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1-1-2015

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Original Citation

Substrate-borne Marking in the Parasitoid Wasp *Urolepis rufipes* (Hymenoptera: Pteromalidae)

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ABSTRACT Many animals use pheromone marking as a way to identify their territory or other resources. Among insects, substrate-borne marking is frequently reported for females, which in many species make marks containing oviposition deterring pheromone, which other females avoid. However, there are fewer reports of substrate-borne marking for males. Here marking in males of the parasitoid wasp *Urolepis rufipes* (Ashmead) is described. The conditions under which males mark and whether males and females respond to the males' marks were examined using behavioral observations. Males marked by dragging the tips of their abdomens across a substrate. They marked much more after mating and after consuming honey. They also marked more when with a female irrespective of copulation, although not when with a male. Females spent more time on or near marked substrates, and males also responded to their own marks. Although males aggressively and successfully defended areas that they had marked against other males, males did not respond to another male's marks in the conspecific's absence. In contrast to males, females did not mark, either on the surface of hosts or on other surfaces; and males showed no detectable response to surfaces which females had recently occupied.

KEY WORDS marking, parasitoid, pteromalid, substrate-borne, *Urolepis*

Pheromone marking is one way that animals identify their territory or other resource and keep other animals away. Among insects, most studies of marking have been of females (Shelly 2004). For example, in some insects, after oviposition, the female marks the host with oviposition-deterring pheromone to prevent successive oviposition by conspecific females (Nufio and Papaj 2001). By responding to the marks, a female can avoid her offspring being crowded during development, which might reduce their size and survival. Among parasitoid wasps there are numerous examples of females marking their hosts with oviposition deterring pheromones (Nufio and Papaj 2001, Mehrnejad and Copland 2007, Stelinski et al. 2007). However, there are fewer reports of females leaving behind substrate-borne pheromones to which males respond (Fauvergue et al. 1995, Pompanon et al. 1997, Bernal and Luck 2007) or of substrate-borne marking by males (van den Assem et al 1980, Ruther et al. 2009). Whether the relative scarcity of the latter two types of marking reflects their being uncommon or their not having been thoroughly studied is unclear. However, in one of the most studied parasitoids, *Nasonia vitripennis* (Walker), which has been referred to as the *Drosophila* of the Hymenoptera (Pultz and Leaf 2003), males make substrate-borne marks with the tips of their abdomens, and these marks contain pheromones that attract female and male conspecifics (Barrass 1969, Ruther et al. 2007, 2011; Steiner and Ruther 2009). The present study examines whether another parasitoid wasp, *Urolepis rufipes* (Ashmead), also exhibits substrate-borne marking behavior, whether both sexes mark or just males, and whether females and males respond to the markings.

Urolepis is in the same family as *Nasonia* and closely related (Burks 1979, Burks 2009, McAllister and Werren 1997). However, *Urolepis* has been relatively little studied, especially behaviorally, although *U. rufipes*

began to receive some attention as a potential biological control agent after being found in association with livestock production (Smith and Rutz 1985, Gibson 2000, Floate and Skovgård 2004). Close relatedness to *Nasonia* would predict similarity in marking behavior; however, their differences in habitat could have selected for differences. Both parasitize the pupal stage of certain fly species, although not the same species (Rueda and Axtell 1985). *Nasonia* are found in association with carcasses and bird nestlings, as well as in poultry and livestock manure; whereas *U. rufipes* appear to have recently made a transition from a very aquatic existence to a more terrestrial one (Smith and Rutz 1985, Gibson 2000). Brine flies (Ephydridae), which have aquatic larvae and semi aquatic adults, are thought to be the original natural hosts of *U. rufipes* (Petersen et al. 1985). However, *U. rufipes* has also been reported from certain synanthropic fly species such as house flies, *Musca domestica* L., and stable flies, *Stomoxys calcitrans* (L.) (Rueda and Axtell 1985). House fly and stable fly pupae are found in manure or rotting vegetation, whereas brine fly pupae are along shores (e.g., Collins 1980). The history of *U. rufipes* with brine flies may explain why, in synanthropic habitats, they are found in somewhat wetter microhabitats than are other closely related parasitoid wasp species (Smith and Rutz 1985). Gibson (2000) even suggests that their hairiness may be to trap air when searching under water for brine flies.

