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## **Inhibition of male sexual behavior after interacting with a mated female**

*Running head: Male sexual inhibition: behavioral hypotheses*

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### **Summary**

A male's reproductive success is generally highly dependent on his mating success. Nevertheless, there may be times during a male's life when his sexual responsiveness ebbs. In the parasitic wasp *Spalangia endius*, after a male mates, he shows a temporary decrease in his sexual responsiveness to even virgin females. We examined behavioral mechanisms for the decrease. 1) Decreased sexual responsiveness is not simply a result of habituation to attractive features of females during mating. If it was, males that had courted dead virgin females for substantially longer than during a normal mating should have been detectably less sexually responsive than males that had not courted, but they were not. 2) Decreased sexual responsiveness also is not a learned aversion to females that results from female brush-off, in which females use their hind legs to dislodge mounted males after copulation. Sexual responsiveness was reduced by mating regardless of brush-off. 3) Male *S. endius* are known to avoid mounting mated females, and surprisingly, the responsiveness of a virgin male decreased even when he had briefly approached and then retreated from an already mated female, i.e., even in the absence of mounting or copulation. This suggests that the aversive stimulus that causes males to retreat from mated females is also involved in the male's subsequent sexual inhibition.

*Keywords:* habituation; male mating experience; male mating history; parasitoid wasp; refractory period; sexual inhibition

### **Introduction**

Historically, males were depicted as the more interested and assertive sex when it came to mating (Darwin, 1871; Dewsbury, 2005). Sexual selection theory suggests why such a difference in the sexes might evolve (Bateman, 1948; Trivers, 1972). A greater expense of producing eggs relative to producing sperm results in a female's fitness being limited by the number of offspring that she can produce and a male's fitness being limited by the number of mates that he can obtain. This expectation may explain why decreases in sexual responsiveness among sexually mature individuals have been more often studied and reported in females (e.g., Huck & Lisk, 1986; review of insects by Ringo, 1996; Wedell, 2005) than in males (e.g., Yilmaz & Aksu, 2000; Gadenne et al., 2001; Sakai et al., 2007; Lehmann and Lehmann, 2007). Decreases in sexual responsiveness may occur in both sexes (Hoefler et al., 2011) and may be temporary or permanent. In males, decreases often occur when males have less to give females, or are perceived as having less to give females, e.g., less sperm, nutrients or manipulative seminal fluids (e.g., Markow et al., 1978; Dewsbury, 1982; Wedell & Ritchie, 2004). The proximal mechanisms leading to loss of sexual responsiveness of males are not well-understood in any animal (Barrozo et al., 2010).

In the parasitic wasp *Spalangia endius*, a male's sexual responsiveness decreases immediately after mating (King et al., 2005). Because the decrease has been demonstrated in tests with dead females as well as with live females, the effect must result from a change in the male, not just from females responding less to mated males than to virgins (King & Fischer, 2010). The decreased responsiveness appears to be a decrease in response to female odors (King, 2006). The decreased responsiveness to females is temporary, being no longer detectable by 5 min after mating (Fischer & King, 2008). The decrease appears to benefit the male by preventing him from

immediately trying to remate with the same female before she disperses in search of hosts (King, 2002; Fischer & King, 2008). Females rarely remate (King et al., 2005; King & Bressac, 2010). Thus his decreased responsiveness may decrease the chance that he spends time courting such an unreceptive female (King et al., 2005). This study examines several behavioral hypotheses for how his decreased responsiveness develops.

*S. endius* is a small wasp that parasitizes the pupae of certain fly species that develop in carrion, manure, or rotting vegetation (Rueda & Axtell, 1985). Hosts are usually patchily distributed. Males emerge before females (Napoleon & King, 1999) and linger in the host patch waiting for females to emerge (King personal observation). Sex ratios are female-biased at about 85% females (Napoleon & King, 1999). Males show no obvious aggression or dominance behavior toward each other (King, 2008).

