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Post-Mating Changes in Restlessness, Speed and Route Directness in Males of the Parasitoid Wasp *Spalangia endius* (Hymenoptera: Pteromalidae)

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Running head: Locomotor Changes and Male Mating Status

Abstract Changes in movement patterns can affect the probability of encountering resources, including mates. This study examined movement in males of the parasitoid wasp *Spalangia endius*, specifically changes in locomotion after mating that could be responsible for males' post-mating sexual inhibition to approach a female. In the presence of a female, mated males were faster moving than virgin males, which by itself would make them quicker, not slower, to reach her. However, mated males also tended to be less restless (i.e., spent less of their time locomoting) and their paths were less direct, both of which would make them slower to reach her. In contrast, in the absence of a female, having recently mated had no significant effect on restlessness, speed or path-directness. Thus the post-mating locomotor changes in males appeared not to be intrinsic changes but rather changes in how they responded to females. Video recordings were corrected for aspect ratio prior to analyses.

Keywords Circuitry; Courtship conditioning; Restlessness; Kinesis; Locomotion; Sexual inhibition; Parasitoid

Introduction

Changes in movement patterns are important in a broad array of behaviors. For example, optimal foraging theory predicts when animals should leave resources (Charnov 1976; Stephens and Krebs 1986), and leaving a resource may be accomplished by changes in turn rate, speed (reviewed in Bell 1991; van Alphen et al. 2003) or restlessness (King and Ellison 2006). Changes in movement may also be associated with changes in reproductive status, with many studies viewing such changes as a cost of reproduction (e.g., Shaffer and Formanowicz 1996; Miles et al. 2000; Veasey et al. 2001). For example, females that are gravid may have reduced locomotor performance, and reduced locomotor performance may increase predation. However, locomotion can also change with reproductive status in an adaptive manner. For example, locomoting more after mating will allow for female dispersal to oviposition sites; and locomoting less after giving birth will keep a female with her young, which may increase their fitness.

Here we look at changes in movement associated with mating in a wasp that exhibits postmating sexual inhibition of males. Most studies of sexual inhibition, also called mating inhibition or nonreceptivity or reduced sexual responsiveness, have focused on females (e.g., Huck and Lisk 1986, review of insects by Ringo 1996, Wedell 2005), rather than males (e.g., Gadenne et al. 2001, Ureshi and Sakai 2001). This may be because males are often viewed as more ardent in the pursuit of mates than females (Dewsbury 2005) because initial differences in male versus female investment often lead to male, but not female, fitness being limited by the number of mates (Bateman 1948; Trivers 1972). Nevertheless, even in polygynous species there may be temporary periods in which males do not mate despite opportunities to do so. In species with male parental care, these periods may coincide with time spent caring for young. However, temporary male sexual inhibition also occurs in species without male parental care, e.g., the cricket *Gryllus bimaculatus* (Ureshi and Sakai 2001), the moth *Agrotis ipsilon* (Gadenne et al. 2001), the wasp *Spalangia endius* (King et al. 2005) and guppies (Jirotkul 1999). Sexual inhibition immediately after mating may be adaptive if males must replenish their sperm supplies or sperm accessory supplies (Gadenne et al. 2001) or as a mechanism to avoid attempts to remate with the same female, by providing time during which she may leave.

Sexual inhibition may occur at any stage of mating from initial attraction to mounting to copulation. Inhibition to even approach the opposite sex could come about through a simple change in locomotor activity. For example, all else being equal, a male will not be as quick to contact a female if there is a decrease in his restlessness (proportion of time spent locomoting), a decrease in his speed, or a decrease in the directness of his path (see Appendix). Any change in locomotor activity may occur irrespective of a female's presence or only in the presence of a female. There have been so few studies of male sexual inhibition that little is known about the role of locomotion, outside of studies on *Drosophila melanogaster*, where changes in overall activity level do not appear to play a role in the courtship inhibition seen after a male has courted a mated female (Siegel and Hall 1979). Here we compare locomotion of virgin males versus mated males of the parasitoid wasp *Spalangia endius*.

Mated *S. endius* males exhibit sexual inhibition in the sense that they are slower than virgin males to contact and mount females (King et al. 2005; King and Fischer 2010). The inhibition appears to be adaptive by preventing males from immediately trying to remate the same female, rather than being adaptive by providing time for replenishment of ejaculate (Fischer and King 2008). Trying to remate the same female would clearly be a waste of time and energy because females seldom remate (King et al. 2005). The inhibition results directly from a change in some aspect of the male, rather than just a change in the female's response to him, because the inhibition occurs even in the absence of female behavior, i.e., even with dead females. In the present study, we compared the locomotor activity of virgin and mated males, specifically their restlessness, speed, and the directness of their path. We observed males both in the presence and in the absence of a female.