In *U. rufipes* usually only one adult wasp develops per host. However, hosts can be numerous and sometimes highly clumped (e.g., Collins 1980, King 1990). Males begin emerging from their hosts as adults a day or two before females (Powell et al. 2003), and sex ratios are generally female-biased (Stenseng et al. 2003). Females mate only once in many situations but will sometimes mate with a second male if he is encountered simultaneously or very soon after the first male (Kuban and King 2012, Cooper et al. 2013). Males are polygynous.

The present study used behavioral observations of *U. rufipes* to 1) describe male marking, 2) identify the conditions under which males mark and 3) determine whether male and female conspecifics respond to marked substrates. Specifically, does the presence of a conspecific, a male's own mating status or the consumption of food or water affect the number of marks that a male makes? Does a female's mating status affect the number of marks that a male makes? Are females and other males attracted to marked substrates, and are males attracted to their own marks? Do males defend marked areas against intruding males? In addition, we also examined whether females mark and how males respond to substrates that were previously exposed to females.

Materials and Methods

General Methods

The *U. rufipes* were a Canadian strain that originated from cattle feedlots in southern Alberta. The wasps were maintained using a natural host, *M. domestica* (house fly) pupae, which were reared following the methods of King (1988), except pine shavings were used instead of vermiculite in the fly media. Pine shavings may facilitate fly pupae production by introducing air spaces (Boire et al. 1988, Machtinger et al. 2014). Parasitized hosts were individually isolated in glass test tubes prior to the wasps' emergence in order to obtain virgin wasps. Wasps were 0-1 day old from emergence at testing. Mated wasps were obtained by placing a virgin male and a virgin female together and observing copulation. Each wasp was only used once and was given honey prior to the experiment unless noted otherwise. Behavioral data were collected by direct observation, except the Observations of Females with Hosts was videotaped.

Analyses were with PASW Statistics (2009). Each experiment included two treatments. When different wasps received different treatments, means were compared by independent *t*-test (Zar 2010). When each wasp had received both treatments, comparison was by paired *t*-test. The exceptions were that when assumptions of normality were strongly violated ($P < 0.001$), nonparametric equivalents were used instead, Mann-Whitney *U* tests in place of independent *t*-tests and sign tests in place of paired *t*-tests. Tests of categorical data were by chi square tests. Two-tailed *P*-values are given throughout.

Male Marking Conditions

Effect of Conspecifics. This experiment examined whether males mark more in the presence of another male than when alone, whether males mark more in the presence of a female, and whether her mating status

mattered. A focal virgin male was observed either: 1) alone, 2) with a virgin female, 3) with a mated female or 4) with a virgin male, $n = 20$ per treatment. The wasps were placed in a sand dish, which was a small blue plastic dish (1.5 cm diameter, 1 cm height) three-fourths full of sand with a glass cover slip as a lid. For 10 min the number of total marks that the focal male made was noted as was any female marking. Perhaps because wasps exhibit negative geotaxis, i.e., going up until they reach a lid, marks were almost exclusively on the lid; wasps were never observed trying to mark the sand.

To improve statistical power and because we were interested in particular comparisons, planned comparisons were used rather than doing an overall analysis followed by post hoc comparisons (Ruxton and Beauchamp 2008). First, number of male marks was compared between the alone treatment and the with-another-male treatment. Then the virgin versus mated female treatments were compared. Because neither of these comparisons was statistically significant, the two treatments within each comparison were then pooled and the two treatments without females were compared to the two treatments with females.

Effect of Male's Own Mating Status. This experiment examined whether a male's prior mating status affects the number of marks that he makes. A mated male ($n = 15$) and a virgin male ($n = 15$) were each placed in separate sand dishes for 24 h. The number of marks was recorded at the end of: 10 min, 30 min, 60 min, 2 h, 4 h and 24 h.

Effect of Honey. This experiment tested whether fed males ($n = 15$) mark more than unfed males ($n = 15$). In the fed treatment, each virgin male was given a small dot (about 3 mm in diameter) of honey in a test tube (12 mm diameter, 75 mm height) plugged with cotton and let sit for 1 h. After 1 h with or without honey, each male was allowed to mate with a virgin female and then placed in a sand dish. The number of marks was recorded 24 h later. The honey had been dyed green with food coloring to test whether food subsequently showed up in male marks. No male completely consumed the honey; however, all fed males consumed honey as evidenced by the greenness of their fecal matter.

