Post-mating enhancement of fecundity in female *Lygus hesperus*

_Colin S. Brent, Matthew P. Fasnacht, and Timothy M. Judd_

1Arid Land Agricultural Research Center, USDA-ARS, Maricopa, Arizona, U.S.A., 2Department of Chemistry, Southeast Missouri State University, Cape Girardeau, Missouri, U.S.A. and 3Department of Biology, Southeast Missouri State University, Cape Girardeau, Missouri, U.S.A.

**Abstract.** Although mated females of the western tarnished plant bug *Lygus hesperus* Knight are known to produce more eggs than virgins, the nature of the inducing stimuli and the specific changes occurring in the female require additional elucidation. Compared with virgin females isolated from males, those exposed to male precopulatory behaviours produce similar numbers of eggs, whereas inseminated females produce 50% more during the observation period. Although the quantity of seminal fluids received by a female does not influence egg number, mating twice within a 10-day span causes a 16% increase in fecundity, on average. Females mating more than twice during the same period do not exhibit additional increases in egg number. Because virgin females contain more chorionated eggs than are laid, mating appears to enhance the rate of oviposition. However, to achieve a sustained increase in fecundity, an augmented rate of oocyte maturation would also be required. Male-derived spermatophores lack substantive quantities of nutrients that might otherwise have enhanced female fecundity. The total amounts of carbohydrate, protein and lipid, as well as eight essential minerals transferred by the male, are insufficient for producing even a single egg, and the female has already produced a large number of chorionated oocytes before she mates. Collectively, the data suggest that seminal fluid contains one or more activational molecules, such as a peptide, which triggers an increase in egg deposition. A prolonged increase in oviposition rate may be achieved through multiple matings to ensure a supply of sperm or to offset the degradation of the putative activational factor.

**Key words.** Accessory glands, fecundity, *Lygus hesperus*, Miridae, spermatophore.

**Introduction**

Egg production by female insects usually depends on the availability of a mate. For some species, precopulatory behavioural interactions between the sexes can promote oogenesis but, in many other insects, virgin females refrain from ovipositing or at least greatly reduce their deposition rate until they have copulated (Ridley, 1988). On occasion, the neuronal stimulus of intromission can be sufficient to activate oogenesis and promote egg laying (Engelmann, 1959; Roth & Stay, 1961; Saunders & Dodd, 1972; Gillott & Langley, 1981; Melo et al., 2001) but, more commonly, the contents of the seminal fluid are crucial for enhancing female fecundity (Gillot, 2003). Not only do mated females receive the sperm necessary to fertilize their eggs, they may also receive additional compounds from the male. These products of the male accessory glands and testes can act as allohormones to influence female reproductive behaviour and physiology. These compounds have an activational effect in promoting female oviposition rates and therefore the effects are independent of spermatophore size. Alternatively, spermatophores may enhance fecundity by supplying the female with a limited resource necessary for oogenesis (Vahed, 1998; Arnqvist & Nilsson, 2000). In this case, larger spermatophores have a greater effect on female oviposition rates. When female fecundity is sensitive to such specific exogenous stimuli, a
unique opportunity is presented to develop highly targeted population control measures. Among the mirids, there is considerable variation in the responses of females to mating stimuli. In some species, mating has no effect on the number of eggs produced, whereas, in others, it can enhance fecundity or may be essential for oviposition to occur (Wheeler, 2001). Polyandry is also common in this group (Wheeler, 2001). The stimulatory effects of insemination are often transitory (Arnaqvist & Nilsson, 2000); therefore, multiple matings may be necessary to maximize lifetime fecundity. In one of the major pest mirids, the western tarnished plant bug *Lygus hesperus* Knight, females produce eggs regardless of their mating status but they oviposit more when allowed to mate (Strong et al., 1970; Strong & Sheldahl, 1970; Brent, 2010a). The size of spermatophores produced by *L. hesperus* males varies by age and recent mating activity but can represent up to 16% of male body mass (Brent, 2010b). Females may mate multiple times during their lives, although the effect on polyandry on their fecundity is unknown. Multiple copulations could ensure a steady supply of sperm, although Strong et al. (1970) conclude that only a single mating was necessary for a female to produce viable eggs throughout her life.