Methods

Biology of *Spalangia endius*

S. endius parasitize the pupae of various dipterans found in manure and rotting organic matter (Rueda and Axtell 1985). Usually only one wasp develops in each host. However, because hosts have a clumped distribution, multiple wasps are likely to emerge in temporal and spatial synchrony. Males emerge from their hosts before females and wait at the hosts for females to emerge (King 2006). Males will mate multiply (King et al. 2005), and there is no obvious aggression among adults (personal observation).

General Methods

The *S. endius* were from a colony established from wasps collected in 1996 from Zephyr Hills, Florida, USA and maintained using a natural host, *Musca domestica* pupae (King 1988). To obtain newly emerged virgin wasps, parasitized hosts were individually isolated in test tubes. Wasps had emerged within less than a day and been given honey prior to use in experiments. Wasps were assigned to treatments at random. Mated

wasps were produced by placing a virgin male and a virgin female into a test tube and watching for copulation to occur.

We tried to avoid differences in handling disturbance between mated males and virgin males. To minimize disturbance when creating mated males, the female was tapped out onto a table, and the male in his tube was placed over her. When the tube had to be tapped to remove the female, the virgin male's tube was tapped the same number of times.

Just-Mated Males in the Presence of a Female

Males were examined using the paired virgin male – mated male test protocol that was initially used to establish sexual inhibition in mated males (King et al. 2005), although the inhibition also occurs when a male is alone (King 2006). Pairs of a virgin and a mated male were videotaped together in the presence of a dead virgin female ($n = 77$). The dead virgin female had emerged and been frozen the previous day. Such females are attractive to males, at least partly due to female-specific pheromones (Nichols et al. 2010). For testing, the dead female was placed in a small petri dish (3.4 cm diameter by 1.1 cm high) filled about three-fourths of the way with off-white sand that had been wetted with water to keep humidity high, which reduces static. She was placed near the edge, dorsal side up. A live mated male and live virgin male were tapped out of their test tubes into the dish, opposite the female. Males were not marked, but the male that was introduced first was alternated and recorded. A glass cover was placed on the dish. A clean dish and cover and new wasps were used for each test.

Restlessness was defined here as the proportion of time males spent locomoting, i.e., walking or hopping, although locomotion was almost exclusively by walking. Because males sometimes started and stopped walking repeatedly in a short period of time, restlessness data were collected from the videotapes by watching the tapes in real time and using a stopwatch. Restlessness was measured during a simultaneous time interval for both males, which was until one of the two males mounted the female or for approximately 10 min, whichever came first ($n = 75$ because restlessness could not be measured for two pairs). Whether virgin males were more restless than mated males was tested with a sign test rather than a paired t-test because the assumption of normality of the difference between treatments was not met and standard transformations did not normalize the data.

Speed and path-directness were also examined. Because the measurements for these calculations were very time consuming, they were taken from a subset of the replicates, the first 41 in which a male mounted within about 5 min. (Temperature and relative humidity of this subsample matched that of the larger sample used to determine restlessness and who mounted first.) Distance moved was measured during the same time period for both males within a replicate, specifically, for about the first 60 s or until one of the males contacted the female prior to mounting, whichever came first. Our distance measurements were of horizontal changes in position for practical reasons. However, the dish was relatively shallow, the depth being less than 6% of the circumference of the dish; and very little of the males' time and distance involved vertical movement.

The software Image Pro Plus 6.3 was used to collect x, y coordinates of each male's location every third of a second, which corresponded to every tenth frame (a total of 5384 coordinates across all males). We then calculated the distance between consecutive points using the Pythagorean Theorem and then summed these individual distances for each male ($n = 41$ pairs of males). Videos widen images, so prior to collecting coordinates, we had calibrated the software to take this into account, i.e., we had included aspect ratio in our calibration. We had confirmed our calibration technique by taking measurements from a video of a string marked at known intervals; we confirmed time units given by the software by analyzing a videotape of a stopwatch.

Speed was calculated as the summed distance of a given male divided by the duration of the sampling period. Except where noted otherwise, speed was calculated irrespective of whether or not a male was still during part of that time, i.e., the male's overall speed, not speed only when moving.

An index of the directness of the path that each male traveled (his path-directness index) was calculated as the straight line distance between the point where he started and the point where he ended

divided by the distance of the actual path taken during that same time. Thus, a directness index of 1 is the most direct path, a straight line with no back tracking.

