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Antennae Enable Male Copulation Initiation in American Oil Beetle (*Meloe americanus*)

Blister beetles are important in agriculture and veterinary medicine. We observed the mating behavior of an understudied blister beetle in western New York State. Copulation was always initiated by males and included a several-minute dorsal phase and a several-hour linear phase. We were able to prevent male sexual behavior by removal of both antennae, possibly due to loss of chemicals normally secreted by those organs. Males with two legs removed were equally able to mate as unmanipulated males, although they may have been slower to initiate copulation. Future work could examine the specific mechanism whereby antennae enable male mating behavior.

Introduction

Blister beetles (Family: Meloidae) comprise some 2,500 species and occur across the globe with the exception of New Zealand (Bologna & Pinto 2001; Pinto & Bologna 1999). They are named for their defensive secretions containing cantharidin. Blister beetles arouse human interest due to their importance for veterinary medicine and agricultural communities (Schoeb & Panciera 1978; Capinera et al. 1985; Horsfall 1943). Common features include gregarious behavior and adaptation to arid conditions (Selander 1960; Cohen & Pinto 1977). Meloidae also share a hypermetamorphic ontogeny, usually including an active first instar called a triungulin (Pinto, Bouseman, & Bologna 1996). Triungulins are typically phoretic on host insects such as bees or other beetle species (Bologna et al. 1990; Saul-Gershenz & Millar 2006).

Courtship behavior is highly variable among subfamilies of Meloidae. This has been used to inform phylogenetics within Meloidae (Selander 1964; Adams & Selander 1979; Turco, Di Giulio, & Bologna 2003). Four subfamilies are recognized: Electinae, Meloinae, Nemognathinae, and Tetraonycinae. Courtship in Nemognathinae and Tetraonycinae occurs dorsally without a display, and copulation (also dorsal) lasts only a few seconds (Bologna 1991; Selander & Martinez 1984). Some Electinae species add a complex frontal courtship which incorporates humeral palpation (Pinto, Bouseman, & Bologna 1996). In Meloinae dorsal courtship includes elaborate display, during which the male stimulates the female using legs and antennae. In addition, copulation can last several hours and incorporates both a short dorsal and a longer linear stage (Bologna 1991). Tribes within Meloinae further diverge. Some utilize a posterior courtship as well as dorsal. Males use specialized structures of the antennae, abdomen, legs, palpi, and genitalia in different combinations to stimulate females (Turco, Di Giulio, & Bologna 2003).

The American oil beetle (*Meloe americanus*) is a poorly studied member of the Meloini tribe, native to the northeastern United States. Males of Meloini are known to employ maxillary palpi, antennae, and genital structures to physically manipulate females during courtship and copulation (Turco, Di Giulio,

& Bologna 2003). However, the specific mating behaviors of *M. americanus* have not, to our knowledge, been characterized.

The first goal of this study was to observe and characterize courtship and copulation initiation in *M. americanus.* We also sought to understand the importance of the antennae in courtship behavior. Based on sexual dimorphism (Figure 1), we hypothesized that antennae play a critical role in male mating success. Specifically, we hypothesized that removal of male antennae would decrease copulation initiation.

Figure 1: a) Female *M. americanus* individual. b) Male *M. americanus* individual. Note the difference in antennal structure: Female antennae are straight and simple. Male antennae exhibit a distinct hook-like structure near the midpoint.

Methods

Our study area was a ~5,000 m^2 meadow in the Mt. Hope Cemetery, Rochester, New York (43° N, 78° W). The experiment took place over several weeks in October, 2015. Temperature ranged from -1° C to 29° C. Beetles were most active in sunny weather, from late morning until late afternoon.

To investigate whether mate-guarding might be influencing male sexual behavior, we first performed a mark-recapture in the meadow to estimate *M. americanus* population density and sex ratio.

We observed, captured, and manipulated *M. americanus* individuals in the field. To investigate the role of male antennae in copulation initiation, we used scissors to remove both antennae of each experimental male at the base. Control males were picked up and held to induce commensurate handling stress, but their anatomy was not modified. As a further control, we a used a third treatment wherein two of each male's limbs (middle leg, both left and right) were removed with scissors at the lowest joint. This

controlled for reduced sexual performance due simply to pain and shock. It also allowed us to test whether loss of legs could alter male courtship.

