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# Behavioral response of Japanese beetles (*Popillia japonica*) to sex pheromone: Exploring factors of social situation and recent mating experience

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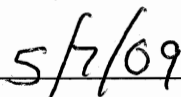
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Behavioral response of Japanese beetles (*Popillia japonica*) to sex pheromone:

Exploring factors of social situation and recent mating experience

(TITLE)

BY

Carissa A. Schoenick

**THESIS**

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
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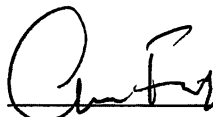
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## Abstract

Insects in have several methods of advertising for mates; one important and common method is via the use of chemical pheromones. Insects release pheromones into the environment to communicate information about their sex, mating status and receptivity to others. For example, Japanese beetles (*Popillia japonica*) produce a very volatile sex pheromone conspecifics can readily detect. This pheromone is released by virgin females only, and triggers scramble competition among the males that detect and follow the signal for access to these females. Because Japanese beetles are an invasive species and an important pest of ornamental and agricultural plants, there is strong incentive to develop an effective trap to manage their populations and to reduce the damage they cause. The beetles' sex pheromone has been incorporated along with floral compounds into lures for these traps because of its strong attractive ability; however traps are still not completely effective. Some individuals fly to the trap's location but do not enter the trap itself, and still others fail to respond to the trap lures at all. Another limitation of beetle attraction via pheromone is that because the pheromone origin is female-specific, for the most part only males are pulled into traps by this additional lure. Occasionally, females have been observed to respond to sex pheromone, however, and investigations of *P. japonica's* antennal structures have shown the presence of pheromone-detecting pores on females' antennal lamellae. This evidence of female response to pheromone and the observed differences in individual males' responses are interesting for their behavioral and economic implications, and this thesis consists of two experiments that attempt to investigate factors influencing individual responses to



pheromone for both sexes and the effect, if any, the social situations of male beetles might have on their pheromone response patterns.

In the first experiment, trials were performed on individuals of both sexes to investigate the behaviors displayed during pheromone exposure and determine the effect of recent mating experience. Beetles were kept either with or without mates for ~24 hours before testing, and their behaviors were recorded as they were exposed to odorless (control) or pheromone-laden air inside an acrylic testing chamber. We found that female beetles do indeed appear to respond to the presence of pheromone; they fly more often in its presence and rub their abdomens more frequently. Males also displayed clear pheromone response behavior, especially through rubbing behaviors. Recent mating experience appeared to have an effect on an individuals' latency to start flying during a trial in conjunction with the presence or absence of pheromone; unmated beetles flew more quickly during the pheromone exposure trials, and had greater latency to flight during control trials.

Beetles were also tested in group trials to investigate pheromone response behaviors in differing social scenarios; several trials were run on groups of 5 male beetles and mixed-sex groups of 3 males and 2 females. Each group was exposed to either a control of normal outdoor air or a plume of pheromone. While patterns did not emerge as clearly in these experiments, a significant interaction between the number of pairs formed during a trial and the ambient light level was observed that suggests that pheromone presence could have an effect on the level of an individuals' arousal level and the instigation of mating interactions.

## Chapter 1

### **Insect Mating Strategies and Signals *and* The Reproductive Biology of the Japanese Beetle**

Reproduction is a crucial facet of any organism's life, and as such, individuals will often expend great effort in order to create viable offspring. In sexual reproduction, sperm and ova from separate organisms must come together to form a zygote. In sexually reproducing animals where fertilization takes place internally via copulation, such as with all insects, the male and female must make physical contact with one another in order to exchange gametes (Thornhill and Alcock 1983). Therefore, a critical requirement for these animals is that the two sexes must somehow locate one another in order to mate. This is facilitated by attracting the opposite sex through the use of advertisement signals, wherein there is much variation in strength, effectiveness, and behavioral responses across species.

#### *Advertisement signals*

To advertise for a mate, an insect may use one or more of three primary signaling modes: visual, auditory and olfactory. A useful and effective way for insects to communicate their position and intention over long distances is through the use of sound. Sophisticated physical features like tymbal (drum-like) organs or stridulatory plates (grooved plates rubbed together) produce and amplify sound that radiates in all directions around the calling males of crickets and grasshoppers (Hoffart *et al.* 2002). The sound production

may last for an extended period of time, e.g. 3-4 hours a night as in the case of the short-tailed cricket *Anurogryllus muticus* (Lee and Loher 1993), and calling males may be isolated or aggregated. For example, male cicadas will gather together or position themselves apart, depending on the species, and employ differing calling strategies to maximize mating success (Sueur 2002). Insects using auditory signals like these incur energetic costs and enable the possibility of exploitation of their call by a rival, a predator or a parasite. For example, parasitoids are known to hone in on the long calls of the field cricket *Teleogryllus oceanicus* (Simmons *et al.* 2001). The benefits of allowing for long range and low-visibility/nighttime advertising, however, make it obvious why many auditory-signaling species exist.

The use of visual signaling for mates is the medium-to-short range method of choice for many insects. Males of the blue butterfly *Polyommatus icarus*, for example, assess the ultraviolet patterns on the wings of females to make mating decisions (Knüttel and Fiedler 2001). Visual advertisers are easy to locate by conspecifics and their message is immediately delivered. The detriment of visual signals come about if the insect cannot conceal its bright visual display from predators, has difficulty getting its signal to the receiver due to darkness or obstructions, or because of some energetic cost, especially if the display utilizes certain physical actions like dancing. This energetic cost is apparent in the platystomatid fly *Euprosopia subula*, who must expend physical energy when they wave their distinctively marked wings in various patterns while courting a mate or signaling aggression to a rival (Burk 1981). The more vigorous the dancing the more victories the male attains in mating and in male-male contests, but also the more energy

he must expend. The usually relatively low cost of producing a static or constant visual signal, however, results in many species using this as their main mode of mate advertisement, especially butterflies and fireflies (Vencel and Carlson 1998).

Olfactory or pheromone-based signaling is perhaps the most common mode of communication in insects. The use of chemical pheromones dispersed through the air is energetically cheap compared to the energetic costs of sound, and can be used in low-visibility situations just as readily. An additional benefit to the use of olfactory signals is the temporal advantage; pheromone cues may remain active for much longer than another type of signal, especially when applied to a solid surface as is the case with many ant species. Leafcutter ant foragers (*Atta cephalotes*) rely on these long-lasting scent trails to follow scouts to high-quality leaf sources (Hubbell *et al.* 1980).

Pheromone cues could potentially betray the signaling insect to a predator, but they are the most difficult type of signal to track back to the sender. While perhaps making tracking difficult for the intended receiver, this attribute does offer some measure of protection to the advertiser. More importantly, detection of pheromone requires that the receiver have the sensory structures to be able to detect the chemical in the first place, and there is only one known instance of a predator using insect sex pheromone in this manner; bark beetles (Scolytidae) attract predatory clerid beetles and dolichopodid flies with their sex pheromones (Greenfield 1981). A less obvious physiological cost of pheromone production may be the compromise of the emitter's immune function (Rantala *et al.* 2003). Thus, those individuals that can emit the most pheromone are more

likely to be physiologically robust, which reinforces pheromone-based sexual selection through female choice.

Both male and female insects may emit sex pheromones (termed “calling”); males of the mealworm beetle species *Tenebrio molitor* actively emit a sex pheromone to attract females, who in turn assess the superiority of the male via his pheromone quality (Rantala *et al.* 2003). Females of other beetle species, such as the oriental beetle *Anomala orientalis*, are the ones who produce sex pheromone to attract dispersed males and gain matings (Wenninger and Averill 2006).