We compared speed and the directness index between virgin and mated males using paired t-tests. The assumption of normality was met for both variables ($P > 0.05$). We then assessed the importance of correcting for aspect ratio, by mathematically removing the correction and then rerunning the calculations and analyses.

Restlessness of Just Mated Males in the Absence of a Female

This experiment was the same as the previous one, except for the absence of a female, and pairs were taped for 5 min. Restlessness was measured over the entire 5 min trial as well as for just the first minute. Each male's x, y coordinates were determined as in the previous experiment except that they were measured up to the end of the 300th frame. Restlessness, speed and directness were each compared between virgin and mated males with a paired t-test because the assumption of normality was met.

Results

In the Presence of a Female

The first male mounted within 1 min in 82% of trials, within 5 min in 91% of trials and within 6 min in 92% of trials ($n = 66$ trials in which a male mounted the female out of 77 trials). The first male to mount was the virgin male in 70% of pairs with a mounting (King et al. 2005), even when analysis was restricted to trials where a male mounted within 1 min ($n = 54$).

Mated males were usually less restless than virgin males (Fig.1; sign test: 45 versus 27, 3 ties, $n = 75$ pairs, $P = 0.045$). When the data were analyzed according to which male mounted first, the virgin, the mated or neither, the mated male was usually less restless than the virgin male in those replicates in which the virgin was subsequently the first to mount the female (sign test, $n = 51$ pairs, $P = 0.030$) but not when the mated male was first to mount (sign test, $n = 14$ pairs, $P = 1.00$) or when neither male mounted (sign test, $n = 10$ pairs, $P = 0.75$). However, differences in restlessness between mated and virgin males did not totally explain which male mounted first because even when both males were active the entire time from the beginning of testing until the first male mounted, the virgin male was usually the first to mount, not the mated male (19 versus 3 pairs, binomial test, $P < 0.001$).

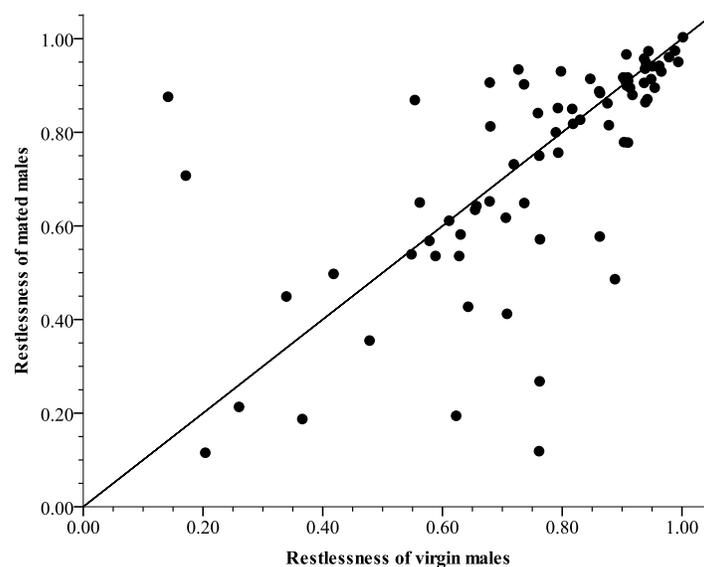


Fig. 1. Restlessness (proportion of time locomoting) of mated males versus virgin males in the presence of a female; reference line of no difference.

Mated males moved almost 20% faster than virgin males (Fig. 2; 4.96 ± 0.20 mm/s versus 4.18 ± 0.17 ; $t = 3.51$, $df = 40$, $P = 0.001$). The mated male was faster than the virgin male even when the analysis was restricted to replicates in which the virgin male was first to mount ($t = 3.01$, $df = 34$, $P = 0.005$) or replicates in which both males were active the entire time from the beginning of testing until the first male mounted ($t = 4.12$, $df = 14$, $P = 0.001$) or when speed was calculated only for periods in which a male locomoted ($t = 3.47$, $df = 40$, $P = 0.001$). The mated male was faster than the virgin in 68% of the 41 replicates (sign test: 28 versus 13, $P = 0.029$).