A typical mating encounter in *S. endius* begins with the male contacting a female with his antennae, then briefly fanning his wings, often as he chases after her (King et al., 2005). Then the male mounts the female and begins to vibrate on her (court her). Following copulation, the female begins brush-off, in which she appears to try to dislodge the male by repeatedly stroking her hind legs up and over her dorsal abdomen, between her body and his, as he continues vibrating on her (postcopulatory courtship). Wing-fanning and mounting are responses to female-derived pheromones (Ruther et al., 2000; Nichols et al., 2010). Both virgin males and mated males almost always wing-fan and pursue females (King et al., 2005). However, the likelihood of mounting when a male reaches the female depends on her mating status. When he reaches a mated female, he often abruptly retreats without mounting (video at King, n.d.). This retreating appears to result from the female releasing an aversive pheromone upon contact or near contact by a male (King & Dickenson, 2008). Identity of the pheromone remains unclear. Having received postcopulatory courtship is the aspect of mating that makes mated females more repulsive than virgin females to males (King & Fischer, 2005). Postcopulatory courtship is the period of male vibration after copulation and prior to dismount.

To begin to uncover the behavioral mechanisms involved in a male's postmating decrease in sexual responsiveness, the present study investigated what types of experiences with females are sufficient to decrease male responsiveness.

**1) The habituation hypothesis** proposes that the postmating decrease in responsiveness results from habituation to attractive features of females during mating. Male attraction to dead females allowed us to test this. Males readily fan and mount dead females and often court them for longer than live females. If the habituation hypothesis is correct, then prolonged exposure to a dead female should be sufficient to cause a decrease in sexual responsiveness.

**2) The brush-off hypothesis** proposes that the postmating decrease in responsiveness results from female brush-off during mating. Whether brush-off is unpleasant for males is difficult to assess; however, brush-off does hasten a male's dismount (King, 2010). Brush-off might decrease subsequent male sexual responsiveness either directly or by becoming associated with attractive features of females. Brush-off can be prevented by using females whose hind legs have been removed.

**3) The aversive mated-female hypothesis** proposes that males become less responsive after exposure to an aversive stimulus from mated females. This hypothesis predicts that retreating from a mated female will be sufficient to decrease responsiveness even for virgin males.

**4) If retreating from a mated female is sufficient, is it also necessary, or is mating by itself sufficient?** The original study reporting a postmating decrease in sexual responsiveness of males did not control for whether males had only mated or also subsequently reapproached and retreated from their mates (King et al., 2005). These data are reanalyzed here controlling for that. These different hypotheses for male decreases in sexual responsiveness are not mutually exclusive e.g., the decrease might result from a combined effect of habituation and brush-off.

## Materials and methods

The *S. endius* were from a laboratory colony that was initiated in 1996 with wasps collected in Zephyr Hills, Florida (King et al., 2005). The colony was maintained on *Musca domestica* pupae. Parasitized pupae were placed singly in glass test tubes in order to isolate virgin adult wasps, which were fed honey on the day of emergence. When a mated wasp was needed, virgins were paired and observed until mating was complete. Clean containers were used for each test. Wasps were moved, including separating males and females after they interacted with each other, by tapping them out of their test tubes, rather than with forceps.

Each experiment involved a male receiving an initial experience with a female, followed immediately by a test of his sexual responsiveness (Table 1). Each female was used only once.

Except in experiment 2b (see below), the test of sexual responsiveness was essentially a race of the experienced male against another male, usually a virgin male, to see which male would respond more quickly to a female. The female was freshly dead and was placed at one edge of the test arena (3.4 cm diameter by 1.1 cm high). The two males to be tested were introduced from the opposite edge, and a separate observer kept track of each male. In each experiment, an experience is considered to have decreased sexual responsiveness if the experienced males lost to the virgin male more than half the time (tested with chi-square goodness-of-fit test within each experiment). This test protocol was chosen to match that first used to demonstrate a post-mating decrease in sexual responsiveness in *S. endius* (King et al., 2005). We cannot rule out the possibility that testing a male's responsiveness in isolation might have given different results. However, mated males exhibit lower sexual responsiveness than virgin males both in two-male tests (King et al., 2005) and when males are tested singly (King, 2006). Furthermore, because this species is highly monandrous, risk of sperm competition is nearly zero. Thus males are not expected to respond to a male's experience in order to avoid sperm competition.

