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Physiological Status of Male and Female *Popillia japonica* (Coleoptera: Scarabaeidae) Affects Mating and Grouping Behavior

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ABSTRACT Because mating may be costly, sexually active males or females are predicted to be in relatively good physiological condition and may preferentially direct their mating behavior toward relatively high-quality mates. We tested this hypothesis in Japanese beetles (*Popillia japonica* Newman), a pest species in which males and females may be either isolated or in aggregations while feeding on host plants. We examined male size and lipid content and female size and egg load with respect to both their pairing status and whether they were isolated or in aggregations. Males that were paired had the highest lipid levels, and single, isolated males had the lowest. Paired females had the highest egg loads and single, isolated females had the lowest. Neither male nor female size was related to pairing status. Females captured during the times of relatively high pairing frequency (i.e., morning and evening) had higher egg loads than females captured at times of lower pairing frequency (i.e., afternoon). These results suggest that mating and aggregative behaviors in Japanese beetles are dependent on the physiological status of males and the reproductive condition of females.

KEY WORDS Japanese beetle, aggregation, lipid, fecundity, mate choice

For males, reproduction may be costly in terms of energy expenditure, risk of injury, predation, diseases, and missing the opportunity of searching for other mates (Thornhill and Alcock 1983, Bonduriansky 2001). These costs can lead to variation among males in their participation in reproduction at a particular time. For example, a male's nutritional state can be associated with its participation in leks (Warburg and Yuval 1997, Yuval et al. 1998), and energy reserves may determine a male's success when mate searching (Otronen 1995) and influence his ability to mate guard (Sparkes et al. 1996). Additionally, costs of mating may lead those males that are trying to mate to direct mating behavior preferentially toward relatively high quality females (e.g., more fecund females). Accordingly, males of some species are more likely to mate with, or devote more resources toward, females with more eggs (Parker 1983, Bonduriansky 2001). Thus, one would predict that, when reproductive behavior is costly for males, the males that are actively participating in mating behavior (e.g., those present at mating sites or actively courting and pairing with females) would be in relatively good physiological condition (e.g., high lipid levels) and would be directing mating behavior toward relatively high-quality females (e.g., females with more eggs).

We tested this prediction in Japanese beetles, an introduced pest in the United States at both the larval and adult life stages (Fleming 1972, Potter and Held 2002). Both male and female Japanese beetles may copulate with multiple mates during their lives (Fleming 1972, Tigreros and Switzer 2009). On emergence, virgin, adult females emit a sex pheromone to attract males (Ladd 1970a). Mated females stop producing sex pheromone but continue to mate on the food plants, on which adults aggregate as a result of plant kairomones (Ladd, 1970a, Loughrin et al. 1995, Tigreros and Switzer 2009). Males guard females after copulating, sometimes for many hours (Barrows and Gordh 1978; Saeki et al., 2005a, b; Switzer et al. 2008), and guarding behavior seems to be costly, affected by a male's energy stores and temperature (Saeki et al. 2005a, Switzer et al. 2008). Also, there is evidence that males prefer to pair with relatively large females and guard relatively large females for longer periods of time (Saeki et al. 2005c, Switzer et al. 2008) and that female body size is positively correlated with number of eggs (Saeki et al. 2005c). Females apparently do not need to mate repeatedly to obtain the sperm necessary for fertilizing eggs over their lifetime (Ladd 1987); however, they may copulate many times even within a day (Tigreros and Switzer 2009) and the last male to mate with the female fertilizes most of her eggs (Ladd 1970b).

In the field, beetle aggregation and mating behavior varies among individuals and over time. For example,

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although beetles may exist in large aggregations, considerable spatial heterogeneity in beetle presence exists within infested plants. That is, even within the same plant, beetles may be tightly grouped with other individuals or may be relatively isolated (Fleming 1972, Iwabuchi and Takahashi 1983, Switzer et al. 2004). Although the function of such aggregations is still unclear for Japanese beetles, in other insect species, aggregations are known to result in increased encounters between males and females and therefore increase the likelihood of finding potential mates (Wells et al. 1990, Loughrin et al. 1995). Additionally, the mating behavior of Japanese beetles seems to change temporally within a day. Female–male pairings are more common in the morning and the evening (Switzer et al. 2001), whereas male–male pairings and physical contests for females are more likely to occur in the late afternoon (Switzer et al. 2004, Kruse and Switzer 2007), without significant changes occurring in sex ratio or density during the day (Switzer et al. 2001, Kruse and Switzer 2007). These mating patterns could be driven either by changes in male's physiological capability, changes in female quality or willingness to mate, and/or changes in which individuals are present on the food plants at different times of the day (Switzer et al. 2001, 2004, 2008; Saeki et al. 2005a; Kruse and Switzer 2007).