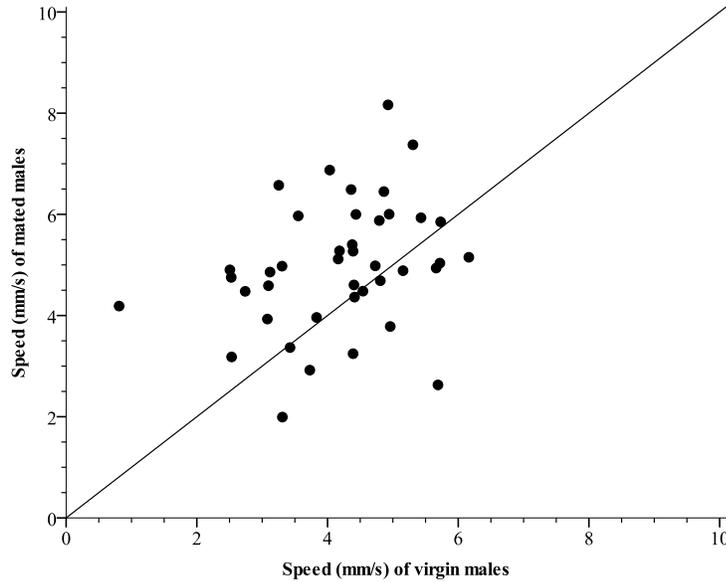


Fig. 2. Speed (mm/s) of mated males versus virgin males in the presence of a female (overall speed, not just when moving); reference line of no difference.

The paths of mated males were significantly less direct than the paths of virgin males as determined by their directness index (Fig. 3; 0.35 ± 0.04 versus 0.44 ± 0.04 ; paired t-test: $t = 3.83$, $df = 40$, $P < 0.001$). As with speed, conclusions were the same when the analysis of directness index was restricted to replicates in which the virgin male was first to mount or replicates in which both males were active the entire time ($t = 4.60$, $df = 34$, $P < 0.001$; $t = 2.75$, $df = 14$, $P = 0.016$).

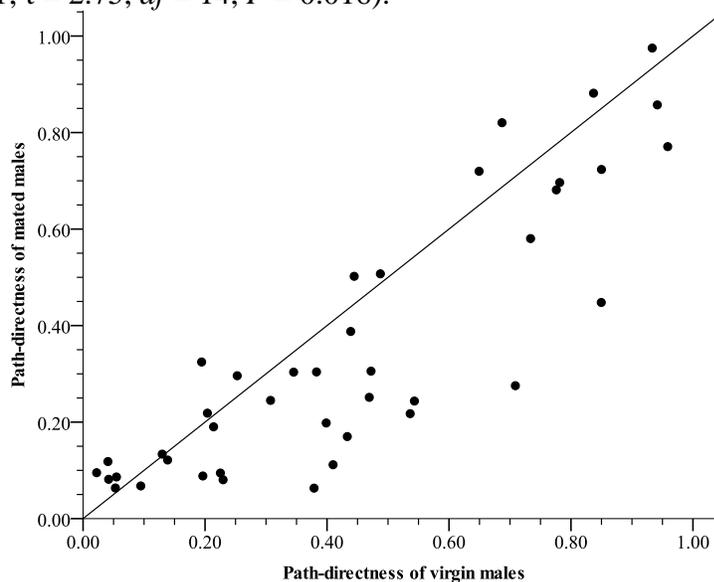


Fig. 3. Directness index of mated males versus virgin males in the presence of a female; reference line of no difference.

If we had not corrected for aspect ratio, we still would have concluded that mated males moved faster and less directly than virgin males.

In the Absence of a Female

In contrast to when a female was present, the mated male was not the less restless one more often than the virgin male was regardless of whether restlessness was measured for the first minute (sign test, $n = 41$ pairs, $P = 0.87$) or over the entire 5 min trial (sign test, $n = 41$ pairs, $P = 0.53$). Likewise, the mean difference between the mated male's and the virgin male's restlessness was not significantly different from zero for the entire 5 min trial ($t = 1.27$, $df = 40$, $P = 0.21$). (Normality was not met for the first minute data.)

In the absence of a female, the mean speed of mated males was only 8% faster than that of virgin males, a difference which is not significant (6.55 ± 0.27 mm/s versus 6.04 ± 0.27 ; $t = 1.69$, $df = 40$, $P = 0.10$). The mated male was faster than the virgin in 61% of the 41 replicates, which is not significantly different than half of the replicates (sign test: 25 versus 16, $P = 0.21$). The paths of mated males were not significantly less direct than the paths of virgin males as determined by their directness index (0.29 ± 0.02 versus 0.31 ± 0.03 ; $t = 0.58$, $df = 40$, $P = 0.57$).

Discussion

Variation among individual male *S. endius* was high, but a few trends were found. Multiple changes in movement followed mating even though a single change would theoretically be sufficient to cause sexual inhibition. In the presence of a female, relative to virgin males, mated males tended to be less restless and less direct in their path but were faster moving. In other words, given the same time as virgin males, mated males tended to traverse a less direct path at greater speed yet spent a smaller proportion of their time locomoting. It can be shown that the postmating sexual inhibition of males was not solely caused by the decrease in restlessness. In pairs where restlessness was identical because both males moved the entire time, virgin males still tended to reach the female first. In these cases, the more direct path of virgin males was presumably responsible.