For each copulation initiation trial, we placed one male and one female individual in a 2.1-cup plastic container and recorded the amount of time until copulation began. We defined copulation initiation as the point at which the male mounted the female dorsally and hooked his legs onto her abdomen. If copulation was not initiated with 1 hr, the trial was recorded as a failure to copulate.

To statistically compare probability of initiation success, we used a pairwise Fisher's exact test for each pair of treatments. We also used an unpaired two-sample Student's *t*-test (unequal variances) to test for difference in initiation time between control treatment and leg removal treatment. Antenna removal treatment was not included in analysis of initiation time because zero copulation successes were observed for that group. Analyses were conducted in *R* (R Core Team 2015).

Results

Mark-recapture

We sampled a total of 2,241 oil beetles in mark-recapture (Table 1). We estimated there to be 3,462 individuals in the population. Overall population density was roughly 0.69 individuals per m^2. The sex ratio of our sample was 48.0% male.

Table 1

	Captured, Initial	Recaptured, Marked	Recaptured, Unmarked
Male	421	204	441
Female	432	138	605
Total	853	342	1,046

Table 1 We used the mark-recapture formula $N = \left(\frac{Kn}{k}\right)$. N = estimated total population size. K = total captured and marked initially. k = recaptured marked individuals. n = total recaptured individuals.

Sexual behavior

All of the ~150 courtship events which we observed were initiated by the male. Males climbed atop the female dorsally and caressed the abdomen with their legs. The male then stimulated the female's antennae with his own, and waved his antennae vertically without touching his partner. The tip of the male's abdomen was repeatedly pressed against the female's abdomen. After several seconds, the male used the hooks on the tips of his legs to grasp the female along the edge of her thorax and abdomen. After this point copulation was always successful, unless another male interfered. This dorsal stage continued from 15 seconds to 15 minutes before entering a linear phase of copulation in which the individuals were connected end-to-end at the tip of the abdomen. We did not observe the linear phase for its full duration,

but observations of other Meloid beetles suggest that it can last up to eight hours (Turco, Di Giulio, & Bologna 2003).

Experimental manipulation

Nearly all control (49 of 52) and leg removal (27 of 29) males initiated copulation in less than one hour (Table 2; Figure 2). Copulation was frequently (Control, 30 of 52; Leg removal, 13 of 29) achieved in less than five minutes. We ran 32 antenna removal trials and did not observe a single mating attempt. Antenna removal males ignored the female partner, instead resting or attempting to escape the container.

Table 2

	Control	**Leg removal**	**Antenna removal**
Success < 1 hr	49	27	0
Success < 5 min	30	13	0
No success < 1 hr	3	2	32
Total	52	29	32

Table 2 Results of experimental treatments. Antenna removal severely impaired male ability initiate copulation, while leg removal had little effect.