The present study begins to elucidate how egg production is regulated in female *L. hesperus* and how this regulation is influenced by males. Females might respond to the mere presence of sexually active males or they may require factors delivered in the spermatophore. To test these alternative mechanisms, the fecundity of virgin, singly- and multiply-mated females is assessed. The macro- and micronutrient content of eggs and spermatophores is determined and the sperm content of spermatophores from virgin and remated males is compared. Recent results (Brent, 2010a, b) suggest that spermatophores contain factors that influence a female’s sexual receptivity and attractiveness to other males. A similar seminal factor regulating fecundity is anticipated.

**Materials and methods**

**Insects**

The *L. hesperus* used in the present study were obtained from a laboratory colony maintained at the USDA-ARS Arid Land Agricultural Research Center (Maricopa, Arizona). The individuals in this colony are periodically outbred with locally-caught conspecifics. The stock insects were given unrestricted access to a supply of green beans (*Phaseolus vulgaris* L.) and an artificial diet mix (Debolt, 1982) packaged in Parafilm M (SPI Products, Westchester, Pennsylvania) (Patana, 1982). Both food sources were replenished as needed. Insects were reared under an LD 14 : 10 h photocycle at 25 °C and 20% relative humidity.

Adults were produced from groups of nymphs reared in 1890-mL waxed chipboard cup (Huhtamaki, De Soto, Kansas) at a density known to have minimal effect on *L. hesperus* development (≤100 nymphs/container; Brent, 2010c). Nymphs in each container were provided with approximately 20 g of fresh green beans and 12 g of artificial diet, which was replaced every 48 h. Rearing cups were covered with a nylon mesh to ensure adequate air circulation and light exposure. Daily monitoring allowed adults to be collected within 24 h of emergence. Cohorts of adults of the same age and sex were reared under conditions matching those for nymphs but with population densities in the range 50–120 adults/container.

**Fecundity effects of mating versus noncopulatory interactions with males**

To determine whether the increase in female oviposition rate is caused by exposure to male-specific behaviours or the stimulus of copulation/insemination, oviposition rates of females exposed to males with and without copulation were compared. At the start of the experiment, these females were virgins aged 7 days post-eclosion. One group was left in isolation for the duration of the experiment. Additional females were allowed to interact individually with a 7-day-old virgin male for 1 h. Females that interacted with males and were exposed to their precopulatory behaviour but did not mate composed the second test group. Mated females generated from those pairings comprised the third group. The females in these three groups were all from the same cohort and emerged as adults on the same day, so that their developmental and physiological states were comparable. The proportion mating in heterosexual pairings was similar to that observed in previous studies (Brent, 2010a, b). After treatment, females from all three groups were reared individually in covered glass Petri dishes (60 × 15 mm) with a 50-mm section of green bean. Egg counts were made every 24 h for 5 days, and the beans were replaced each day. At the end of the observation period, all females were dissected to determine whether they were inseminated.