Effect of Water. This experiment tested whether males that consume water then mark more. Set up was identical to the previous experiment except males had either consumed water that had been dyed green ($n = 15$) or were not given water ($n = 15$).

Female Response to Male Marks

This experiment examined whether virgin females respond to substrates marked by mated males. To generate marks, a mated male was placed in a sand dish for 24 h. The cover slip (22 mm^2) was then removed, and the number of marks on it was recorded. Cover slips are referred to as "exposed" even if the male did not mark them; however, all but 3 of 44 exposed cover slips were marked. To test female response, each test female ($n = 44$) was presented with an exposed and an unexposed (control) cover slip side by side, with about 8 mm between them. The cover slips were attached to the underside of the lid of a medium sized petri dish (3.4 cm diameter, 1.1 cm height) with drops of water. The exposed cover slip was placed on alternate sides of the dish between tests. The test female was added to the dish and observed for 10 min. The total time in contact with each cover slip was recorded. Contact is defined as having at least half of the body on the cover slip.

Preference was calculated as the difference between (total time on the exposed cover slip) and (total time on the control cover slip). If a sign test showed that time on the exposed slip was greater than time on the control slip, a preference was considered present. To test for an effect of number of marks on preference, preference was regressed against the number of marks on the exposed cover slip (Zar 2010).

Male Response to Male Marks

Male Response to Self, to Another Male. This first male-response experiment examined whether mated males respond to their own marks ($n = 22$) and whether they respond to marks made by another mated male ($n = 22$). The set-up was the same as in the female response experiment. In the self treatment, the male was placed in a test tube while the test area was prepared, i.e., in between letting him mark a cover slip and testing him with it.

Defense Experiment. This experiment examined whether a male will defend a previously occupied substrate against another male. The set-up was similar to the previous experiment except the test arena did not include a control cover slip, and the exposed cover slip was on the bottom, not the lid, of the dish. There were

two treatments ($n = 22$ per treatment), a one male treatment and a two-males treatment. For both treatments, a marked cover slip was generated by placing a mated male in a sand dish for 24 h. The cover slip, with the male still on it, was then removed and placed in the center of an arena. For the two-males treatment, a second mated male was introduced. In both treatments, the first male in, that is the male that had been allowed to mark the slip prior to observations, is referred to as the first-male.

Then for 10 min, the amount of time that each male was in contact with the cover slip and the number of times each male contacted the cover slip was noted, as was whether the second-male ever contacted the cover slip and whether aggression by the first-male seemed to prevent the second-male from contacting the cover slip. For each measure, first-males were compared between the one male treatment and the two-males treatment, and first-males and second-males were compared within the two-males treatment.

Male Response to Substrate Visited by a Female

This experiment examined whether females mark or otherwise leave a scent behind that is attractive to virgin males. The set-up was the same as in the first male-response experiment with cover slips, except the exposed cover slip had been exposed to a virgin female and the response of a virgin male was tested ($n = 22$).

Observations of Females with Hosts

This experiment examined whether females mark hosts after drilling into them. Prior to observations, each mated female was given 20 hosts for 24 h in the hopes of increasing the chance that she would parasitize hosts during observations. Then she was transferred to a sand dish with one unparasitized host laid horizontally on the surface of the sand with spiracles exposed. The dish was videotaped for 6 h ($n = 10$ females, each on a different day). Tapes were then observed for any instances of marking behavior and of completed drilling into the host.

Results

Male Marking Conditions

Effect of Conspecifics. Marking by *U. rufipes* males resembled that of *N. vitripennis* males as described by Barrass (1969). The male bent the tip of his abdomen under his body as he walked forward, and this left behind a whitish streak.