#### *Response to pheromone*

There are varying levels of response that insects may produce when presented with a stimulating pheromone. When a pheromone elicits an immediate, often behavioral response in the receiver, it is considered a “releaser pheromone,” and when it causes a relatively more long-lasting physiological change in the receiver it is considered a “primer pheromone” (Shorey 1973). Social insects are frequent users of this second type of pheromone; queens of harvester ant colonies are believed to release pheromones that suppress workers’ fertility in order to prevent worker reproduction from occurring in the colony (Smith *et al.* 2007). Conversely, non-social insects often use releaser pheromones to communicate with and influence the behavior of other members of their species for short-term needs such as mating, oviposition, and aggregation (Shorey 1973).

There are many pheromones produced by insects that can be considered “sex pheromones.” These are typically releaser pheromones that stimulate behaviors in conspecifics that either directly or indirectly lead to mating. Many insect species have a sex pheromone the female releases to advertise her receptivity to conspecific males. When this pheromone is released, it may trigger a hierarchy of response in males. The behavioral steps in this hierarchy are firstly stimulation from the resting state, then initiation of flight and approach to the pheromone source, and finally a copulatory attempt (Shorey 1973). This sequence of events has been demonstrated for many species of Coleoptera including the oriental scarab *Exomala orientalis* (Facundo 1999). Quantitative studies have shown that increasing concentration of pheromone is typically required for each step in the male response hierarchy; at the correct high concentration, however, the female sex pheromone can directly stimulate male copulatory behavior in absence of all other female-based cues; this has been directly observed in the Osaka beetle *Anomala osakana* (Leal 1996). Interestingly, in some species, females have also been observed detecting and responding to the female-produced sex pheromone of their own species (hereafter referred to as autodetection). This phenomenon is common in the Lepidoptera (Ochieng *et al.* 1995; Schneider *et al.* 1998), and has been observed in some species the Coleoptera as well; for example, female individuals of some scarab species that utilize female-produced sex pheromones such as *E. orientalis* (Leal *et al.* 1994) and *Holotrichia consanguinea* (Leal *et al.* 1996b) have been caught along with males in traps baited with pheromone. In some cases, females exposed to conspecific pheromone will even display mating-related behaviors. For example, females of the eastern spruce budworm *Choristoneura fumiferana* respond to their species' pheromone with behaviors

that include antennal grooming, flexation of the body and ovipositing motions; electroantennogram measurements place the amplitude of the females' antennal responses at about two-thirds that of the males (Palanaswamy and Seabrook 1978). Autodetection can also have inhibitory effects; for example, females of the leafroller species *Choristoneura rosaceana* and *Argyrotaenia velutinana* will delay and/or reduce their performance of calling behaviors in the presence of conspecific pheromone (Gokce *et al.* 2007).

### **Japanese beetle biology**

The Japanese beetle (*Popillia japonica*, hereafter JB) is an introduced pest with what can best be described as a scramble competition mating system. In the scramble competition system, receptive females are located randomly over wide areas, and males search competitively for them without defending individual territories (Thornhill and Alcock 1983). Because JB have the potential for long-range flight and are not particularly large (approximately 1cm in length), olfactory signaling is used throughout the adult life cycle as the main means of communication and orientation.

JB were introduced to the U.S. from Japan in the early 1900's and have since spread across the eastern U.S. as a successful invasive pest of many kinds of plants, including agricultural and ornamental species (Fleming 1972; Potter and Held 2002). The aggregative behaviors of these beetles and their relative lack of natural predators have allowed them to grow in number to proportions that can be highly damaging to valued

plants; this has spurred much interest in developing a method for controlling their populations (Potter and Held 2002).

The JB has an annual life cycle that starts with oviposition in soil by the female during the summer. The larvae remain in the soil and feed on the roots of a wide variety of plants which include agricultural crops, ornamental plants and grasses (Fleming 1972), making them a pest of many important types of plants even at this stage. Larvae will over-winter in the soil, continue development in spring, then pupate and emerge as sexually mature adults in late spring/early summer (Fleming 1972; Potter and Held 2002).

Male JB are highly attuned to their species' sex pheromone, which is emitted by virgin females as they emerge from the ground (Ladd 1970; Fleming 1972, Potter and Held 2002). The pheromone disperses through the air with the aid of wind. For successful detection, males must have molecules of the pheromone make contact with the sensilla placodea (sensory pores) on the lamellae (thin branched plates) at the end of their antennae. The most numerous and largest type of sensory pore the male beetle has is the pheromone-detecting placodea. Interestingly, female beetles have been shown to have these same pores, although in half the number possessed by males (Kim and Leal 2000). After entering the sensilla placodea, the pheromone is encountered and bound by a Pheromone Binding Protein (PBP). Signals to the brain of the beetle are triggered by chemical messengers generated by the interaction of the pheromone and the PBP. The signals are transmitted to the portion of the brain that deals with primary processing and



spatial and temporal encoding (Kim and Leal 2000). Reaction to the pheromone will vary based on unknown (probably physiological) factors, but it is safe to say it behaves as a releaser pheromone and is a very attractive olfactory stimulus for most males. Once pheromone is detected, males will typically initiate flight quickly to try and locate the source and mate with the virgin they hope to find (Potter and Held 2002).

Males can be so tenacious in response to the female sex pheromone that they will form "mating balls," which are clusters of males surrounding the newly emerged virgin female, all attempting to copulate with her (Ladd 1970; Fleming 1972). Virgins are especially valuable as mating partners because they carry with them a full load of mature eggs, and they tend to oviposit soon after this first important mating (Potter and Held 2002), although in some cases it has been observed that a female may not oviposit for several days after emergence (Van Timmerman *et al.* 2002). A male who can mate with a virgin female likely gains a high probability of fertilizing at least some of her many (30+) eggs, especially if she does not mate with another male before she burrows back underground to commence oviposition. Female beetles cease to produce the sex pheromone after they are mated for the first time (Ladd 1970); why and how this occurs is unknown. It is possible the males themselves stop production of the pheromone by passing accessory gland proteins to the female during mating, as is the case with *Drosophila melanogaster* (Tram and Wolfner 1998). Such a change would benefit the male by reducing the female's attractiveness to other males, which would result in less competition between the male's sperm and that of his rivals. It is also possible that the cessation of pheromone production is caused by the female, perhaps to avoid continued inundation by males

while she is trying to accomplish other behaviors, or to simply avoid the other costs of copulating and being guarded. Regardless, the fact that pheromone production by females ceases has important implications for the significance of aggregations and mating balls for the JB, whose non-virgin females will continue mating and ovipositing during their adult life and so may need to secure more matings.

The mating behavior of JB consists of the male approaching and mounting the female from behind, copulating by inserting his aedeagus, and remaining coupled in this manner for an average of 2 minutes (Barrows and Gordh 1978). The male then withdraws the aedeagus and usually remains mounted on the back of the female for anywhere from a few minutes to several hours, presumably to guard her against other potential matings, thereby increasing his chances of fertilizing some of her eggs (Thornhill and Alcock 1983). Some incidental behaviors relevant to this study that occur during the mating process are the actions of rubbing and of abdominal undulation. These behaviors have been observed by multiple experimenters in the lab, and have also been reported in the oriental scarab *Anomala orientalis* (Facundo *et al.* 1999). Rubbing in this case occurs in both sexes when the beetles are copulating or in a mate-guarding mounted pair (occasionally it is observed in unmounted males in group situations), and is described as raising “either (or both) hindleg above the caudal end of the elytra and/or the exposed tergites and then stroked it (them) down with varying speed” (Barrows and Gordh 1978; Facundo *et al.* 1999). Undulation of the abdomen has been observed in both sexes in mounted pairs, and is described as “rhythmic anteroposteriad ‘pumping’ or pulsation of the abdomen” (Barrows and Gordh 1978; Facundo *et al.* 1999). What purpose these

behaviors may serve is unclear, however they occur with a high frequency and are associated with the mating process or the mating-related physiological state.