**Fecundity effects of varied spermatophore size**

To determine whether the effect of insemination on female oviposition rate was activational (induced by allohormones) or graded (influenced by a limiting resource), egg production rates were measured daily for females exposed to one of four treatment groups: virgin; mated to a male that had mated 24 h previously (small spermatophore); mated to a virgin male (large spermatophore); or paired with a male for the duration of the experiment to become multiply inseminated. Mated females were obtained from 1 h pairings with males. This duration was particularly important for males used to produce the small spermatophore. Over a longer period of pairing, the males could have greatly replenished the contents of their accessory glands thereby increasing the size of the spermatophore delivered to the females (Brent, 2010b). All individuals were aged 5 days at the start of the experiment and were housed in glass Petri dishes with a section of green bean. At this age, most females will not have yet exhibited ovipositional behaviour (Strong et al., 1970; Brent, 2010a). Egg counts were made along with bean replacement every other day until the females were 15 days post-eclosion.
A 10-day sample period was used to ensure that females paired with a male would have the opportunity to remate. Normally females enter a 5-day refractory period after mating during which they are un receptive to courting males (Brent, 2010b). The extended period also permitted determination of the duration of any changes in egg production induced by mating. All females were dissected at the end of the experiment to determine mating status and the number of spermatophores present. An additional group of 50 females were dissected at 5 days post-eclosion to determine the number of vitellogenic and chorionated oocytes (Ma & Ramaswamy, 1987).

To determine the relative sizes of the spermatophores being contributed, a separate group of newly-mated females were prepared using the same mating scheme. The spermatophores were immediately dissected after insemination occurred and were weighed on a microbalance (model TE153S; Sartorius, Germany). A subset of these was used to determine the number of sperm per spermatophore. After each was homogenized in 20 μL of distilled water, the number of sperm in a 10 μL aliquot was counted on a haemocytometer under a compound microscope.

Macronutrient content of the egg and spermatophore

To determine whether males contributed any nutrients to the female that might be limiting to oocyte production, the macronutrient content of eggs and spermatophores were determined and compared. Total protein, carbohydrates and lipids were determined using a procedure similar to that outlined by Judd et al. (2010). For egg and spermatophore macronutrients, 15 and 14 samples were prepared, respectively. Each sample was pooled from tissue dissected from ten to 12 females. The wet mass of each sample was determined immediately after tissue collection. Samples were homogenized in 150 μL water and then centrifuged at 14 000 g for 2 min.

Proteins. For each sample, a 50-μL sample was used in the Bradford assay (Bradford, 1976). The aliquot was added to 1.0 mL of Bradford reagent, vortexed and allowed to sit for 3 min. The absorbance was measured at 595 nm and compared with 2.7, 3.35, 10.5, 23.0, 41.5 and 53.5 μg bovine serum standards. The results obtained were adjusted based on the percentage of the total sample used.

Carbohydrates. Total carbohydrates was measured using the anthrone assay (Van Handel, 1985a). For each sample, a 50-μL aliquot was added to 12.5 μL of 18% Na2SO4; 1.25 mL of anthrone reagent was added and the sample was heated at 100 °C for 12 min. The samples were allowed to cool to room temperature and then the absorbance was measured at 625 nm and compared with 10, 25, 50, 75 and 100 μg glucose standards.

Lipids. Total lipid content was measured using the phosphovanillan assay (Van Handel, 1985b). After the aliquots for the protein and carbohydrate samples were removed, 50 μL of 1 : 1 chloroform : methanol was added to the tube. The sample was vortexed and then centrifuged at 14 000 g for 2 min. The chloroform layer was removed and placed in a new tube and allowed to dry. Once dried, the sample was resuspended in 200 μL of concentrated sulfuric acid then heated at 100 °C for 10 min. The sample was then added to 3 mL of phosphovanil- lans reagent and allowed to develop for 30 min. The absorbance was measured at 525 nm and compared with 18, 45, 72 and 90 μg corn oil standards.

Statistical analysis

After an initial analysis of variance (ANOVA) for overall significance, each comparison of total egg production used a Student’s t-test, followed by pairwise multiple comparisons of egg production between treatment groups was conducted using the Holm–Sidak method. Overall significance level for Holm–Sidak tests was P = 0.05. The same test was used to compare masses of spermatophores from virgin and recently mated males. Because of non-normally distributed data, a Mann–Whitney rank sum test was used to compare
the number of sperm ejaculated by virgin and remated males. Pearson’s correlation was used to test the relationship between spermatophore number and egg production. All statistical analyses were performed with SIGMAPLOT, version 11.0 (Systat Software, Point Richmond, California).