The number of marks that a male made did not differ significantly if the male was alone or if he was with another male (Fig. 1; Mann-Whitney $U = 45.00$, $n_1 = 20$, $n_2 = 20$, $P = 0.74$). The number of marks also did not differ depending on whether he was with a virgin or a mated female (Mann-Whitney $U = 42.50$, $n_1 = 20$, $n_2 = 20$, $P = 0.58$). However, males made more marks when with females than when not with females (i.e., than when alone or with another male) (Mann-Whitney $U = 49.30$, $n_1 = 40$, $n_2 = 40$, $P = 0.001$). Likewise, the percent of males that did not mark at all was greater in the treatments without females compared to the treatments with females (75% versus 43%; chi-square test of independence: $\chi^2 = 8.72$, df $= 1$, $P = 0.003$). None of the females marked. All males started the 10 min marking observation period as virgin, but during observations, 90% of the males mated in the virgin female treatment and none of the males mated in the mated female treatment.

Effect of Male's Own Mating Status. A difference in the number of marks that mated males made versus the number that virgin males made was statistically detectable by 1 h (Table 1; Mann-Whitney $U = 56.50$, $n_1 = 15$, $n_2 = 15$, $P = 0.019$) and was still detectable at the end of 24 h (independent t-test: $t = 2.22$, df $= 28$, $P = 0.035$).

Effect of Honey. Males that were fed honey made significantly more marks (Fig. 2; independent t-test: $t = 3.29$, df $= 28$, $P = 0.003$). The green dye showed up in marks of all males that had been fed honey.

Effect of Water. Consuming water did not significantly increase the number of marks (Mann-Whitney $U = 73.00$, $n_1 = 15$, $n_2 = 15$, $P = 0.11$).

Female Response to Male Marks

Virgin females showed a preference for, i.e., spent significantly more time on, the cover slip that had been exposed to a mated male compared to the control cover slip (Fig. 3; sign test $P = 0.003$). Female preference for the exposed cover slip was not significantly related to the number of marks on the exposed cover slip ($R^2 < 0.001$, $F < 0.001$, df $= 1, 42$, $P = 1.00$).

Male Response to Marks

Male Response to Self, to Another Male. Response to a self-marked substrate is shown in Fig. 4. When the exposed cover slip had his own marks, the male showed a preference for it (i.e., spent more time on it) relative to the control (sign test $P = 0.035$); and the magnitude of the preference (y) was greater when there were more marks (x) on it ($R^2 = 0.25$, $F = 6.80$, df $= 1, 20$, $P = 0.02$; $y = 5.26x - 14.66$).

Response to another male is shown in Fig. 5. When the exposed cover slip had another male's marks, males did not spend significantly more time on the exposed cover slip or on the control (paired t-test: $t = 0.39$, df $= 21$, $P = 0.70$); and there was no significant relationship between the magnitude of the preference (y) and the number of marks (x) on the exposed cover slip ($R^2 = 0.017$, $F = 0.34$, df $= 1, 20$, $P = 0.56$).

Defense Experiment. There was no evidence that a cover slip being more heavily marked made the second-male avoid it more. Specifically, there was no significant relationship between total time that the second-male spent on the cover slip and the number of marks that the first-male had made on it prior to observations (Spearman rank correlation: $r_s = 0.26$, $n = 22$, $P = 0.24$), and second-males were not slower to contact cover slips that started with more marks ($r_s = -0.10$, $n = 22$, $P = 0.67$).

First-males that had made more marks did not spend more time on their own cover slips than males that had made fewer marks (one male treatment: Spearman rank correlation: $r_s = -0.04$, $n = 22$, $P = 0.86$; two males treatment: $r_s = -0.21$, $n = 22$, $P = 0.34$). Comparing the first-male in the one male treatment versus two male treatment, there was no significant effect of the second-male's presence on the first-male's total time on the cover slip or the number of times that he contacted the cover slip (Table 2; Mann-Whitney $U = 224.0$, $P = 0.67$; $t = 1.71$, df $= 42$, $P = 0.10$).