Because of the high importance and frequency of mating in JB, researchers have designed a highly attractive trap by combining an artificial form of the JB sex pheromone (japonilure) with a second lure that contains attractive floral compounds mimicking host plant volatiles. These traps can potentially collect hundreds of adults of both sexes; however despite this strong response there are beetles that do not react as expected/desired to these traps and will either ignore them or aggregate on nearby plants instead of entering them (Ladd 1970; Gordon & Potter 1985, 1986; Switzer *et al. in press*). Trapping success has been noted to decline throughout the adult season as well (Ladd 1970, Klein *et al.* 1981, unpublished data). Why these changes in response occur is unknown; recent mating experience, social situation, and seasonal effects may all come into play. A male that is actively engaged with a female, either copulating with or guarding her, may make a different decision about how to react to virgin pheromone than a male who is otherwise unoccupied. This could also be true for males who have very recently mated or, conversely, have had no recent mating encounters.

Finally, there is also the question of why female JB have sensilla pores capable of detecting their species' sex pheromone. No study to date has explored possible consequences for JB females' ability to detect the pheromone; some studies, however, have provided evidence of response including non-virgin females involved in mating balls and females that have been caught in traps containing virgin females as the only bait

component (Ladd 1970, Klein *et al.* 1972). This evidence is all circumstantial however; as no controlled experiment has been performed to purposefully or conclusively determine that JB females will react in a significant way to the sex pheromone itself.

### **Thesis Experiments**

The following experiments have attempted to address the questions, outlined above, regarding individual response differences in JB; (1) Why do males have a variable response, or fail to respond, to japonilure? What factors, such as their recent mating experiences or their current social situation, influence their reactions? and (2) do female JB respond to japonilure? Which females respond, and what factors (such as recent mating experience, size or egg load) might influence their response? Knowledge gained from investigating these questions could lead to more effective trapping and/or control practices for JB and potentially reduce the often devastating impact they can have on important economic and ornamental plant species across the U.S.

## Chapter 2

### Effects of sex and social experience on response to synthetic sex pheromone in the Japanese beetle (*Popillia japonica*)

#### Abstract

The Japanese beetle (*Popillia japonica*) is an invasive scarab that eats a wide variety of plants of ornamental and economic value in the USA. It uses a virgin-female-produced sex pheromone to facilitate its scramble competition mating system, in which males respond readily to this pheromone and attempt to locate and copulate with the female. There is evidence of intriguing individual response variation where this pheromone is concerned, however; not all males respond to the pheromone in the same way, and some females will respond for unknown reasons. Two experiments were conducted that investigated possible causes for variation in response. In the first experiment, we examined whether sex and recent social experience affected responses to pheromone, using the synthetic form (japonilure). Male and female beetles were tested individually in trials that exposed them to a pheromone-laden or a blank (control) air stream. Each sex treatment included two groups; those who had been kept only with the same sex and thus had experienced no mating in the last 24 hours, and those who had been kept with equal numbers of the opposite sex and thus had the opportunity to mate. In the second experiment, groups of beetles were tested in two social situations; all male groups of 5 beetles, and mixed-sex groups of 3 male beetles and 2 female beetles. All beetles in the

second experiment were kept with the same sex and were therefore without matings for ~24 hours. The groups were exposed to either control (ambient outdoor) or pheromone-laden air streams, and pair formation frequency as well as pairing duration were recorded. Results of the first experiment indicated that both sexes respond to the presence of pheromone. For females, both flight behavior and rubbing behavior increased in the presence of pheromone. For males, individuals were more likely to rub in the presence of pheromone but did not differ in their flying frequency. Both sexes showed that recent mating experience had an interactive effect with the presence of pheromone; beetles who had recent mating opportunities had a shorter latency to their first observed flight during the trial than unmated beetles when exposed to pheromone, but a longer latency than unmated beetles during control trials. Results of the second, social experiment indicated that although the presence of pheromone did not influence pair duration, pair formation frequency was affected by a combination of light levels and pheromone exposure. Individuals exposed to pheromone in higher-light situations had a significantly higher pair formation rate than those in control trials. The opposite was true of beetles in low-light situations however, with control trial beetles showing the higher frequency of pair formation, suggesting a connection between responsiveness or arousal level and environmental factors when responding to pheromone. Future studies to investigate the effects of different levels of pheromone exposure and other physiological factors such as level of hydration or satiation would be appropriate to further investigate the individual response variables discovered in this study.

## Introduction

The Japanese beetle (*Popillia japonica*) is a highly successful invasive species and a significant pest of many important economical and ornamental plants in the USA (Fleming 1972; Potter and Held 2002). Consequently, understanding the mating behavior of the Japanese beetle will be crucial to our ability to manage this pest. Japanese beetles are known to use a sex pheromone, (*R,Z*)-5-1-decenyl)-dihydro-2(3*H*)-furanone (Tumlinson *et al.* 1977) that is only produced and released by virgin female beetles once they emerge from the soil for the first time (Ladd 1970; Fleming 1972; Potter and Held 2002). Male beetles are strongly drawn to this volatile pheromone and will even form “mating balls” of several scrambling males around a single virgin (Ladd 1970; Fleming 1972). Because of the highly attractive nature of the pheromone, designers of Japanese beetle traps have included its synthetic form (japonilure) in their trap lures. In practice however, while many beetles end up in the traps, many others linger around the trap without entering it, or do not appear to respond to the trap’s nearby presence (Gordon and Potter 1985, 1986; Switzer *et al. in press*). These observations hint at an underlying mechanism to decide, at the individual level, whether or not to respond to pheromone, and to what degree. Evidence of a ‘response hierarchy’ based on pheromone signal strength has been found in behavioral studies dealing with insect pheromone detection that could help explain some of this variation (Shorey 1973), but does not help explain these individual differences between beetles apparently exposed to the same level of pheromone.

We know surprisingly little about what may affect pheromone-elicited behaviors in the Japanese beetle, especially given the importance of pheromone in current trapping approaches. Heath *et al.* (2001) showed that environmental conditions such as temperature and light levels played an important role in the beetles' response to pheromone in a wind tunnel. Other factors that could affect response have not been investigated, however; these could include the mating history of the beetle and the current social environment in which the beetle finds itself when the pheromone is detected. Such factors are known to be important in other species. For instance, unmated males of the red flour beetle *Tribolium castaneum* were more responsive to pheromone-baited traps than their mated counterparts (Fedina and Lewis, 2007). Studies investigating neuronal responses to sex pheromone in the sphinx moth *Manduca sexta* have shown that increased levels of serotonin in the brain can amplify neural responsiveness to sex pheromone (Kloppenborg *et al.* 1999); serotonin levels are influenced by activities such as mating, feeding and other interactions, indicating that the moth's recent behaviors have some effect on the strength of the response to sex pheromone. Finally, the recent mating history of the individual may influence its likelihood of performing mating-related behaviors (Browne 1993).

Although female Japanese beetles produce pheromone to attract males (Ladd 1970; Fleming 1972, Potter and Held 2002), response to pheromone may not be limited to males alone. Female Japanese beetles also have pores on their lamellae that are capable of detecting their species' sex pheromone (Kim and Leal 2000). Females have approximately half the number of pores that males have, but the pores are otherwise



structurally identical. Furthermore, females have been captured in traps baited solely with virgin females (Klein *et al.* 1972), with japonilure (Klein *et al.* 1981), and have also been occasionally found involved in mating balls (Switzer, unpublished data). Pheromone self-detection or 'autodetection' (the sensing of female-produced sex pheromone by conspecific females) has been demonstrated in a variety of insects, most notably Lepidoptera (Ochieng *et al.* 1995; Schneider *et al.* 1998), but also in at least some scarab beetles. For example, females of the scarab *Holotrichia consanguinea* are attracted to traps baited with female sex pheromone almost in the same numbers as males (Leal *et al.* 1996). Very little has been investigated regarding female Japanese beetle response to conspecifics' sex pheromone, however. These observations about their incidental behavior and basic physiology raise interesting questions about the role of autodetection in the mating system of the Japanese beetle, and more specifically, what sort of behavioral responses might actually occur in females and in what relation to the responses of the males.