Except in experiment 2b, during the test, we recorded which male was first to contact the female, first to mount completely, first to court her (vibrate), and first to attempt copulation. Occasionally one of the response variables could not be determined for a pair of males, so see Figures 1-3c for sample sizes for each variable. Replicates in which neither male exhibited any of these responses within 10 min have been excluded but were uncommon (2 of 174 replicates across experiments for which it was recorded). Only the first-to-mount data were formally analyzed; the data on which male was first to contact the female, first to court, and first to attempt copulation were not. This was done to match an earlier study (King et al., 2005) and because the male that is first to mount a female is usually also the first to contact, court, and attempt copulation (King et al., 2005; Figures 1-3c below).

Wasps were 0 – 2 d old, and each male was tested against a male that was the same number of days old and the same size to our eyes, although even very obvious differences in male size do not appear to affect mating success in this species (King & Napoleon, 2006). The males were assigned to treatments (e.g., virgin versus experienced) at random.

### *The habituation hypothesis*

**Experiment 1:** the experienced male was a virgin male that had just courted a dead virgin female. During a normal mating, a male is on the female for about half a minute, range 24 – 64 s (King, 2010). If the male had not dismounted the dead female after 5 min, his container was tapped to dislodge him from the female; whether he dismounted or had to be tapped off, and how long he was on the dead virgin female, were recorded. He was then tested against a virgin male.

### *The brush-off hypothesis*

**Experiment 2a:** If males that mate without brush-off are less responsive than virgin males, then brush-off is not necessary for decreased responsiveness, i.e., brush-off is at least not the only aspect of mating that contributes to postmating decrease in sexual responsiveness. The experienced male had just mated, but he had not received brush-off because his mate lacked hind legs. Prior to mating, her hind legs had been ablated using a sharpened insect pin after she had been immobilized on a cryolizer (a cylinder enclosing a cold-retaining medium that holds a

temperature of about  $-18^{\circ}\text{C}$ , BioQuip Products, Inc.) (following Edvardsson & Tregenza, 2005; King, 2010). She had been placed with the male for mating only after she recovered as evidenced by her walking. As they mated, any brush-off with the female's middle legs had been noted. He was then tested against a virgin male.

**Experiment 2b:** If mated males that received brush-off are subsequently less responsive than mated males that did not, then brush-off contributes to a postmating decrease in sexual responsiveness. In this experiment, all males had mated prior to testing; but half had mated with a hindlegless female, and half had mated with a middlelegless female (to control for any surgery effect on the vigor of mating), and whether the male received brush-off during this mating was recorded. The test setup was the same as in the other experiments, except that every male in both treatments had his responsiveness tested in the absence of another male. This was because we could not control precisely when a male got around to mating, and the first male's sexual responsiveness might return while we waited for a second male to mate. During testing, the duration until a male mounted the dead female was recorded. Duration until mounting was compared between males that had received brush-off and males that had not. This comparison was with survival analysis, specifically Cox's regression, with males that failed to mount within 10 min as uncensored events. Survival analysis accounts for the possibility that if allowed a longer testing period, some males that did not mount within the 10 min test period eventually might (review of survival analysis in van Alphen et al. 2003).

### *The aversive mated-female hypothesis*

**Experiments 3a and 3b:** the experienced male had interacted with an already mated female. To create such males, a freshly mated female was allowed to enter a test tube containing a virgin male, and they were observed until a clear interaction occurred. The type of interaction was recorded. Usually the male would fan the female, make contact, and then back away. However some males mounted, and in some cases then courted before dismounting; none copulated. In experiment 3a, testing was against a virgin male. In experiment 3b, testing was against a mated male; and for each pair of males, the mated male's mate had been used to give the experienced virgin male his experience.

**Experiment 3c:** the experienced male had interacted with a virgin female, and he was then tested against a mated male. This experiment, along with experiment 1, addressed whether the outcome in experiment 3b was a result of the experience being with a mated female rather than just any female. The interaction in experiment 3c was just fanning ( $N = 13$ ) or more than fanning ( $N = 40$ ): fanning and chasing, or in three cases the male then started to mount before they could be separated.