First-males showed aggression toward second-males. In many of the trials, the male on the cover slip lunged at the new male and chased him away from the cover slip. Some of the first-males left contact with their cover slips; however, these males did not go far from the cover slip. Several second-males contacted the cover slip; however, they only did this when the first-male was not on the cover slip. These behavioral observations suggest that the first-male's presence kept the second-male from the cover slip. In the two males treatment, the second-male spent significantly less time on the cover slip than the first-male did (Table 2; sign test $P = 0.002$) and contacted the slip less often, about four times less (paired t-test: $t = 7.32$, df $= 21$, $P < 0.001$). This pattern is probably not explained by the fact that first-males, but not second-males, started the observation period on the cover slip. The reason this explanation is insufficient is that the amount of time that a second-male spent on the exposed cover slip in this experiment was also considerably less than for males exposed to another male's cover slip even in the absence of another male (this experiment versus the another-male treatment in the Male Response to Self and to Another Male experiment above: 3.64 ± 1.66 s, $0 - 31$ s versus 81.18 ± 31.36 s, $0 - 532$ s; Mann-Whitney $U = 158.0$, $n_1 = 22$, $n_2 = 22$, $P = 0.03$).

Male Response to Substrate Visited by a Female

The females did not leave any visible marks on the cover slips, and males showed no obvious response to female cover slips. Males did not spend significantly more time on the female cover slip than the control (sign test $P = 1.00$) and did not contact the female cover slip significantly more often than the control cover slip (paired t-test: $t = 0.44$, df $= 21$, $P = 0.67$).

Observations of Females with Hosts

Within the 6 h observation period, 60% of females host-drilled at least once, and 20% host-drilled twice. None of the females showed any behavior indicative of their marking the host, i.e., none dragged their abdomen across the host.

Discussion

In *U. rufipes*, male marking seems to function in intersexual communication and in self navigation, but not in intrasexual communication. Males marked even without having fed and without having mated. However, males marked more when they were with females, when they were mated and when they had been given honey. In all of these cases, the effect on number of marks was large.

The increased marking by fed males suggests that marking is energetically expensive or makes use of a component of honey. Marks by *U. rufipes*, like those of *N. vitripennis*, contain traces of what they ate (Barrass 1969). In *N. vitripennis*, the effect of the adult male's diet on amount of marking has not been examined. However, the larval diet of a male, i.e., of his host, affects the amount of pheromone that he produces and releases, as well as his sperm production and attractiveness to females (Blaul and Ruther 2011).

In contrast to the effect of a female's presence, males did not mark more when with another male. That males responded to females but not to other males by increased marking makes sense given that females, but not other males, responded to marked surfaces. Marking more after encountering a female may increase a male's chances of attracting that female. Furthermore, the clumped nature of *U. rufipes*' hosts means that multiple females are likely to emerge in the same location; thus if a male has encountered one female, other females may be nearby. The present study measured marks. However, it should be noted that a male sometimes extrudes his aedeagus into the air in temporal association with his marking (personal observation). Whether this releases pheromone and whether it adheres to substrates and contributed to the responses to marked substrates that were seen in the present study is unclear. Responses to marked substrates are responses to pheromones in *N. vitripennis* (Steiner and Ruther 2009, Ruther et al. 2011).

Although male *U. rufipes* showed no apparent response to substrates marked by other males, males appeared able to detect marks in that they responded more to their own marked surfaces. This suggests that males may use marking as a means of returning to a site, e.g., one in which they have encountered a female. Males also stayed longer on substrates on which they had put more marks, although only when the experimental design involved their leaving and then reencountering the substrate (Mated Male Response to Self and to Another Mated Male experiment), not when the experimental design involved their having never left the substrate (Defense experiment). Marking as a means of relocating a resource may be especially helpful in species like *U. rufipes* that exhibit male-male aggression, where a male may run off in an unexpected direction to chase off another male (van den Assem et al. 1980).

The male substrate-marking behavior seen in *U. rufipes* appears to be similar to that of the well-studied confamilial *N. vitripennis* in form, in context, and in the function of attracting females. Specifically, like in *U. rufipes, N. vitripennis* males mark more after interacting with a female (Steiner and Ruther 2009), and virgin females are attracted to marked substrates (Ruther et al. 2009, Steiner and Ruther 2009). In contrast to *U. rufipes*, in *N. vitripennis* a male's marks are reported to be attractive not only to himself but also to other males (van den Assem et al 1980, van den Assem 1986, Ruther et al. 2011).

Marking to attract females may be an effective male mating strategy because it allows a male to attract additional females while still defending a host from which a female is in the process of emerging. Chewing out of a host can take an hour in a related parasitoid wasp species that also parasitizes house fly and stable fly pupae (King 2006).