In this study, I investigated whether an individual's social situation or recent mating opportunities might affect pheromone-elicited response behaviors in Japanese beetles. I tested both male and female beetles, which allowed me to determine whether females alter their behavior in response to pheromone, and if so, whether their response is similar to that of males. Rather than using a large wind-tunnel apparatus to test for simple directed flight behavior as is common in pheromone response studies (e.g. Allison and Cardé 2007; Lelito *et al.* 2008), we examined more subtle behaviors by performing pheromone exposure trials in a smaller test chamber that was designed not only for the

expression of some directed locomotion, but also for allowing the trials to be conducted within an environmental chamber which provided opportunity to control temperature and light conditions while allowing close observation of the individual beetles. Consequently, an associated goal of this study was to examine whether such smaller scale methodology would be successful in eliciting behavioral responses to pheromone in the Japanese beetle.

## **Methods**

The experiments described below took place during July and August of 2007 in Coles Co., Illinois, USA. Beetles were captured on food plants at approximately 1000 hr, with the exception of the females used in the solitary pheromone experiment who were captured using Tanglefoot™ Japanese beetle field traps. Trapped females were not allowed to remain in the trap for more than 30 min. After capture, beetles were sexed using characteristic foreleg morphology (Smith and Hadley 1926) and separated into their respective treatments. Beetles being held were provided with leaves from little leaf linden, *Tilia americana* L., for food.

### *Solitary testing*

#### Procedure

For the solitary experiment, beetles were separated into plastic screen-topped containers and provided with leaves for food and damp paper towels for moisture. To vary mating

opportunity, we adjusted whether only members of the same sex or some members of the opposite sex were present in the holding container. For the 'both-sexes' treatment, we placed equal numbers of each sex in the container (these individuals are hereafter referred to as 'mated' beetles), and for the 'same-sex' treatment, containers only contained a single sex overnight before being used for the following day's trials (hereafter referred to as 'unmated' beetles). The beetles were therefore held in these social conditions for approximately 24 hr prior to testing.

Trials were performed inside an environmental chamber to control for light, temperature and humidity levels. The chamber was kept at 50% relative humidity and 26°C inside the testing boxes with a light level of 3300 fc for all trials.

The trials were performed in two 15 cm x 15 cm x 15 cm acrylic boxes connected in series (Fig. 1). Air flow was made unidirectional by having tubing hooked up to an inflow and outflow port on each box. To this tubing, a small aquarium pump was attached to pull air through the box. The first box in the series (from which the air was being drawn) was considered the stimulus box, and for pheromone treatments, the japonilure section of a Japanese beetle trap lure (Tanglefoot, Inc.) was placed in the box. The second box in the series, considered the testing box, held the beetle for observation. This testing box had a 15 cm x 6.5 cm x 6.5 cm rectangular arm connected 'upwind' of the main box. The purpose of this arm was to allow directional movement toward the air source. On the bottom of each box we taped a single layer of paper towel for traction and absorption of any excretions by the beetles, and boxes were made relatively air tight by

sealing the lids and around the tubing with Blu-Tack adhesive putty by Bostik™. In inflow tube drew air from the chamber to maintain constant humidity and temperature. The outflow tube was run outside the laboratory window to expel any pheromone-tainted air from the testing chamber and the laboratory. Between trials, the tubing was disconnected from the test box and clamped to prevent air from the stimulus box escaping.

Trials were run between 1000 h and 1600 h each day. One trial from each pheromone treatment (japonilure or control with no japonilure) was run simultaneously in different setups. To begin, a beetle was placed into the testing box, the lid was sealed with putty, and the tubing between the stimulus and test box was clamped and disconnected. The beetle was allowed to acclimate for 10 minutes with air flow occurring only through the testing box. After the acclimation period, the tubing was connected to the test box and un-clamped so air then pulled first through the stimulus box before entering the testing box. Observations of the location (i.e. testing box main chamber or arm) and activity of each beetle were made at 30-second intervals for 10 minutes during this “exposure period.” Activity consisted of specific behaviors, e.g. not moving, crawling, flight or rubbing. ‘Rubbing’ was defined as one or both of the hind tarsi being drawn or stroked repeatedly down the beetle’s side, sometimes including the elytra.

The paper towel on the bottom of each testing box was replaced at the end of each trial, and all testing boxes were washed out at the end of each day (approximately 10 trials). A box’s assignment to control or japonilure trials was random between days but consistent

during the day's trials.

### Analysis

A simple chi-square test was performed for each sex to compare the number of beetles that flew at least once during the exposure period to those that did not fly at all with respect to recent mating experience and trial type. In addition, for each replicate, we determined their latency to first flight, with the initial time starting at the beginning of the exposure period, and their frequency of rubbing behavior. Only beetles that flew were used in the analysis of flight latency, and rubbing frequency was calculated as the number of post-exposure observational passes (behavioral records taken every 30 seconds) that a beetle was noted to be performing the rubbing behavior divided by total number of post-exposure observational passes. We used two-way ANOVA to compare flight latency and rubbing frequency with factors of recent mating experience and trial type for both sexes. Two additional ANOVAs were performed to investigate the effects of the factors of size and egg load on flight latency. Size was divided into two classes; above and below the mean. Egg load was divided into three classes; zero mature eggs, and above and below the mean number of eggs for those beetles that had mature eggs. Only 13.2% (16) of the females we were able to obtain egg load and size data for displayed the rubbing behavior, which was not a large enough sample for a sufficient analysis of the possible effects of the factors of egg load and size on rubbing frequency. The same was the case for males, with only 17.8% (26) having a measurable rubbing frequency.

## **Group Testing**

### Procedure

For the group experiment, following capture and sex identification, beetles were prepared for the following day's experiment by being separated into containers by sex, provided with food, and individually marked by gluing queen bee marking tabs of different colors (E.H. Thorne, Ltd.) on the pronotum.

For testing, we constructed boxes that allowed for directional air flow and easy viewing. The frame of these boxes was cardboard measuring 25 cm long x 20 cm wide x 20 cm tall that had four large windows (15 x 20 cm on the larger sides and 15 x 15 cm on the smaller sides) cut on the four sides of the box. On the two facing ends, we applied plastic window screen mesh with silicone caulk; this allowed for directional air flow through the box. To facilitate viewing, we covered the windows on the two sides with thin acrylic, and fit the lid of the box with a hinged sheet of clear acrylic. The bottom was covered with a thin acrylic sheet for durability and ease of cleaning. Inside the box, we placed a leafless stick for climbing substrate.

Two types of group treatments were conducted. Both of these treatments consisted of putting five beetles into a box and allowing 15 minutes for acclimation in the shade. The all-male treatment used five males. The mixed-sex trial used three males and two females, which approximates the natural sex ratio on food plants (Switzer *et al.* 2001). An all-male treatment was used in order to determine if males behaved differently when

exposed to pheromone in social situations were females were not present, as well as to investigate the instances of male-male interactions such as attempted mounts.

Trials were run between 1000 h and 1400 h each day in light shade. This was intended to provide relatively high, natural light levels which may be important for response to pheromone (Heath *et al.* 2001) and mating behavior (Switzer *et al.* 2008) without overly heating the interior of the box or triggering phototaxis behavior in the beetles. Light levels were recorded with a hand-held light meter (Apogee Instruments, Inc.) and temperature was recorded at the beginning of each trial (temperature ranged from 25°C to 32.5°C, average temperature  $29.6 \pm 1.65^\circ\text{C}$ ; light levels ranged from 230 to 800fc, average light level  $451.48 \pm 155.30\text{fc}$ .)

After the 15 minute acclimation period, the box for a given trial was set carefully on a solid surface and a PVC pipe (13 cm in diameter, 91.5 cm in length) was set in place to direct airflow into the box through one of the screened sides. A portable fan was positioned at the other end of the tube and used to blow a steady stream of air (1.5 m/s) through the tube and the box, respectively. For the pheromone treatment, the japonilure portion of a Trece Japanese beetle trap lure (Tanglefoot, Inc.) was hung 4 centimeters inside the tube opening downwind of the fan. For control trials, the tube was left empty.

