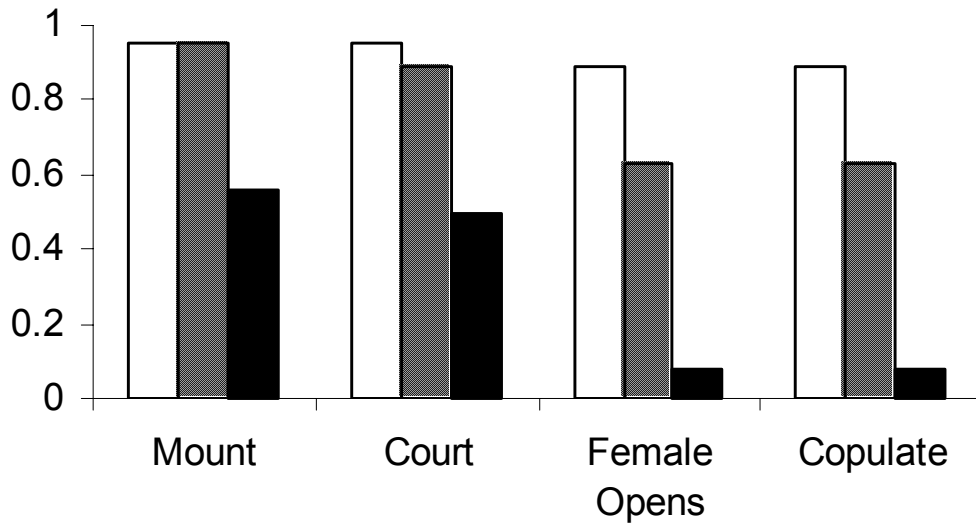


Fig. 1. Proportion of pairs in which mounting, precopulatory courtship, female genital opening and copulation occurred for different treatments of females:

□ female whose previous mating had been interrupted before copulation, i.e., she received precopulatory courtship and opened her genital orifice;

▨ female whose previous mating had been interrupted after copulation, i.e., she received precopulatory courtship, opened and copulated;

■ control female whose previous mating had been allowed to complete, i.e., she received precopulatory courtship, opened, copulated, and received postcopulatory courtship.

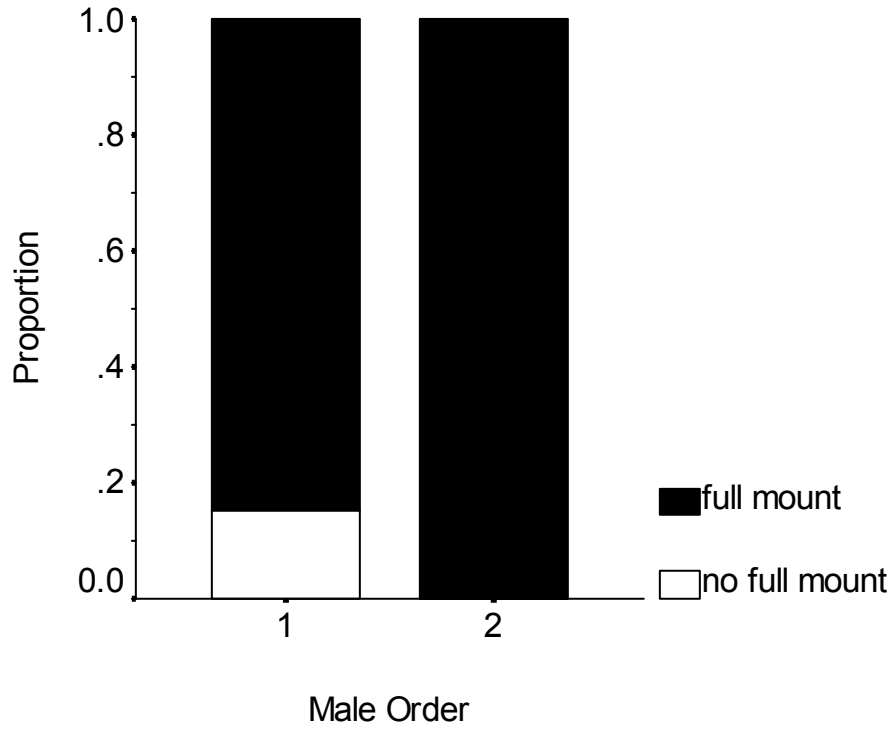


Fig. 2. Proportion of pairs in which the male fully mounted the female when a virgin female was presented with mated males in succession until she was fully mounted; number of pairs tested at each male order: 1st male (n = 20), 2nd male (n = 4).



Fig. 3. Proportion of pairs in which the male fully mounted the female when a mated female was presented with many mated males in succession; number of pairs tested at each male order: 1st - 7th male (n = 13), 8th male (n = 12), 9th - 12th males (n = 11), 13th - 15th males (n = 10), 16th - 19th males (n = 9), 20th male (n = 8), 21st - 22nd males (n = 7), 23rd male (n = 5), 24th - 25th males (n = 3). (Sample sizes decreases with order because the number of males available for testing varied among females.)