**Experiment 4** was simply a reanalysis of data from the original report that mated males are less responsive than virgin males in two-male tests. In this analysis, we only included a mated male if he had been removed from his mate before he had the opportunity to recontact and retreat from her ( $N = 60$ ). This is the last experiment described in King et al. (2005). In other words, this analysis examines whether mating by itself is sufficient to reduce a male's sexual responsiveness.

## **Results**

Results are summarized in Table 1.

### *The habituation hypothesis*

**Experiment 1:** Across all replicates, experience with a dead virgin female did not significantly decrease sexual responsiveness (Figure 1): 40% were first to mount, which was not significantly less than chance ( $\chi^2_1 = 2.12$ ,  $P = 0.15$ ). Duration of the experience, i.e., how long the male was mounted on the dead female, had not been significantly greater in replicates in which the experienced male lost (Mann-Whitney  $U = 379.00$ ,  $N_1 = 34$ ,  $N_2 = 23$ ,  $P = 0.81$ ). Most (36 of 57) experienced males had not dismounted voluntarily by the end of the 5 min on her. Restricting analysis to these 36 males, sexual responsiveness of experienced males was still not significantly decreased relative to virgin males: 47% were first to mount, which was not significantly less than chance ( $\chi^2_1 = 0.11$ ,  $P = 0.74$ ).

### *The brush-off hypothesis*

**Experiment 2a:** Brush-off was not necessary for a postmating decrease in sexual responsiveness. The experience of mating with a hind-legless female decreased sexual responsiveness relative to a virgin male (Figure 2): 21% of experienced males were first to mount, which was significantly less than chance ( $\chi^2_1 = 9.14$ ,  $P = 0.002$ ). On three occasions the hind-legless female touched the mated male with her middle legs while he was mounted, although it did not appear to be a typical brush-off. Conclusions are the same when these three males are excluded ( $\chi^2_1 = 9.00$ ,  $P = 0.003$ ).

**Experiment 2b:** Brush-off did not significantly contribute to the postmating decrease in sexual responsiveness. Whether a male had mated with ( $n = 19$ ) or without brush-off ( $n = 34$ ) had no significant effect on how quickly he mounted the test female (Cox's regression:  $\beta = 0.28 \pm 0.36$ ;  $\chi^2 = 0.63$ ,  $df = 1$ ,  $P = 0.43$ ) or whether he mounted (63% of brush-offed males and 71% of unbrush-offed males were mounted within 10 min ( $G = 0.31$ ,  $df = 1$ ,  $P = 0.58$ )). As in experiment 2a, conclusions are the same when middle-leg brush off cases ( $n = 2$ ) are excluded.

### *The aversive mated-female hypothesis*

**Experiment 3a:** Despite the absence of copulation, interacting with a mated female reduced a male's subsequent sexual responsiveness (Figure 3a): 30% were first to mount, which was significantly less than chance ( $\chi^2_1 = 8.32$ ,  $P = 0.004$ ). When the analysis was restricted to males that clearly had not fully mounted, i.e., the male simply retreated ( $n = 39$ ), the result was the same: 31% were first to mount, which was significantly less than chance ( $\chi^2_1 = 5.77$ ,  $P = 0.016$ ).

**Experiment 3b:** When males that had interacted with a mated female were tested against mated males, 46% of the former were first to mount, which was not significantly less than chance (Figure 3b;  $\chi^2_1 = 0.26$ ,  $P = 0.61$ ).

**Experiment 3c:** Interacting with a virgin female did not decrease male sexual responsiveness to the level of mated males (Figure 3c): 64% of such experienced males were first to mount, which was significantly more than chance ( $\chi^2_1 = 4.25$ ,  $df = 1$ ,  $P = 0.039$ ) and this (which male was first to mount) was independent of whether the experienced male had just fanned or more ( $\chi^2_1 = 0.05$ ,  $df = 1$ ,  $P = 0.82$ ).