Based on these observations, we studied Japanese beetles on food plants and addressed the following questions. First, is the physiological status of males and females (i.e., lipid level in males and egg load in females) related with their participation in pairings and aggregations? Second, are the temporal patterns in Japanese beetles mating correlated with physiological status of males and females?

Materials and Methods

We collected beetles from a pesticide-free soybean field located on the campus of the University of Illinois, Champaign, IL on 5 d between 20 and 28 July 2006. Collections were made at 0700, 1400, and 1900 each day, and we rotated collection sites within the field between each sampling to avoid collecting from the same area on the same day. Beetles were collected according to three categories of "social status": (1) paired beetles that were part of a group (we considered beetles to be part of a group, or aggregation, when they were with at least one other beetle on their leaf), (2) single beetles that were part of a group, and (3) single beetles that were isolated (we considered beetles to be isolated when no other beetle was within at least one leaf-length; i.e., at least 10 cm away on a different leaf). Approximately 30 beetles were collected for each category. We limited our collection of paired beetles to just those in aggregations because paired, isolated beetles were relatively uncommon, making it difficult to obtain a sufficient sample size during each collection period. Beetles were immediately put on ice after collection. After all collections for that time period, beetles were placed in a freezer at -17°C .

Assessment of Beetle Characteristics. In the laboratory, we separated males from females on the basis of foreleg morphology (Smith and Hadley 1926). Body size of males and females was determined by measuring the maximum body width (which occurs just posterior to the pronotum) at $12.5\times$ using a dissection scope (Olympus SZX7; Olympus America, Inc., Center Valley, PA) with an ocular micrometer. This measure of body size correlates well with other morphological measurements and thus is a good measure of overall body size (Van Timmeren et al. 2000).

To assess the physiological condition of males, we measured their total lipid content using a modified Bligh and Dyer (1959) procedure as follows. Masses were determined using a Mettler-Toledo XS205 (Mettler-Toledo, Inc., Columbus, OH) analytical balance to 0.01 mg. Each beetle was weighed twice for each mass determination, and the average of these two measurements was used as the individual's mass. We first determined a male's wet mass, dried the individual in an oven for 72 h at 60°C , and weighed him again to determine his dry mass. For lipid extraction, each dry male was placed in a 1.5-ml microfuge tube, and we homogenized the individual using a Dounce homogenizer in $500\ \mu\text{l}$ of 2:1 chloroform and methanol solution for 5 min, followed by centrifugation at 84,000 rpm for 5 min. The supernatant was removed and placed in a clean centrifuge tube, and the pellet was rehomogenized in $300\ \mu\text{l}$ chloroform and methanol solution and centrifuged for an additional 3 min. The resultant supernatant was combined with the original supernatant and air dried for 24 h, followed by oven drying at 60°C for 24 h. The mass of the remaining solid was defined as the "total lipid content" for that individual.

To assess the fecundity level of females, we dissected them and counted the number of mature oocytes (Saeki et al. 2005c).

Statistical Analysis. In some animal taxa, lipid content may not increase as an isometric function with body size (Post and Parkinson 2001). Consequently, we examined the nature of the relationship between lipid content and body size using a reduced major axis or geometric mean regression (Ricker 1973) of $\ln(\text{total lipid content})$ with $\ln(\text{body size})$, using RMA software for reduced major axis regression (Bohonak 2002). Second, to compare the lipid content of males, we used an analysis of covariance (ANCOVA) with time of day and social status as factors and body size as a covariate. $\ln(\text{body size})$ and $\ln(\text{lipid content})$ were used to improve the normality of the residuals. The assumptions of normality, homogeneity of variances, and heterogeneity of the slopes were met for the ANCOVA (Huitema 1980). We evaluated the homogeneity of the slopes by both using formal significance tests and looking at the individual regression slopes (Quinn and Keough 2002). To test for differences in body size depending on social status and time of the day, we used a two-way analysis of variance (ANOVA) for each sex. The residuals for this ANOVA were normal and the variances were homogenous. For females, data on egg loads were analyzed using a