In *U. rufipes*, being with a female increased male marking regardless of female mating status. There is little benefit to attracting a mated female because such females are rarely receptive (King, unpublished data). However, marking after encountering a mated female may still be beneficial to males if the presence of a mated female is generally associated with virgin female(s) also being present. Whether such an association occurs is not yet known. Mated *N. vitripennis* females are not attracted to the major chemical component in the marks (Ruther et al. 2007). Response of mated females to marked substrates has not yet been examined in *U. rufipes*, but given their lack of receptivity, they would not be expected to respond. The degree of interaction with a female that is needed to trigger increased marking in *U. rufipes* remains to be tested. Since males marked even when they were with a mated female and did not copulate, copulation is not necessary. Perhaps female pheromones trigger males to mark.

Responding to marked substrates may benefit females by helping them find a mate quickly, so they have more time to search for hosts. Females did not respond to the number of marks that males made, under the

conditions tested; whether they respond to quality remains to be explored. In *N. vitripennis*, females respond to both quantity and quality of pheromone, with the result that they are attracted to marks of mature virgin males, larger males, and males that developed on hosts that had a higher quality diet, all of which are indicative of males with more sperm (Ruther et al. 2009, Blaul and Ruther 2011, 2012).

 U. rufipes females showed no evidence of marking their hosts externally. Bosque and Rabinovich (1979) suggest that external marking of hosts is relatively unusual among parasitoids of host stages other than eggs, in part because such hosts tend to be less accessible. The hosts of *U. rufipes* are often buried and often in moist substrates (Smith and Rutz 1985, Gibson 2000). Tests of what cues females use to avoid superparasitism suggest that external host cues are not used by other confamilials whose hosts include *M. domestica*, such as *N. vitripennis* (King and Skinner 1991), *Muscidifurax zaraptor* Kogan and Legner (McKay and Broce 2004) and *Pachycrepoides vindemiae* Rondani (Goubault et al. 2004). The confamilial *Dinarmus basalis* (Rondani) parasitizes bruchid beetle larvae hidden within cowpea seeds, which seem accessible, yet females do not appear to mark seeds (Gauthier et al. 2004). Gauthier et al. (2004) suggest that in this case, marks might be costly by increasing attraction of another parasitoid species whose larvae are better competitors. Host marking by females has been documented in the pteromalid *Halticoptera laevigata* Thomson; females parasitize a tephritid larva in a honeysuckle fruit and mark the fruit (Hoffmeister and Roitberg 1997).

 U. rufipes females also showed no evidence of substrate marking to attract males; males did not respond to substrates that a female had previously occupied. Van den Assem et al. (1980) suggest the same is true of *N. vitripennis*, although formal data were not provided. In contrast, males respond to substrates that were previously visited by females in several parasitoid wasps in other families, *Aphelinus asychis* (Walker), *Trichogramma brassicae* (Bezdenko) and *Aphytis melinus* (DeBach), although visible marks and obvious marking behavior were not reported (Fauvergue et al. 1995, Pompanon et al. 1997, Bernal and Luck 2007).

 In summary, the present study showed that in *U. rufipes,* substrates marked by males are attractive to females, and males mark most often after mating, both of which have also been shown in the closely related and well-studied species *N. vitripennis*. In *U. rufipes,* male marks also appear to be attractive to the male that made the marks, although not to other males.

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Table 1. Mean ± SE (minimum – maximum) number of marks, and the number of males that did not mark, at the end of each time period

	Number of marks by virgin males	Number of marks by mated males	Number of nonmarking virgins	Number of nonmarking mated males
10 min	0.20 ± 0.16 (0- 2)	0.33 ± 0.13 (0 – 1)	10	13
30 min	0.33 ± 0.16 (0 - 2)	0.93 ± 0.27 (0 - 3)	7	11
1 h	0.40 ± 0.19 (0 - 2)	1.93 ± 0.60 (0 - 9)	5	11
2 h	0.67 ± 0.25 (0 - 3)	2.27 ± 0.69 (0 - 9)	5	9
4 h	1.27 ± 0.30 (0 - 4)	3.00 ± 0.82 (0 - 11)	4	4
24 h	3.27 ± 0.51 (0 – 7)	6.33 ± 1.28 (0 - 15)	2	1

n = 15 mated males; *n* = 15 virgin males

Table 2. Time (s) that each male spent on the cover slip and the number of times that each male contacted the cover slip in the defense experiment