Results

Effect of mating on female fecundity

There was significant variation in the number of eggs oviposited by females in different test groups (ANOVA: $F = 7.758$, d.f. = 2, $P < 0.001$; Fig. 1). Egg production was significantly higher in mated females compared with those left isolated from all male contact (Holm–Sidak: $t = 3.592$, $P < 0.001$) or exposed to male courtship behavior but unmated (Holm–Sidak: $t = 3.161$, $P = 0.002$). However, simply being allowed to interact with a male without the added stimulus of copulation failed to enhance female output beyond that observed in isolated females (Holm–Sidak: $t = 0.372$, $P = 0.711$).

Although egg production was influenced by the number of times a female was inseminated (ANOVA: $F = 22.693$, d.f. = 3, $P < 0.001$; Fig. 2), it was unaffected by the mass of the spermatophore received. The spermatophores of 5-day-old virgin males were almost twice as heavy (mean ± SE: 0.216 ± 0.008 mg; t-test: $t = 9.141$, d.f. = 78, $P < 0.001$) as those from males that had mated 24 h earlier (0.122 ± 0.007 mg), and contained a five-fold greater number of sperm ($1713 ± 285$ versus $320 ± 84$, respectively; Mann–Whitney test: $t_{12,12} = 215.5$, $P < 0.001$), although there was no difference in the total number of eggs produced by females in these two groups over 10 days (Holm–Sidak: $t = 0.316$, $P = 0.753$). By contrast, females allowed to mate multiple times during this period produced more eggs than those inseminated with a single large (Holm–Sidak: $t = 2.369$, $P = 0.019$) or small spermatophore (Holm–Sidak: $t = 2.460$, $P = 0.015$). For both mated and unmated females, egg production started high then dropped steadily (Fig. 3). Females allowed to mate multiple times sustained a consistently higher rate of egg production, particularly during the later days. Throughout the 10-day sample period, egg output was significantly lower for virgins compared with those receiving small (Holm–Sidak: $t = 6.106$, $P < 0.001$), large (Holm–Sidak: $t = 5.215$, $P < 0.001$) or multiple spermatophores (Holm–Sidak: $t = 7.650$, $P < 0.001$). For at least the first few days of the experiment, this rate was not limited by the number of eggs available for
oviposition; virgin females sampled at 5 days post-eclosion were found to have $9.1 \pm 0.5$ vitellogenic and $29.3 \pm 1.57$ chorionated oocytes in their ovaries. The latter is 50% greater than the number of eggs laid during the first 2 days. Despite the additive effect to female fecundity of mating more than once, there was no difference in the total number of eggs produced by females mated twice $(139.06 \pm 7.00)$ or three or more times $(140.73 \pm 8.14)$; $t = -0.156$, d.f. = 29, $P = 0.877$ and no significant correlation between spermatophore number and total egg production (Pearson: $r = 0.196$, n = 36, $P = 0.245$; Fig. 4).

Macronutrient and mineral content of eggs and spermatophores

Spermatophores had substantially less of every mineral included in this study on a per mg wet mass basis than did eggs (Table 1). The smallest differences were for magnesium and zinc (four-fold), and the greatest was for copper (129-fold). Compared with eggs, the spermatophores were also effectively devoid of macronutrients, with greater than 1000-fold differences. Carbohydrates were completely absent from spermatophores.