Each trial lasted 12 minutes. At 30 second intervals, we made observations of the location and activity of each beetle. Behaviors recorded focused on social interactions related to mating and included mounting or being mounted, attempted mounting, and fighting. To

be considered 'mounted' a beetle would need to be successfully perched on the back of another beetle with the tarsi hooked to the ridge of the mountee's elytra in the typical mounted position of these insects (Barrows and Gordh 1978), and would need to cease movement of the tarsi. Attempted mounting was defined as one beetle approaching and crawling up on to the dorsal surface of another beetle but not completing the mounting position due to the mounting individual's disengagement or resistance from the mounted individual. Fighting was defined as a beetle approaching a mounted pair and attempting to wedge the top-mounted beetle off of the bottom beetle by forcing its head between the abdomens of the two (Kruse and Switzer 2007). This is accompanied by pushing and crawling motions of the tarsi as well as resistance by the top-mounted beetle in the form of kicking and sometimes jerking of the body, and sometimes by the bottom-mounted beetle in the form of actively crawling away. After the acclimation period, the fans were turned on and the japonilure baits were affixed to the inside of the tubes for the pheromone treatment. Pheromone and control trials were alternated throughout the day in order to balance the environmental conditions experienced by beetles in the two treatments. To avoid cross-contamination of treatments, boxes and PVC tubes were kept consistent with respect to treatment type and all boxes were wiped with ethanol between trials. Each individual beetle was only used in a single trial.

### Analysis

Light levels during the group trials were variable (range = 570 fc, average light level  $451.48 \pm 155.30$ fc). Because light may be an important variable in the behavioral responses of Japanese beetles, we added light as a factor in our analysis. Light levels



were categorized into two groups, “low” and “high,” below and above the mean. For each all-male replicate, we determined the number of mounts attempted and the latency to the first attempted mount after exposure to either japonilure or control. For each mixed-sex replicate we determined the number of pairs initiated during each trial (pairing frequency) as well as pair durations, defined as the number of observational periods a pair remained in mounted contact. We then used one-way ANOVA to compare attempted mounts and mounting latency between trial types for all-male trials and to compare pairing frequency and duration between trial types for mixed-sex trials.

## **Results**

### Pheromone response in solitary trials

Among females, individuals exposed to pheromone were more likely to fly at least one time during a trial than control females (Fig. 2;  $\chi^2 = 4.85$ ,  $df = 1$ ,  $P = 0.028$ ); the frequency of flight was similar for mated and unmated females. For males, the likelihood of flight was not affected by recent mating experience or pheromone exposure (Fig. 2;  $\chi^2 = 1.04$ ,  $df = 3$ ,  $P = 0.79$ ).

Neither recent mating experience nor pheromone exposure were significant individual factors affecting the latency to flight for females that flew at least once during the trial period after exposure began (mated vs. unmated:  $F_{1,37} = 0.59$ ,  $P = 0.45$ ; control vs. pheromone trials:  $F_{1,37} = 0.83$ ,  $P = 0.37$ ). However, a significant interaction existed between recent mating experience and pheromone exposure in these females (Fig. 3a;

$F_{1,37} = 4.22$ ,  $P = 0.047$ ); unmated females had longer latency to flight in control trials and a shorter latency when exposed to pheromone, whereas mated females had a relatively shorter latency to flight in the control trials and a longer latency when exposed to pheromone (Fig. 3a). Likewise for males who exhibited flight behavior at least once after exposure began, recent mating experience and pheromone exposure were not significant as individual factors (mated vs. unmated:  $F_{1,44} = 0.03$ ,  $P = 0.86$ ; control vs. pheromone trials:  $F_{1,44} = 1.28$ ,  $P = 0.27$ ), but a borderline significant interaction existed in the same direction as for females (Fig. 3b;  $F_{1,44} = 3.30$ ,  $P = 0.076$ ). Neither the physical factors of size ( $F_{1,48} = 0.061$ ,  $P = 0.81$ ) nor egg load ( $F_{2,44} = 0.421$ ,  $P = 0.66$ ) showed a significant effect on the latency to flight for females. Male size appeared to have a borderline significant effect on latency to flight when recent mating experience was considered ( $F_{1,79} = 3.74$ ,  $P = 0.057$ ), however the observed latencies were usually highly variable as a consequence of small sample size (mated males: latency below mean size =  $2.46 \pm 3.24$ ,  $N = 26$ , latency above mean size =  $4.14 \pm 0.21$ ,  $N = 18$ ; unmated males: latency below mean size =  $3.85 \pm 3.69$ ,  $N = 17$ , latency above mean size =  $2.52 \pm 3.38$ ,  $N = 26$ ).

Rubbing behavior was observed in both sexes. For all females tested, recent mating experience did not appear to be a significant factor in rubbing frequency post-exposure (defined as the percent of total observational scans a beetle was observed rubbing) ( $F_{1,149} = 0.001$ ,  $P = 0.97$ ), however rubbing occurred at a higher frequency in female beetles exposed to pheromone than in control females (Fig. 4;  $F_{1,149} = 5.94$ ,  $P = 0.016$ ). Similarly for all males tested, rubbing frequency was much higher for males exposed to pheromone (Fig. 4;  $F_{1,174} = 17.71$ ,  $P < 0.001$ ) but was not affected by recent mating experience ( $F_{1,174}$

= 0.50,  $P = 0.48$ ). For females, neither recent mating experience nor exposure to japonilure significantly affected the latency to the start of the rubbing behavior, however the two factors together produce a significant model ( $F_{3,41} = 2.99$ ,  $P = 0.042$ ). This was not the case for males; latency to the start of rubbing behavior was not affected by recent mating experience or exposure to japonilure ( $F_{3,49} = 0.97$ ,  $P = 0.41$ ).

Other recorded behaviors were rarely observed; undulation of the abdomen, washing movements, and head-bobbing behaviors were all observed during less than 3.5% of the observational periods.

#### Pheromone response in group trials

In the group trials, those beetles in mixed-sex trials did not differ in their duration of pairing with regard to pheromone exposure ( $F_{1,50} = 1.75$ ,  $P = 0.19$ ;  $N_{\text{control}} = 28$ ,  $N_{\text{pheromone}} = 26$ ) or with regard to light levels ( $F_{1,50} = 0.36$ ,  $P = 0.55$ ;  $N_{\text{low light}} = 24$ ,  $N_{\text{high light}} = 30$ ). With regard to number of pairs formed in these mixed-sex trials, exposure to pheromone was not a significant factor ( $F_{1,26} = 0.00$ ,  $P = 0.99$ ), nor were light levels ( $F_{1,26} = 0.57$ ,  $P = 0.46$ ). A significant interaction did exist between light levels and exposure to pheromone with regard to number of pairs formed, however (Fig. 5;  $F_{1,26} = 5.31$ ,  $P = 0.029$ ). On average, more pairs were formed under high light in the pheromone treatment but under low light with the control treatment.

Male beetles in all-male trials often attempted to mount other males; however, the frequency of male-male attempted mounting was not different between control and

pheromone trials ( $F_{3,146} = 0.004$ ,  $P = 0.59$ ). Observed instances of fighting post-exposure to control or pheromone were uncommon and sporadic and did not occur with enough frequency to perform a successful analysis; they are, if anything, slightly biased toward the control trials (all-male control = 6 instances, mixed control = 14 instances, all-male pheromone = 1 instance, and mixed pheromone = 5 instances).

## **Discussion**

### *Solitary trials*

The results of our solitary beetle trials indicate that both female and male beetles show behavioral responses to the presence of synthetic sex pheromone. Females, but not males, flew in the experimental apparatus during solitary trials with higher frequency in the presence of pheromone. Both sexes showed an effect of pheromone on the length of time before their first flight, but only when their overnight exposure to the opposite sex was taken into account. Also, our results clearly displayed a strong trend toward higher rubbing frequency in the presence of pheromone.