Treatment	Male[a]	*n*	Time (s) Mean ± SE (Min – Max)	Number of Contacts Mean ± SE (Min – Max)
1 male	first-male	22	129.60 ± 0.17 (1.00 – 592.00)	1.90 ± 0.21 (1.00 – 4.00)
2 males	first-male	22	112.20 ± 0.17 (2.00 – 600.00)	2.50 ± 0.27 (1.00 – 5.00)
2 males	second-male	22	3.60 ± 0.11 (0.00 – 31.00)	0.55 ± 0.18 (0.00 – 3.00)

[a]Each first-male was with a cover slip that he had previously had 24 h to mark; and then for the 10 min period of observation and data collection, he was either kept alone with that slip (1 male treatment) or a second-male was introduced (2 males treatment).
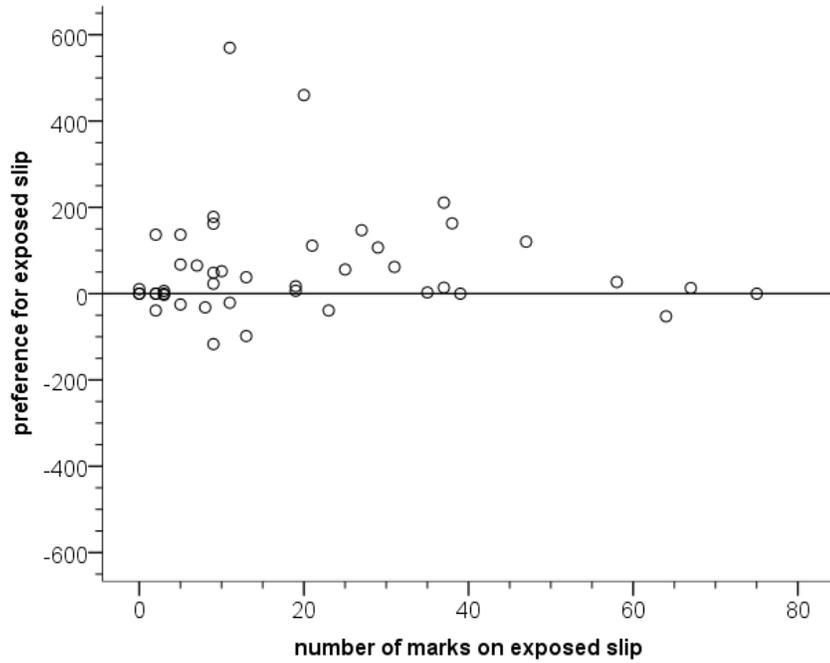
Fig. 1. Number of marks by males in 10 min when alone, with a virgin female, with a mated female or with a virgin male.

Fig. 2. Number of marks by mated males in 24 h when given honey versus no honey for 1 h prior to testing.