Differences in locomotion between virgin and mated males were detected only in the presence of a female, suggesting that these post-mating locomotor changes are not intrinsic changes in males but rather changes in how males respond to cues from females. Postmating changes in the response of males to female cues could result from changes in the physiology of male receptors. For example, exposure to female sex pheromone during mating may cause a temporary saturation of the mated male's receptor sites for that pheromone, thereby obstructing his detection of the female. An arresting female sex pheromone has been identified for *S. endius*, and male antennae have been shown to respond to it (Nichols et al. 2010), but whether virgin and mated male antennae respond differently has not been examined. Alternatively, mating may trigger changes not in the antennal receptors, but rather in the brain's response to signals from the antennal receptors (Gadenne et al. 2001). Baker et al. (1981) report that in oriental fruit moths, increasing concentration of certain components of female sex pheromone cause virgin males to slow down, make fewer turns and change how tight the turns are. Whether mated males respond differently was not tested. These findings seem consistent with the idea that virgin males in our study may have been responding to an attractant female pheromone more than mated males were. From the description of their methods, it seems likely that Baker et al. (1981) failed to account for their monitor's aspect ratio. Our analyses show this may not have affected their conclusions.

Knowing that an organism responds to a pheromone is not the same as knowing how its locomotion is affected. Response to pheromone is generally recognized by an observation of an increase in path directness (positive chemotaxis). However, pheromone could also cause an organism to move more quickly

(chemo-orthokinesis) or slowly (negative chemo-orthokinesis); and a chemical that causes a change in one aspect of locomotion will not necessarily affect another aspect of locomotion (e.g., Visser and Avé 1978).

The present study used a dead female when measuring male locomotor activity. Although this was done to eliminate effects of female behavior; nevertheless, males may sometimes encounter females in roughly a fixed position such as a dead female represents. This would be the case when a male encounters a female chewing her way out of a host, a process that can take an hour (King 2006), or on occasions where a male catches a female resting and unaware of the male's approach. How or if the presence of a live female would change the effect of male mating status on locomotion remains to be tested. However, males exhibit postmating sexual inhibition even with live females (King and Fischer 2010).

This study examined post-mating changes in movement of males. Post-mating changes in movement also occur in females. In *Lobesia botrana*, a European grapevine moth, mated females lift off more quickly and fly for longer periods of time (Hurtrel and Thiéry 1999). *S. endius* females become more likely to burrow after mating, which probably facilitates host finding (King 2002); and in the confamilial *Nasonia vitripennis*, mated females are more restless than virgin females for up to two hours after mating (King et al. 2000). In contrast to these examples of changes in locomotion that may facilitate leaving after mating, in female German cockroaches, locomotor activity decreases after mating (Lin and Lee 1998). Likewise, in the funnel-web spider *Agelenopsis emertoni*, mated females, particularly those that have invested more in their egg masses, are slower than unmated ones (Pruitt and Troupe 2010). This is thought to be a mechanism for reducing the female's likeliness of fleeing her eggs in response to predatory attacks. The decrease in speed occurs even in the eggs' absence. Changes in activity that are associated with reproduction may be adaptive even when they include a cost. For example, mating increases activity in *Drosophila* females, which may facilitate foraging and egg-laying, but it may also contribute to the reduced lifespan of females after mating (Isaac et al. 2010).

The present study shows that although a change in one parameter of locomotion may theoretically be sufficient to achieve an apparently adaptive behavioural outcome, at least in *S. endius*, multiple changes occur.

Appendix:

By definition:

T = time to reach the female

s = path-directness = d / d_p

d = direct distance to the female

d_p = length of path taken to reach the female

r = restlessness = t_1 / T

t_1 = time spent locomoting

v_1 = average speed when locomoting, = d_p / t_1

v_o = overall speed = d_p / T

Then: $T = d_p / (r * v_1) = d / (s * r * v_1)$

Thus, all else being equal (e.g., same d , s , and v_1), but varying restlessness (r), time to reach the female (T) will decrease with increasing restlessness (r). Likewise, all else being equal, T will decrease with increasing speed while moving (v_1) or with increasing path-directness (s).

Also: $T = d_p / v_o = d / v_o s$

Thus, in terms of overall speed, all else being equal (same d and s) time to reach the female will decrease with increasing overall speed.

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