Beetles that had been kept with the opposite sex and therefore had recent mating opportunities, although quicker to initiate flight behaviors in the control trials, appeared to become relatively slower to fly when exposed to japonilure than those held previously in same-sex groups (unmated beetles). What exactly this indicates is unclear; a longer latency to flight may be interpreted as a sign of pheromone detection and response on the

one hand, especially when one considers the fact that high levels of pheromone inundation can bypass seeking behaviors and lead directly to the triggering of copulatory-type behaviors (Shorey 1973) which do not involve flight. This would indicate that beetles with recent mating experience were more stimulated by pheromone in this case, however we cannot be sure of the relative inundation of pheromone with regard to the response hierarchy as no other concentration was supplied for a relative comparison.

Support for the alternate interpretation that initiation of flight is likely the more telling response factor observed in our experiments can be found in the case of the red flour beetle (*Tribolium castaneum*); unmated beetles of this species were more responsive to traps baited with aggregation pheromone than their mated counterparts (Fedina and Lewis 2007). The 'unmated' Japanese beetles held previously in same-sex groups may be more responsive generally to mating-related stimuli, therefore, and vice versa for those individuals that were given mating opportunities. At an ultimate level, this makes sense because recent mating experience has been shown to affect an insect's mating-related behaviors; it has been observed in several species that the act of mating is followed by a period of time in which additional matings are less likely to occur, and that this effect is even more pronounced in females than in males (Browne 1993). At a proximate level, physiological changes related to activity differences between mated and unmated individuals also need to be considered when attempting to explain this pattern. In the moth *Manduca sexta*, serotonin levels are highest in active individuals, and high serotonin levels amplify the neural responsiveness to sex pheromone in the brain (Kloppenborg *et al.* 1999). In Japanese beetles, unpaired beetles tend to be more active

than paired beetles (Tigreros and Switzer 2008), and beetles may remain paired for hours, even through the night for some beetles (Tigreros *et al.* 2007). Therefore, if Japanese beetles have a similar physiological response to activity as *M. sexta*, the relative increased activity in unmated individuals may lead to an increased responsiveness to sex pheromone. This interpretation of flight latency differences may also be supported by the observation that unmated beetles were slower to fly than the beetles with recent mating experience in the control trials; if they are truly more responsive because of higher serotonin levels (or another physiological reason), then this longer flight latency could be considered complementary to their shorter latency to flight during pheromone exposure trials if they are indeed responding to pheromone in a way that involves the initiation of flight. Obviously these potential explanations of our observed patterns are speculative, and future studies would need to examine the effect of feeding and mating on serotonin levels and the resulting pheromone sensitivity levels to provide support for this hypothesis; they should also investigate responses triggered by exposure to differing concentrations of pheromone to clarify the reason for markedly increased flight latency in beetles with recent mating opportunities, and the opposite pattern for those deprived of recent mating activities.

Female, but not male, beetles were more likely to fly at least once during a trial when pheromone was present. This difference in responsiveness could be due to methodological and/or seasonal differences in the male and female trials. Male trials were run first and used beetles that were invariably caught from food plants. Female trials were run two weeks after the start of male trials and, because of relatively low beetle

numbers at that time, females were captured using traps with both the floral and japonilure lure components. Therefore, female subjects, but not male subjects, may have been biased toward individuals that were more likely to be active in the presence of pheromone in the first place. However, even if true, this still would not explain why males were not more likely to fly when exposed to pheromone. At least two potential explanations exist for the lack of a male flight effect. First, high levels of pheromone (such as the full lure used in this study) may indicate a close proximity to the female or be an overwhelming response as postulated in the flight latency interpretation, and therefore flight may not be warranted. or triggered. Second, high levels of pheromone may indicate trigger a response that does not involve flight, as postulated in the flight latency interpretation. Finally, male activity in response to stimuli such as pheromone may not be equally likely throughout the season. Sex ratio in trap captures does seem to become less male-biased as the season progresses (Klein *et al.* 1981). Therefore, while there are a number of alternative explanations for this pattern, a change in pheromone responsiveness is one possibility.

Perhaps the most intriguing result of this study is that females responded to sex pheromone at all. In Japanese beetles, females produce this pheromone and its apparent purpose is as a male attractant. Female pheromone autodetection is present in other insect species including other scarabs (Gokce *et al.* 2007; Ochieng *et al.* 1995; Palanaswamy and Seabrook 1978; Schneider *et al.* 1998), and in Japanese beetles, its effect has been observed during trapping experiments (Klein *et al.* 1972, 1981; Leal *et al.* 1996) and electroantennogram measurements (Kim and Leal 2000). In Japanese beetles, the

situation is somewhat different than in other species, however, because only virgin female Japanese beetles produce pheromone. The non-virgin females in our experiment therefore would be responding to the pheromone of conspecific females who are at an earlier point in their reproductive life. Potentially, non-virgin females may be attuned and attracted to the virgins' pheromone as a way to gain additional matings for themselves at a convenient time (such as directly prior to oviposition), and/or may even be attempting to utilize the competition between males created by the scramble to get to the virgin females to acquire sperm from the most tenacious males. Of course our observations may have been the result of exposing females to levels of pheromone much higher than they would normally experience on food plants, and thus we may have been observing a behavioral artifact of our methods. However the presence of female beetles in virgin female or japonilure-only baited traps (Klein *et al.* 1972, 1981) and in mating balls (PVS, unpublished data) suggest that a response exists under more natural concentrations and conditions, and that the behaviors displayed were not merely reactions to an overwhelming stimulus. Alternatively, because we did not observe a directional or seeking-type response in our experiment, there is the possibility the female behaviors we observed may even have indicated an aversion to pheromone rather than an attraction. Clearly, future studies should address issues of pheromone concentration and investigate the observed behavioral sequences and patterns in greater detail, which would allow for an improved understanding of the response variety among females and better deciphering of the purpose and/or intention of the behaviors displayed.

Rubbing activity for both sexes was notably higher in the presence of pheromone. What



the Japanese beetle accomplishes with rubbing is unclear. Female scarabs of other species such as *Exomala orientalis* display rubbing behaviors linked with “calling,” or pheromone dispersal (Facundo *et al.* 1999); however, because only virgin female Japanese beetles emit pheromone (Barrows and Gordh 1978), it is not clear if it is appropriate to associate non-virgin female rubbing behavior with this explanation. Females of the eastern spruce budworm *Choristoneura fumiferana* have been observed to perform their species’ calling behavior in the presence of female pheromone (Palanaswamy and Seabrook 1978). Likewise, female *Holotrichia consanguinea* (a scarab that utilizes a female-produced sex pheromone) that were attracted to, but not captured by, traps baited with female pheromone performed calling behaviors where they landed (Leal *et al.* 1996). Therefore, in female Japanese beetles we may be observing a similar sort of calling response to pheromone.

Male Japanese beetles also performed similar rubbing motions. In scarab species in which the male is a pheromone producer, such as *Kheper nigroaeneus* and other ball-rolling dung beetles, leg motions similar to those of a calling female have been observed (Edwards and Aschenborn 1988). These motions have been observed in Japanese beetle males when a male is mounted on the back of the female before, during and after copulation, in which case he rubs her abdomen rather than his own (Barrows and Gordh 1978). Considering the absence of a female in these solitary trials, it is possible this rubbing behavior observed in males is similar to the stimulating rubbing behavior he would perform on a female. Alternatively, or perhaps additionally, this behavior could also represent the response behavior in a male that is triggered by intense pheromone

inundation, and may even explain the flight latency and frequency patterns observed. In some insect species, the female sex pheromone present at a high concentration can operate in the absence of all other female-produced stimuli to cause release of male copulatory behavior (Shorey 1973). High pheromone concentration may have triggered behaviors that were further along the pheromone response sequence than flying. That is, while flying might be considered an initial mate-seeking behavior, rubbing may be more associated with males who have located a female. Future studies offering beetles a variety of pheromone concentrations, and exposing mounted males to pheromone, could help determine at what point a male is triggered to begin rubbing and thereby determine whether this observed flight disparity is linked to the increased rubbing behaviors (due to the effects of the response threshold hierarchy) or some other factor, such as simply the absence of a female to perform the rubbing behavior upon.