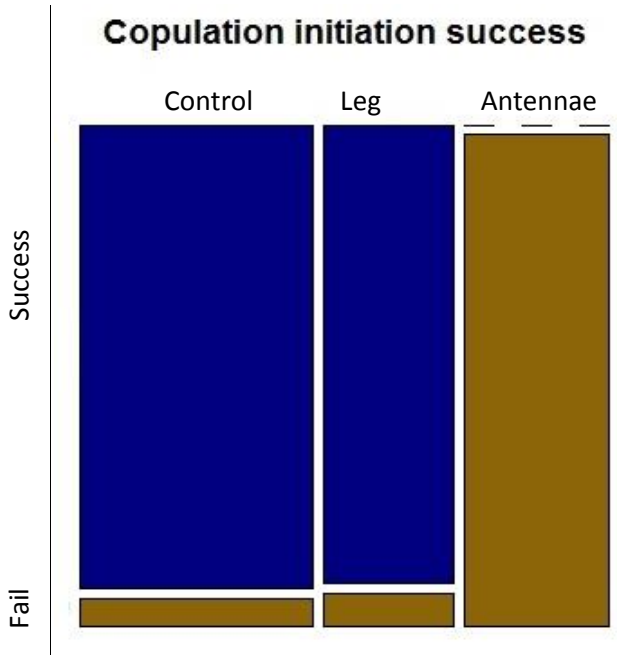

Figure 2 Mosaic plot of proportion of successes and failures for each treatment type. Control and leg removal treatments resulted predominantly in successes, while antennae removal trials all failed to initiate copulation within one hour. Column labels indicate treatment type. Width of a bar is proportional to number of trials of that treatment type. Lengths of the colored stacked bars represent the proportion of successes and failures.

A Fisher's exact test showed a significant effect of antenna removal on failure to copulate, compared to the control (P < 0.00001; $\widehat{OR}$ = ∞; 95% CI: 73.8517, ∞) and compared to leg removal (P < 0.00001; $\widehat{OR}$ = ∞; 95% CI: 45.9502, ∞). Odds of successful copulation were not significantly different between control and leg removal treatments within one hour (P = 1; $\widehat{OR}$ = 1.2070; 95% CI = 0.9535, 11.2238) or within five minutes (P = 0.3536, $\widehat{OR}$ = 1.6674; 95% CI: 0.6101, 4.6428).

In the successful trials, mean time until copulation initiation for control males and leg removal males was 309 s and 621 s, respectively (Figure 3). Because we observed zero successful trials for antenna removal individuals, there is no data for time until initiation. Variances were unequal between control and leg removal treatments (P < 0.00001; F = 0.1580; 95% CI: 0.0766, 0.3043), so we used an unpaired two-sample *t*-test with unequal variances. The effect of leg removal on time until copulation initiation was marginally significant (P = 0.0550; t = -1.9947; 95% CI: -630.14 s, 7.1316 s).

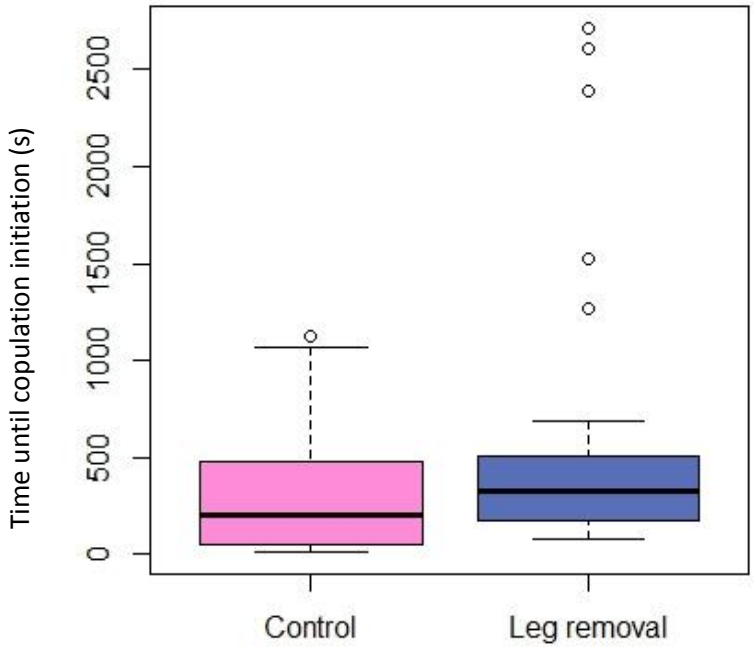

Figure 3 Box-and-whisker plot of time until copulation. Each box is drawn based on the first quartile, median, and third quartile values. Whiskers extend to the most extreme datum no more than 1.5 times the interquartile range from the box. Note the several outliers in the leg removal treatment group.

Discussion

We found a sex ratio of nearly 1 : 1 in the local *M. americanus* population, with slightly more females. Knox & Scott (2006) found that mate-guarding in *Necrophila americana*, another member of Meloidae, was far more prevalent when sex ratio was biased toward males. For our study, this means that the dorsal courtship and long linear phase of copulation we observed are probably intrinsic elements of

the sexual behavior of *M. americanus*, rather than context-dependent mate-guarding strategies. The low population density we found (0.69 individuals per m^2) further supports context-independence.