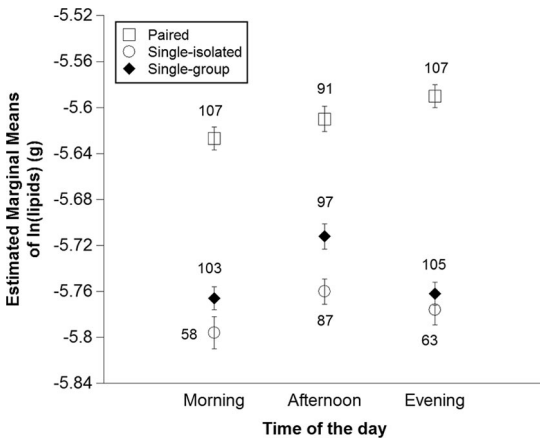


Fig. 1. Estimated marginal means (\pm SE) of $\ln(\text{lipid})$ content of males collected paired, single-isolated, and single-group, at three different times of the day (morning: 0700 hours, afternoon: 1400 hours, and evening: 1900 hours) on soybeans leaves. The covariate is evaluated as $\ln(\text{size}) = 1.81$. Number by points represents sample size.

Kruskal-Wallis nonparametric ANOVA and subsequent Mann-Whitney pairwise tests among categories because of a failure to meet the assumptions of the parametric tests. Data for the 5 collection days were pooled for analysis. Statistics were calculated using SPSS statistical software Version 15.0 (SPSS, Chicago, IL). All means are followed by \pm SE.

Results

Males had an average of $10.2 \pm 0.14\%$ ($N = 819$) total lipid content. However, lipid content varied depending on size, time of the day, and social status. First, for all categories of males combined, larger males had disproportionately higher lipid contents than small males. That is, the slope of the \ln - \ln RMA regression of lipid mass versus body size was significantly higher than three (slope = 6.06 ± 0.10 , $N = 819$, one-sample t -test $t = 29.99$, $P = 0.0001$). Second, the lipid content of males differed depending on time of the day ($F_{2,799} = 7.38$, $P = 0.001$) and social status ($F_{2,799} = 199.74$, $P < 0.0005$), with a significant interaction effect between time of the day and social status ($F_{4,799} = 2.78$, $P = 0.03$). Overall, it seems that paired males had the highest lipid content and single isolated males the lowest (Fig. 1). Also, the lipid content of males for the three social categories was lowest during the morning. Paired males had higher levels when caught in the afternoon and the highest levels when caught in the evening. For single males (either isolated or in a group), those captured in the afternoon had the highest lipid levels (Fig. 1).

Male body size did not differ among males of different social status (paired males = 6.11 ± 0.02 mm, $N = 305$, isolated single males = 6.13 ± 0.02 mm, $N = 208$, single males in a group = 6.13 ± 0.02 mm, $N = 309$; $F_{2,813} = 0.40$, $P = 0.67$). However, size did differ among males captured at different times of day. Males col-

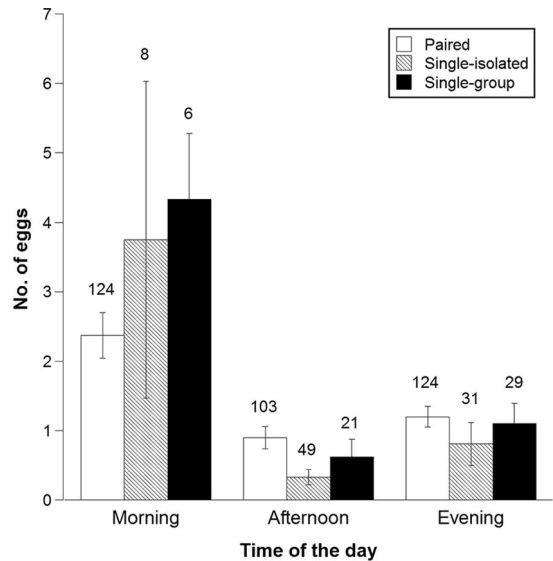


Fig. 2. Mean \pm SE number of eggs of female Japanese beetles collected in 2006 on soybeans. Females were collected paired, single and isolated, and single in a group at three different times of the day (morning: 0700 hours, afternoon: 1400 hours, and evening: 1900 hours). Numbers above bars represent sample size.