Finally, although the functional explanations for our results will require further studies, we successfully elicited responses to sex pheromone in Japanese beetles on a scale that is much more practical for many researchers than a full-fledged wind tunnel, especially considering the importance of light and temperature on the pheromone response of Japanese beetles (Heath *et al.* 2001). Future studies should consider using a similarly-scaled apparatus when examining pheromone-related behavior in this species.

### *Group trials*

Interestingly, in our group trials, we generally found that beetles did not alter their behavioral patterns when exposed to pheromone. The number of pairings that occurred in

mixed-sex trials did differ depending on both the presence of pheromone and light intensity, however. Exposure to pheromone seemed to depress pairing activity in low light but encourage it in high light, while the control trials suggested the opposite pattern; that beetles tended to pair with slightly higher frequency in low light than in high light. This is an unexpected result considering the apparent relationship between high light intensity and higher beetle activity and response levels (Heath *et al.* 2001; Switzer *et al.* 2008), but nonetheless hints at the fact that pheromone presence may have an effect on the level of arousal a beetle experiences and the subsequent instigation of mating-related interactions among individuals.

Our experiment was unable to detect differences in the responses of control-trial beetles and japonilure-exposed beetles in social settings. A possible explanation for this is increased mate guarding tendency; Japanese beetles have been shown to mate-guard for longer in the presence of other beetles (Saeki *et al.* 2005). Because social aggregations are often male-biased, a male may not as easily find another mate if he leaves the one he's found with other competition so nearby. In addition to the possible effects of this increased guarding tendency, males may be less likely overall to respond to stimuli such as pheromone when they find themselves in a social setting; they often spend much of their lifetime locating and persisting in large aggregations (Loughrin *et al.* 1995). Perhaps once they are within an aggregation, males may be less receptive to outside stimuli than they would be if they were solitary. These factors may make it not altogether surprising that the interval of time pairs stayed together was not different between control and pheromone-exposed beetles, or that attempted mountings among-all male groups were

similar in frequency. An additional reinforcing circumstance could be that the high level of pheromone inundation may have resulted in stimulating copulatory behaviors in the beetles (Shorey 1973) to the point where previous mating status was not enough of a factor to influence behavioral patterns. Mountings and copulations were in many cases already underway once the trials started, in which case the pheromone could have acted as a redundant stimulus that would not instigate further behavioral differences.

Investigating the responses of individuals and groups to a gradient of pheromone levels would be the next logical step for investigating response behaviors in these beetles. A controlled range of pheromone concentrations could reveal additional patterns, or better refine those patterns observed in this study. More fine-tuning of the amount of pheromone the beetle is exposed to may also reveal the hierarchical stages of response behavior (Shorey 1973) with greater precision and resolution, shedding more light into the role of physiological and/or social condition in the pheromone response behaviors of this scarab.

Additionally, the responses of naturally occurring aggregations or small groups in the field to pheromone plumes could provide a clearer picture of the social factors involved in pheromone response decisions; questions addressed by this approach could include if individuals in large aggregations respond more or less to pheromone presence than their solitary counterparts, or how the size of the group affects behavior in smaller clusters. Other physiological states such as age and recent feeding activity may also be worthwhile to investigate, as they could play a role in an individual's likelihood of responding to pheromone in some manner. Further studies along these lines would be especially

intriguing in the case of females; as they have been shown to detect and respond to pheromone in this study, further uncovering the strength of females' response patterns and the variation therein would be a very interesting next step in the investigation of the mating system of this beetle and of other scarabs with similar mating systems. A small-scale apparatus for individual behavioral observations was successful in this case and could also prove to be a useful tool for future investigations into the finer details of the behavior of these beetles and other insects' pheromone response behaviors.

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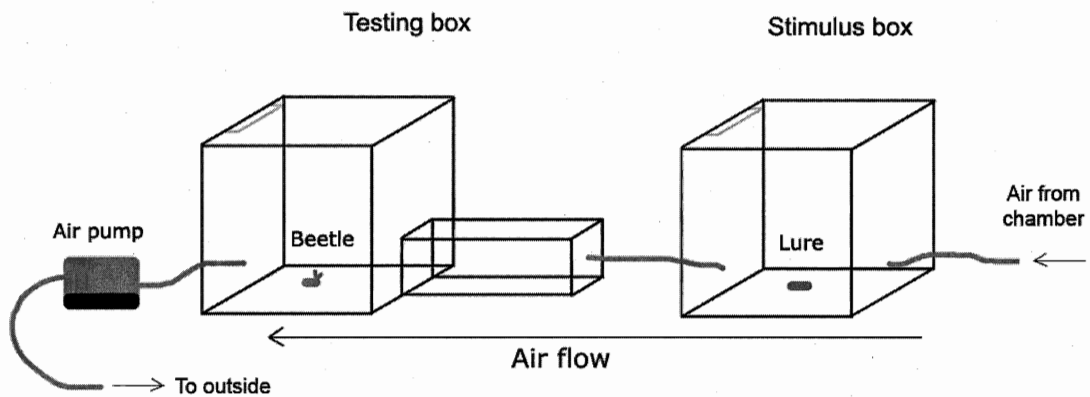
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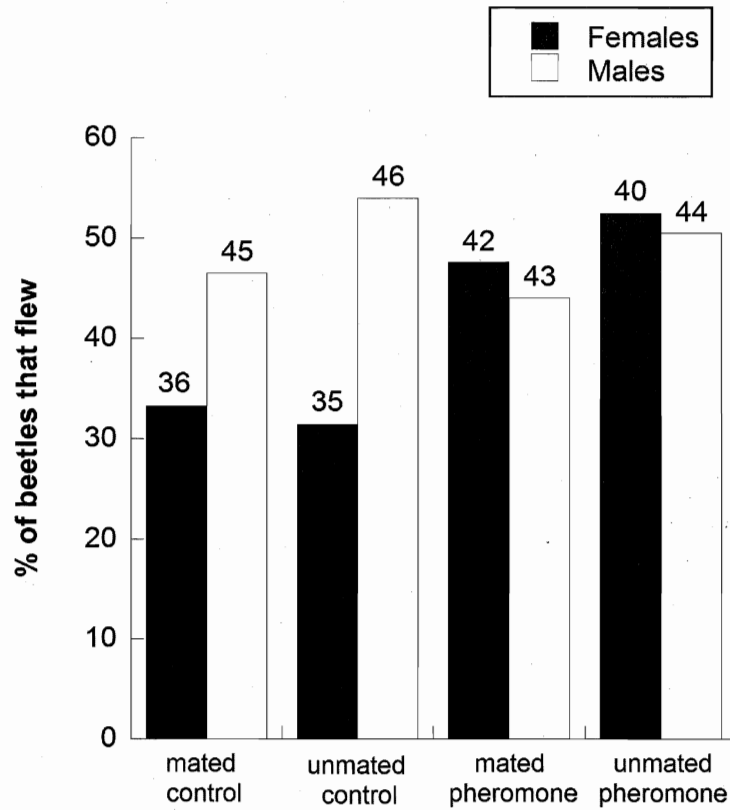
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## Figures