Discussion

As with many insect species (Ridley, 1988), including other mirids (Wheeler, 2001), females of L. hesperus lay more eggs after mating. Specifically, the females require intromission, and not just pre-copulatory behavioural interactions (Fig. 1). This is independent of the size of the spermatophore received by a female. The data of the present study strongly suggest that the short-term effect of mating on fecundity involves enhanced ovipositional activity. Within the first 2 days, mated females oviposit 60–80% more eggs than virgins (Fig. 3). The inducing stimuli could either be physical cues associated with copulation

<table>
<thead>
<tr>
<th>Table 1. Mean ± SD (μg/mg wet mass) of the nutrient content of Lygus hesperus eggs and spermatophores.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minerals</strong></td>
</tr>
<tr>
<td><strong>Egg</strong></td>
</tr>
<tr>
<td><strong>Spermatophore</strong></td>
</tr>
<tr>
<td><strong>Macronutrients</strong></td>
</tr>
<tr>
<td><strong>Egg</strong></td>
</tr>
<tr>
<td><strong>Spermatophore</strong></td>
</tr>
<tr>
<td><strong>Means were calculated from 11 samples for minerals and 15 and 14 samples, respectively, for egg and spermatophore macronutrients.</strong></td>
</tr>
</tbody>
</table>
Most likely, the limited nutrients observed in the seminal fluid females, although their impact is expected to be negligible. The content of a single egg. Some of the nutrients could still be absorbed and utilized by the fat body or ovaries of the females, although their impact is expected to be negligible. Most likely, the limited nutrients observed in the seminal fluid are principally used to enhance the survival of the sperm before fertilization (Neubaum & Wolfner, 1999; Chapman, 2001; Gillott, 2003). Alternatively, they may reflect the nature of the activational compound. The minerals found in highest concentration (i.e. magnesium and zinc) are often associated with enzymatic function, as well as DNA and protein synthesis (Prasad, 1995; Saris et al., 2000). The spermatophore might also deliver other untested for compounds that can influence egg production, such as folic acid, free amino acids, RNA or Juvenile Hormone.

The protein component of the spermatophores may also contain one or more peptides produced by the accessory glands. These can have a variety of activation or inhibitory effects on female reproduction, and are found in a wide range of species (Arnqvist & Nilsson, 2000). They can cross the seminal repository wall to enter the haemolymph (Lung & Wolfner, 1999), travelling to the brain to influence behaviour or to the organs involved in oogenesis (Wolfner et al., 2005). Other post-mating responses by L. hesperus females suggest the presence of such peptides in the spermatophore (Brent, 2010a, b) and, collectively, the suite of changes induced by mating is similar to the peptide-induced responses observed in inseminated D. melanogaster (Gillott, 2003; Wolfner et al., 2005). Peptide expression in male accessory glands and spermatophores is currently being assessed with the intent of devising peptides or their mimics suitable for controlling Lygus reproductive behaviours.

Despite the regularity of polyandry in L. hesperus (Fig. 4) (Strong et al., 1970; Strong & Sheldahl, 1970; Brent, 2010b), Strong et al. (1970) suggest that females may need to mate just once during their lives to produce a full complement of eggs. In terms of the number of sperm delivered to a female, this may be the case. However, the findings of the present study indicate that multiple mating enhances egg production beyond that of a singly mated female, at least during the period observed. This is a common response among insects (Arnqvist & Nilsson, 2000) and is observed in another mirid (Smith, 1977). On the other hand, there are numerous examples of mirids that mate only once or are polyandrous but appear to gain no fecundity benefits beyond that derived from a single mating (Abasa, 1973; Blommers et al., 1997; Groot et al., 2000; Wheeler, 2001; Takahashi & Higuchi, 2006; Gemenio et al., 2007; Okutani-Akamatsu et al., 2009; Siswanto et al., 2009). Because the present experiment only observes part of the adult life of L. hesperus, it is possible that the effect of polyandry on egg production cannot be sustained and does not influence the lifetime fecundity of the female. The induced increase in oviposition rate could also result in a shortened lifespan (Arnqvist & Nilsson, 2000), although the effect in L. hesperus remains unknown. However, the consistency with which females remate together with the resulting persistent high rate of oviposition (Fig. 3) suggests that whatever the male is providing may be important to the female’s fitness. Additional matings may be paced to counter the degradation of the spermatophore, thereby ensuring an adequate supply of viable sperm or maintaining a threshold concentration of seminal factors that promote egg production (Brent, 2010b).
References