lected in the afternoon (6.07 ± 0.02 mm, $N = 276$) were significantly smaller than males collected during the morning (6.15 ± 0.02 mm, $N = 268$) and evening (6.15 ± 0.02 mm, $N = 278$; $F_{2,813} = 5.85$, $P = 0.003$).

Female egg load differed among females of different social status (Fig. 2; Kruskal-Wallis, $\chi^2 = 14.26$, $P = 0.001$, $df = 1$). Females that were single and isolated had fewer eggs (median = 0 eggs, range = 0–18, mean = 0.81 ± 0.25 eggs, $N = 88$) than paired females (median: 0 eggs, range = 0–16, mean = 1.53 ± 1.53 eggs, $N = 358$; Mann-Whitney $U = 11,798$, $P = 0.0001$, $N = 439$) and single females in a group (median = 0 eggs, range = 0–7, mean = 1.25 ± 1.87 eggs, $N = 56$; $U = 1,997.5$, $P = 0.02$, $N = 144$). Female egg load varied depending on time of day (Kruskal-Wallis, $\chi^2 = 29.75$, $df = 2$, $P = 0.0001$), with females collected in the morning having more eggs (median = 1 egg, range = 0–18, mean = 2.54 ± 0.32 eggs, $N = 138$) than females collected in the afternoon (median = 0 eggs, range = 0–10, mean = 0.71 ± 0.11 eggs, $N = 173$; $U = 8158.5$, $P = 0.0001$, $N = 311$) and evening (median = 0 eggs, range = 0–8, mean = 1.11 ± 0.12 eggs, $N = 184$; $U = 10408.0$, $P = 0.003$, $N = 322$), and females collected in the evening having significantly more eggs than females collected in the afternoon ($U = 13380.5$, $P = 0.003$, $N = 357$). Note that, in Fig. 2, the mean for single, isolated females is deceptively high because of a combination of a relatively small sample size and an outlier female with 18 eggs.

Female body size was not related to social status (paired = 6.74 ± 0.02 mm, $N = 351$, isolated singles = 6.65 ± 0.05 mm, $N = 88$, singles in a group = 6.72 ± 0.05 mm, $N = 56$; $F_{2,486} = 1.79$, $P = 0.17$) or time of day

(morning = 6.68 ± 0.03 mm, $N = 138$, afternoon = 6.73 ± 0.03 mm, $N = 173$, evening = 6.74 ± 0.03 mm, $N = 184$; $F_{2,486} = 0.95$, $P = 0.38$).

Discussion

Because mating is costly, beetles in relatively good physiological condition, in terms of energy stores and egg loads, may be the ones involved in behaviors that increase their chances to mate. In this study, we found evidence supporting the prediction that the physiological status of beetles will affect their mating behavior, grouping behavior, and their presence at a mating site during different times of the day.

Mating Status Patterns. We found that paired males had higher total lipid levels than single males, and single-isolated males had the lowest lipid levels overall. This pattern of paired males having the most lipids is consistent with results from studies on other species of arthropods (Otronen 1995, Plaistow et al. 2003). In Japanese beetles, lipid content seems to influence mating success in two different ways. First, males who have the lowest lipid reserves remain isolated on the food plants, thus likely reducing their chances of finding females. These males may be feeding to increase energy stores in preparation for more mating behavior. Second, among the males who are aggregated (and thus likely attempting to mate), those with the largest lipid content appear to be the most successful at pairing, or remaining paired, with females (cf. Plaistow et al. 2003). Mate guarding in Japanese beetles seems to be costly; while they are guarding females, males cannot eat and replenish either their energy or water content, and this affects their ability to guard females for extended periods of time (Saeki et al. 2005a). However, we cannot rule out that the observed patterns were driven by unmeasured variables, such as beetle age. In some insects, lipid levels change predictably with age (Liquido and Irwin 1986, Matsubara et al. 2005).