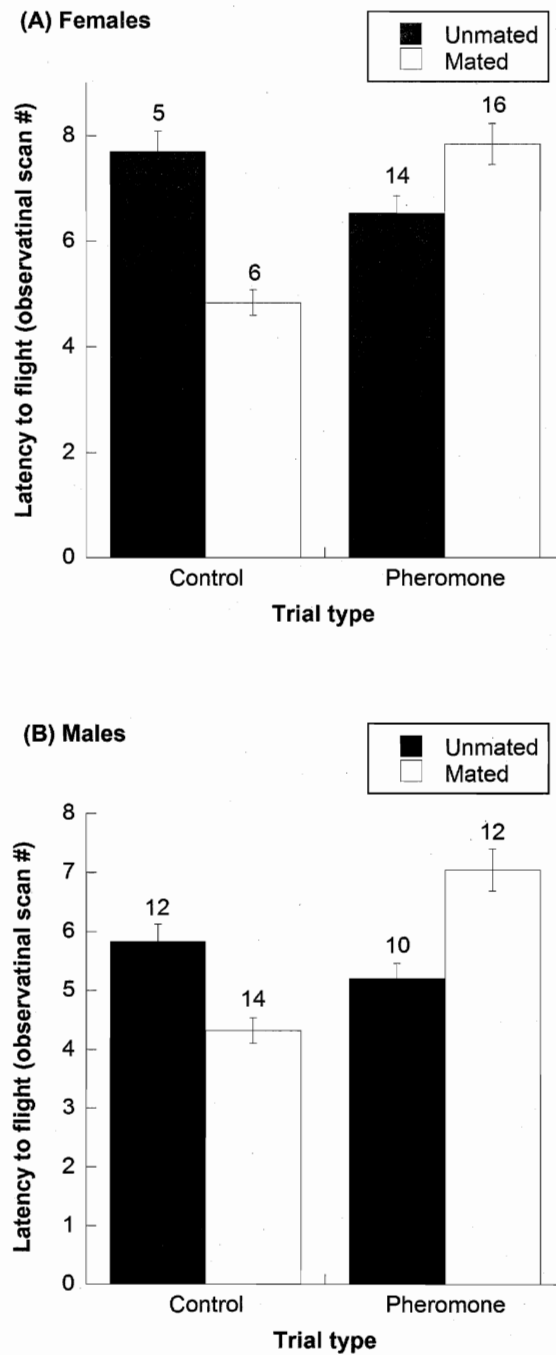


**Fig. 1:** Testing apparatus setup during trials – Air is drawn in from the environmental chamber, through the stimulus box containing a weigh boat holding either a Pherocon lure or nothing (control), then drawn through the testing box containing the beetle and pumped outside.

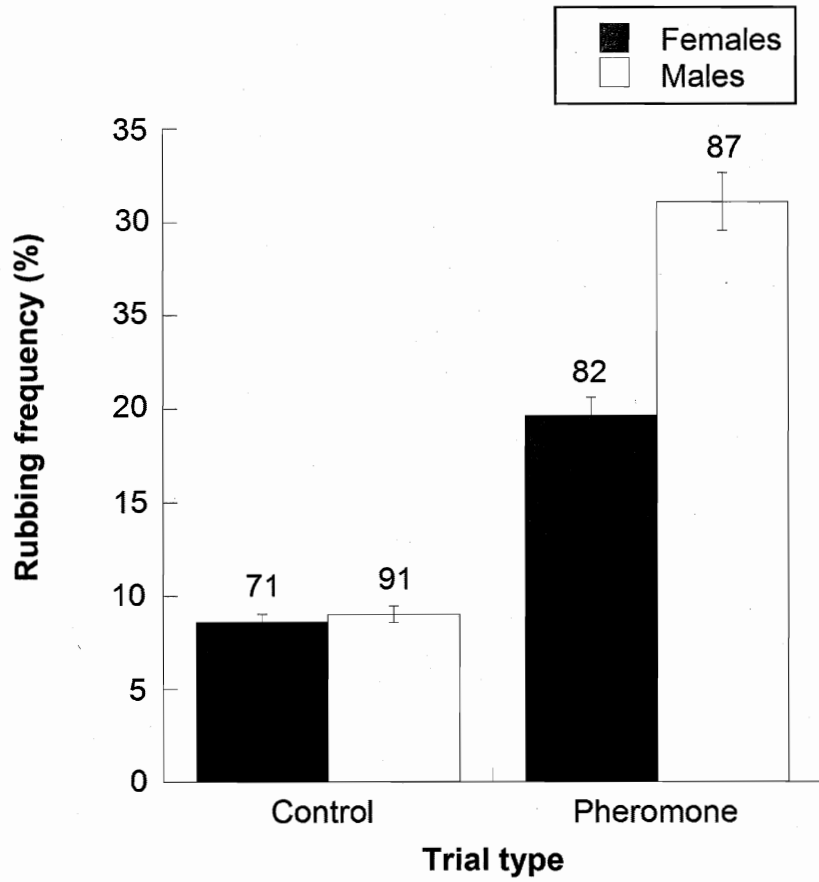


**Fig. 2:** Percentages of those beetles that flew at least once during the trial for female and male mated/unmated and control/pheromone-exposed Japanese beetles tested individually in the environmental chamber. Numbers above bars represent total sample size.

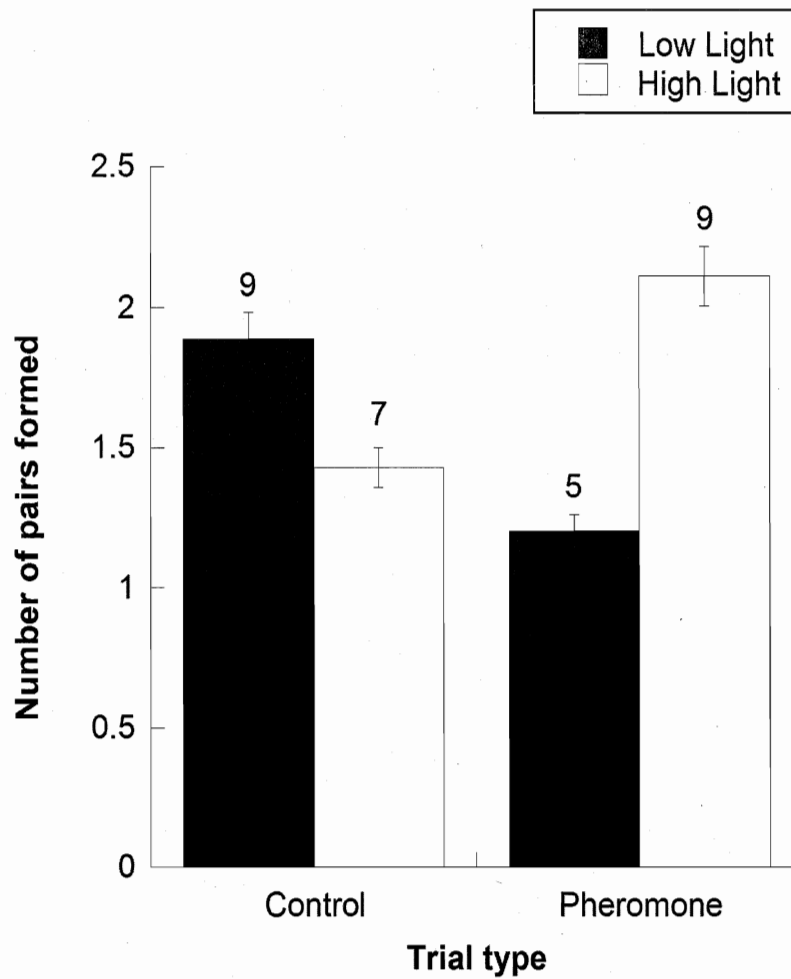




**Fig. 3:** Relative latency to flight (uncorrected  $\pm$  SE) for female (A) and male (B) control-trial and pheromone-trial Japanese beetles tested individually in the environmental chamber (females:  $N_{\text{control}} = 11$ ,  $N_{\text{pheromone}} = 30$ , males:  $N_{\text{control}} = 26$ ,  $N_{\text{pheromone}} = 22$ ). Numbers above bars represent sample size.



**Fig. 4:** Relative rubbing frequency (number of observational scans rubbing was observed over total, uncorrected  $\pm$  SE) for female and male control-trial and pheromone-trial Japanese beetles tested individually in the environmental chamber. Numbers above bars represent sample size.



**Fig. 5:** Numbers of Japanese beetle mounted pairs formed (uncorrected  $\pm$  SE) in mixed-sex trials under high-light and low-light conditions with and without exposure to pheromone. Numbers above bars represent sample size.