**Experiment 4:** Reanalysis of the data from the original report of a post mating decrease in sexual responsiveness of males did not change the outcome. Mated males were less sexually responsive than virgin males even when the former had not subsequently approached and retreated from their mate after mating: 23% of mated males were first to mount, which was significantly less than chance ( $\chi^2_1 = 17.07$ ,  $P < 0.001$ ).

## **Discussion**

The habituation hypothesis and one of the two hypotheses involving rejection, the brush-off hypothesis, was not supported. The other rejection hypothesis, the aversive mated-female hypothesis, was supported. The habituation hypothesis was not supported because sexual responsiveness was not decreased in males that had courted a dead virgin female for considerably longer than would occur during a normal mating. This does not mean that *S. endius* males never habituate in any aspect of mating; in other animals, habituation varies with stimulus and context (e.g., Jenkins & Rowland, 2000; Griffith & Ejima, 2009). However, habituation does not seem to explain the postmating decrease in sexual responsiveness of *S. endius* males. The brush-off hypothesis was not supported in *S. endius* because sexual responsiveness was reduced after mating regardless of the presence or absence of brush-off.

Sexual responsiveness of males decreased even after simply approaching and retreating from a mated female. Mounting or copulation was unnecessary. Sexual responsiveness of these males was indistinguishable from that of mated males. This was not the case when the interaction had been with a virgin female. These outcomes are consistent with the aversive mated-female hypothesis. This suggested that the initially-reported decrease in responsiveness of fully mated males (King et al., 2005) might have the same cause. That is their decrease might be

related to males reapproaching, and then retreating from, their mate after dismounting, rather than from mating per se. However, this was not the case because the decrease in sexual responsiveness was observed even when the analysis was restricted to males that had been removed after dismounting but before they had time to reapproach the female. The decrease in sexual responsiveness of mated males may result from exposure to the same aversive that affects experienced males. In mated males this exposure may occur towards the end of mating, perhaps during postcopulatory courtship. It is during postcopulatory courtship that mated females gain the ability to repulse males (King & Fischer, 2005). Postcopulatory courtship lasts about 22 s.

Decreased sexual responsiveness in *S. endius* males could be just a response to exposure to the aversive by itself. For example, males may take time to recover from the aversive. The decreased sexual responsiveness of experienced males, and perhaps also mated males, could alternatively result from associative learning as has been suggested for *Drosophila melanogaster*. In *D. melanogaster* if a virgin male simply courts a mated female, his subsequent courtship of even virgin females will decrease (e.g., Siegel & Hall, 1979, Ejima et al., 2005). This phenomenon is referred to as courtship conditioning, and the mechanism has been relatively well studied (reviewed in Mehren et al., 2004; Siwicki et al., 2005). Males are thought to come to associate aphrodisiacs, which are present in both virgin and mated females, with aversive stimuli produced by mated females when males approach. Identifying the aversive stimuli has been difficult, with more than a dozen studies over more than 30 years (reviewed in Tram & Wolfner, 1998; Mehren et al., 2004; Ejima et al., 2007; Yew et al., 2009).

That the postmating decrease in sexual responsiveness in *S. endius* is related to an aversive stimulus from mated females is further suggested by data on two confamilials. In *Nasonia vitripennis* and *Urolepis rufipes*, males do not exhibit decreased sexual responsiveness after mating (King unpublished data). Males also mount mated females as readily as virgin females, suggesting that mated females in these two species lack an aversive.

The present study primarily used a binomial measure of responsiveness. This was also used to show the initially reported decreased responsiveness of mated males (King et al., 2005). This difference in responsiveness can also be seen in mated males being less restless (i.e., spending less of their time locomoting) and their paths being less direct (King & Owen, in press).