For females, egg load seemed to be a key factor determining their likelihood of mating. The females with fewest eggs were those who remained single and away from the aggregations. Males may not be attracted to these females, instead preferring to pair with females that are fecund and therefore probably closer to oviposition. Previous studies on Japanese beetles have likewise found that females with more eggs are more likely to mate (Saeki et al. 2005c, Tigreros and Switzer 2008), and a male preference for gravid females has been found in other insects (Bonduriansky 2001). Whether and how male Japanese beetle males might assess female egg load is unknown, but certainly olfactory, tactile, and visual mechanisms are all possibilities (Bonduriansky 2001). However, such fecundity-related pairing could be the result of female behavior. As observed in other insects (Edward and Gilburn 2007), females with fewer eggs may modify their behavior either by not frequenting aggregations or by not attracting males to decrease the chances of being found by males when oviposition is not imminent.

We did not find any relationship between female size and pairing status, although this pattern had been found in a previous study (Switzer et al. 2001). However, Tigreros and Switzer (2008) showed that body size patterns vary according to the nutritional status of females; how frequently a large versus small female mates depends on whether she was well fed. The field-based patterns found in this study, therefore, may be the result of an interaction between size, fecundity, and the energy levels of females.

Temporal Patterns. Male lipid content varied among beetles collected at different times of day, with the lowest lipid levels in the morning, and, for single males, the highest lipid content during the afternoon. These patterns may reflect two aspects of behavior by the males. First, they may be the result of some males flying during mate-searching, which typically takes place during late morning and early afternoon (Fleming 1972). Second, the patterns may result from feeding behavior by the males, with less feeding overnight. Kreuger and Potter (2001) found that Japanese beetles feed much less at night than during the day and may not feed if the night is relatively cool. Active feeding by single males in the morning potentially could explain higher lipid levels in the afternoon (assuming processing is rapid enough), but why single males have lower levels in the evening is unclear. Studies looking at changes in particular beetles as a result of their behavior would help distinguish these possibilities.

The patterns observed for male Japanese beetles relating male body size with time of the day may be a result of their heat budget. It has been shown for insect species with dark coloration, like Japanese beetles, that large individuals reach a temperature excess easier than small insects. Thus, larger insects have an advantage during the coldest time of the day (early morning and late evening), whereas smaller insects can perform better at middle of the day, when temperatures are higher (Willmer and Unwin 1981). Where the males may go during their respective times of physiological stress is not known, but our data suggest they are not on the food plants. However, such size changes through the day were not found in an earlier study on Japanese beetles (Switzer et al. 2001), which indicates that these size relationships may be affected by other factors than those considered here.

The temporal pattern in female fecundity gives some insight into the mating patterns and oviposition dynamics of Japanese beetles. Females in the morning had by far the most eggs on average, with females in the afternoon and evening having significantly fewer eggs. This pattern was robust, as we observed the same result in a sample of 763 females collected in a soybean field in Coles County, IL, in 1999 (unpublished data). Potentially, this could be the result of highly fecund females in the morning leaving the food plants in late morning and early afternoon and/or an influx of less fecund females later in the day. Tigreros and Switzer (2009) found that approximately one half of the females that left food plants during the day left during the period between 1100 and 1300 hours, and Smith

and Hadley (1926) mentioned that females typically entered the soil in the late afternoon. Also, our results support that egg load may be an important component of the female's disposition to mate. Switzer et al. (2001) found the highest mating frequencies were in the morning and evening, which corresponds to the fecundity patterns found in this study.

In conclusion, we found that the lipid levels of males and the fecundity level of females were related to their presence in feeding aggregations and their participation in mating. In addition, we found that male condition and female fecundity varied among sampling periods during the day and that some of these aggregation and mating patterns were dependent on the time of day. The patterns suggest that male mating preferences, the timing of female oviposition, and possible avoidance of male harassment all play a critical role in explaining the mating system and aggregating behavior in Japanese beetles. To the extent that females are driving these patterns, control strategies directed at females may be more effective at not only local population control but also in management of aggregation formation and maintenance on food plants.

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