Antennae removal caused males to cease copulation attempts. However, the mechanism by which antennae facilitate mating behavior is not certain. We observed antennal caressing of females during courtship. It might therefore be expected that antenna removal would decrease male success when copulation is attempted, but our antenna removal treatment males did not even try to mate. It is possible that the hook in the male's antennae (Figure 1b) contains a hormone gland responsible for triggering male sexual behavior. Cantharidin has been found to play a role in Meloid sexual behavior, and in some species cantharidin concentration is as high in the antennal glands as in the genitals (Nikbakhtzadeh et al. 2008). High cantharidin may incite males to mate, and during courtship Meloid males release cantharidin from their antennae onto females' (Nikbakhtzadeh, Hemp, & Ebrahimi 2007). It is also possible that males receive chemical signals from females via antennae, but we suspect this is not the case. When males were confined together, we frequently observed males attempting to copulate with one another, implying that explicit recognition of female oil beetles is not necessary to trigger mating.

We do not believe pain or shock caused loss of mating behavior in antenna removal males. If this was the primary factor influencing motivation and ability to attempt copulation, we should have observed no difference in copulation initiation success between antenna removal and leg removal treatments. However, since there were zero observed successes for the antenna removal treatment, the behavioral differences we observed are exceedingly unlikely to be due to chance ($P = 2.6\times10^{-14}$). The clear difference between antenna removal and leg removal, along with the procedure of equal handling between control males and antenna removal males, strongly supports the hypothesis that behavioral change is due to particular properties of the antennae.

Leg removal treatment did not decrease the proportion of males which attempted to copulate, compared to controls. However, there was a marginally significant increase in the amount of time before copulation was attempted. This could mean that the male's middle pair of legs is important in mating. We observed the male to grip the edge of the female's thorax and abdomen with the tips of his legs during dorsal courtship. This behavior might be mechanically more difficult with missing limbs. Our conclusions, though, are not definitive. The average time until copulation for leg removal males was greatly increased by the presence of three extreme long-duration trials. Removing these outliers from the data rendered the variances equal in an *F*-test ($P = 0.4472$; $F = 0.7733$; 95% CI: 0.3601, 1.5188), and the marginal significance of differences in time until copulation vanished from the *t*-test ($P = 0.4047$; $t = -0.8383$; 95% CI: -229.22 s, 93.55 s). We believe the dataset is more representative of behavior when we do include the outliers. We aborted three control and two leg removal trials when copulation did not occur within one hour and labeled them failures, but the choice of the one hour cutoff was arbitrary. It is possi-

ble that copulation could have occurred after the one hour, and there could therefore exist additional unrecorded long-duration successes in both groups. Overall, though, the datasets with and without outliers are most informative when considered together.

In future trials, we would like to examine the behavior of males with exactly one antenna removed. In one accidental trial, a single-antenna male was able to initiate copulation. If we were to find that single-antenna males are equally capable at initiating copulation as control males, it would further support the hormone hypothesis. This could be due to a compensation mechanism whereby the remaining antenna secretes additional chemical, or perhaps a half-dose would be sufficient to trigger sexual behavior. If the average time until copulation initiation is increased by ~100%, it could support the quantitative half-dose hypothesis. Similarly, we could remove different pairs or single legs and test for differences in time until copulation in order to assess which legs are most important during courtship.

It would also be informative to perform a chemical extraction from antenna tissue and identify the chemicals present with gas chromatograph–mass spectrometry as has been done with Meloid tissue in the past (Saul-Gershenz & Millar 2006). This could more definitively elucidate the mechanism underlying the observed behavior differences.

Conclusion

We examined mating behavior of *Meloe americanus*. Mating included a short- to medium-duration dorsal courtship followed by a long linear phase. Experimental removal of male antennae prevented males from initiating copulation. Removal of the middle pair of legs did not alter male success at copulating, but may have increased the time until copulation. The hypothesis that male antennae are critical to successful mating was supported. A possible mechanism is the secretion of sex chemicals in the antennae.

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