Knowing whether male postmating sexual inhibition is common waits testing in more species. Mating does not always decrease male sexual responsiveness. Courting a mated female decreases a male's subsequent courtship of other females in *D. melanogaster*, but completed mating does not. Specifically, after mating twice, *D. melanogaster* males initiate courtship just as quickly as, and court for as long as, virgin males (Markow et al., 1978). In Caribbean fruit flies and in the spider *Nesticodes rufipes*, recent mating even increases a male's sexual responsiveness (Teal et al., 2000; Molina & Christenson, 2008). In *D. melanogaster*, the effect of having courted an immature conspecific sometimes depends on the duration of that courtship. For example, Dukas (2010) found that having courted females for 30 min increases male courtship of subsequent immature females, whereas having courted for 60 min decreases subsequent courtship. Even experience with a heterospecific of the opposite sex can affect subsequent mating behavior (e.g., *Drosophila* (Dukas, 2004, 2008), guppies (Magurran & Ramnarine, 2004), Syrian hamsters (delBarco-Trillo et al., 2010)). Often rejection or lack of success results in decreased response by males to the species that rejected them (Dukas, 2004, 2008, 2009; Kujtan & Dukas, 2009). However, sometimes decreased male sexual response after exposure to a female is specific to individual females (e.g., LaDage and Ferkin, 2006; Valero et al., 2009).

The present study focused on behavioral mechanisms. The physiological basis of postmating sexual inhibition remains to be examined in *S. endius* and has been examined for males in relatively few animal species (e.g., Prado et al., 2003; Sakei et al., 2007). Even in rats and humans, despite many studies, the mechanisms of post ejaculatory refractory time in males are considered poorly understood (Levin, 2009; Phillips-Farfán & Fernández-Guasti, 2009). Among insects, the physiological basis of postmating sexual inhibition has been most studied in the cricket *Gryllus bimaculatus* and the moth *Agrotis ipsilon*. In the cricket *G. bimaculatus*, male sexual inhibition after mating is partly due to a timer in ganglia of the posterior abdomen (Ureshi & Sakai, 2001; Ureshi et al., 2002). In *A. ipsilon*, after mating a male ceases to be attracted to female sex pheromones (Gadenne et al., 2001). His antennae are still as receptive to the pheromones; however, neurons in the olfactory part of his brain become less sensitive to the pheromone (Barrozo et al., 2010). At the same time, males become more sensitive to plant odor (Barrozo et al., 2011).

Although much remains to be learned about the effects of earlier intersexual experiences on subsequent sexual responsiveness of males in both vertebrates and invertebrates, the present study contributes to a growing literature on this topic and on the effects of past rejections on subsequent attempts.

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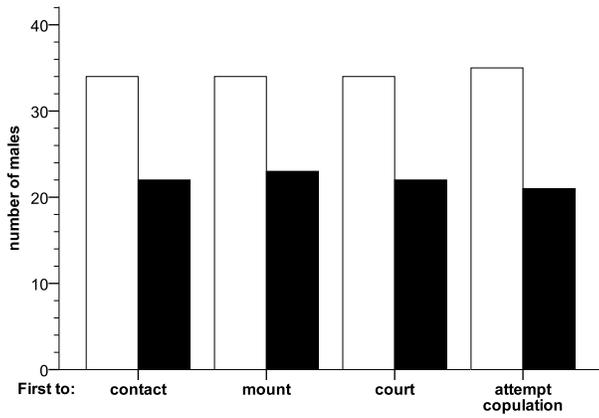
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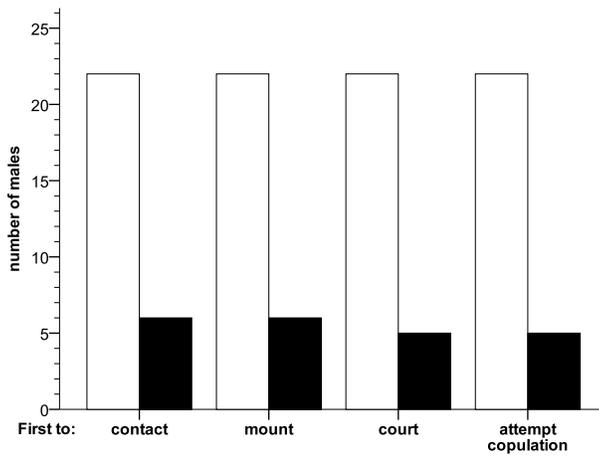
Table 1. Summary of experiments (e♂ = experienced male)