Fig. 3. Female preference for a cover slip that was exposed to a male previously (i.e., time on the exposed cover slip – time on the control cover slip) versus the number of marks on the exposed slip. 0 indicates no preference.
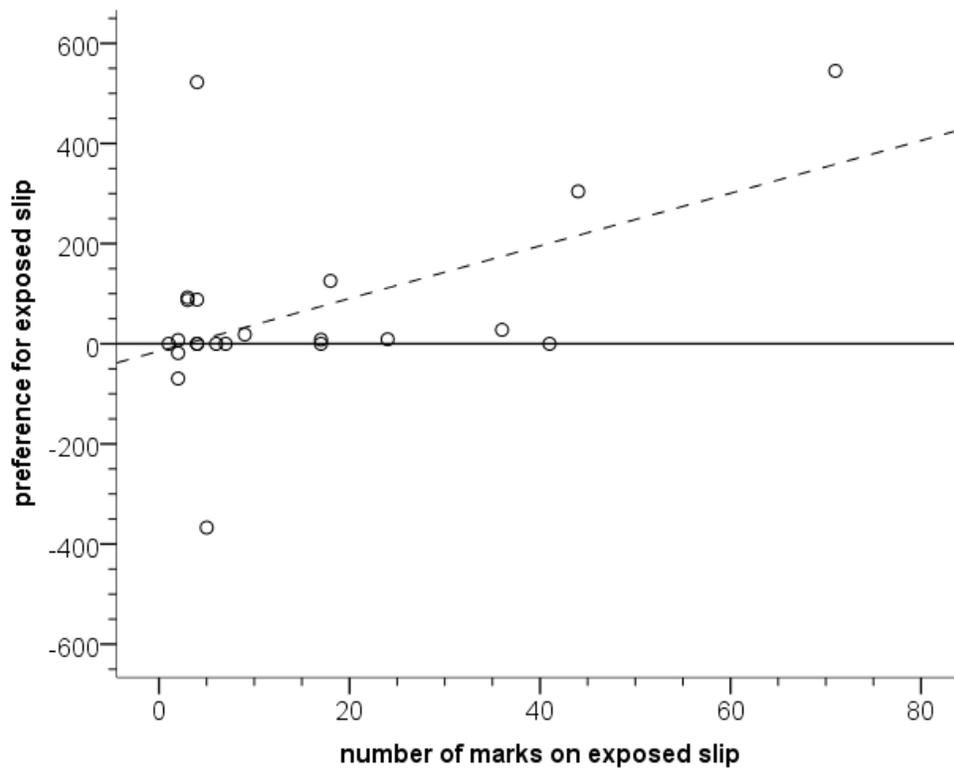

Fig. 4. Male preference for a cover slip that was exposed to himself previously (i.e., time on the exposed cover slip – time on the control cover slip) versus the number of marks on the exposed slip. 0 indicates no preference; - - -, regression line
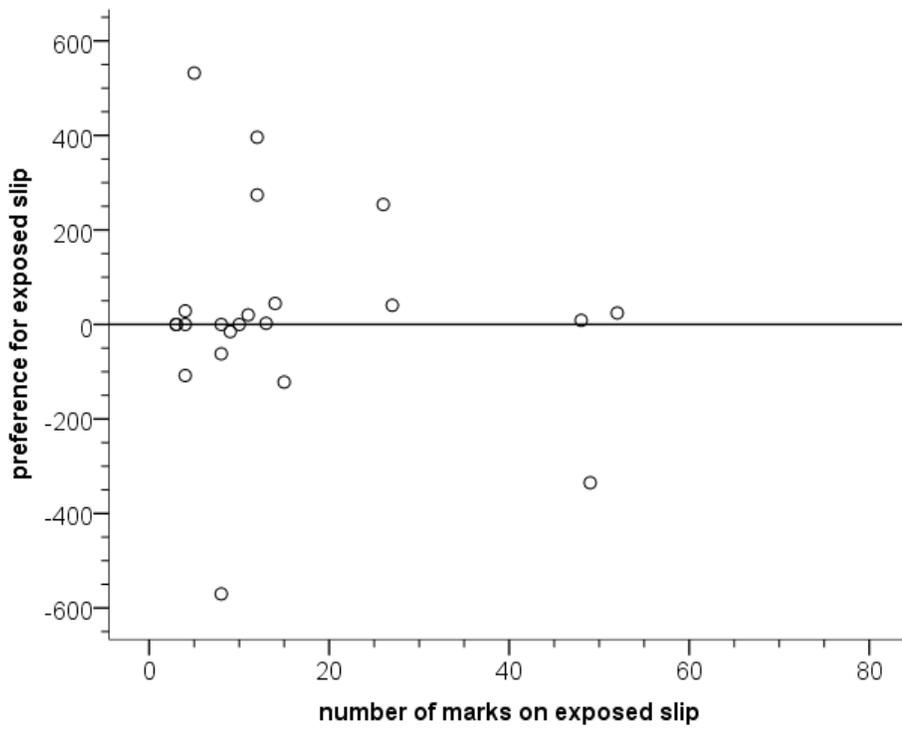
Fig. 5. Male preference for a cover slip that was exposed to another male previously (i.e., time on the exposed cover slip – time on the control cover slip) versus the number of marks on the exposed slip. 0 indicates no preference.