Experiment	e♂	e♂ then tested against:
1	virgin courted a dead v♀	virgin ♂
2a	mated with no brush-off	<b>virgin</b> ♂*
2b	mated with no brush-off	
	mated with brush-off	
3a	virgin interacted with a mated ♀	<b>virgin</b> ♂*
3b	virgin interacted with a mated ♀	mated ♂
3c	virgin interacted with a virgin ♀*	<b>mated</b> ♂
4	mated, but no recontact of ♀	<b>virgin</b> ♂*

\*1<sup>st</sup> to mount significantly more often than other male in that experiment (*P* values in text of Results)

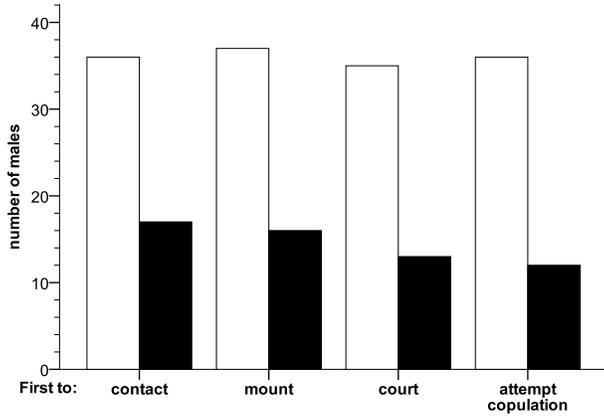


**Fig. 1** Each experienced male (■, had previously courted a different dead virgin female) was tested against a virgin male (□) to see which was first to contact, first to mount, first to court (vibrate on), and first to attempt copulation.

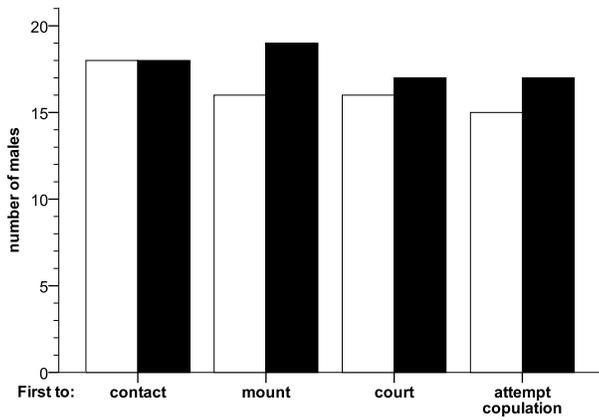


**Fig. 2** Each experienced male (■, had mated a hindlegless female) was tested against a virgin male (□) to see which was first to contact, first to mount, first to court (vibrate on), and first to attempt copulation.

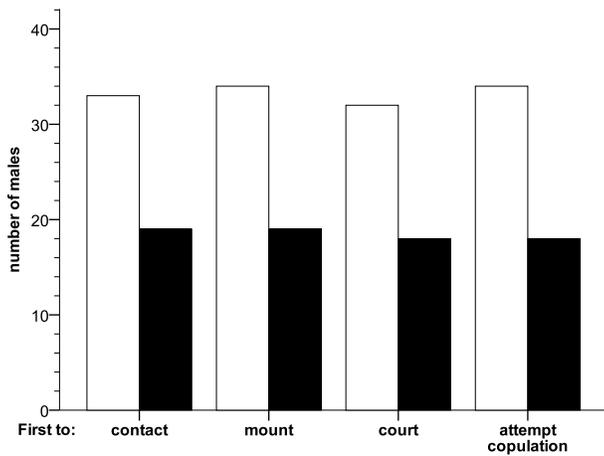
(a) experience was with a mated female, testing was against a virgin male



(b) experience was with a mated female, testing was against a mated male



(c) experience was with a virgin female, testing was against a mated male



**Fig. 3.** Each experienced male (■) was tested against another male (□) to see which was first to contact, first to mount, first to court (vibrate on), and first to attempt copulation