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Effect of diapause status and gender on activity, metabolism, and starvation resistance in the plant bug *Lygus hesperus*

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Abstract

Lygus hesperus Knight (Hemiptera: Miridae), a key pest species distributed throughout the western USA, survives winter in a state of diapause. A laboratory population was examined to elucidate the changes in behavior and physiology linked to this period of dormancy and to determine how these changes are affected by gender. It was found that under starvation conditions, diapausers lived significantly longer compared to non-diapausers in both genders. This may be attributable to the greater lipid reserves that diapausers have compared to non-diapausers. Diapausers also spent more time at rest and less time feeding than non-diapausers. Gender did not impact these behaviors. There was no difference in resting metabolic rate or flight activity between diapausers and non-diapausers, however, there were significant gender differences when the data were pooled. Males had higher resting metabolisms than females, whereas females spent more time flying. Collectively, these results point toward a higher degree of resource conservation in diapausers. These differences may enhance resistance to starvation and dehydration conditions that *L. hesperus* are likely to encounter while overwintering.

Introduction

The western tarnished plant bug, *Lygus hesperus* Knight (Hemiptera: Miridae), is a major pest of cotton and numerous other cultivated crops (Jackson et al., 1995) and is found throughout much of the Western USA (Wheeler, 2001). Like many insects, overwintering lygus bugs face adverse environmental conditions, such as reduced food availability and periodic extremes of cold. Survival can require a reallocation of resources that precludes various activities and developmental pathways until conditions are more favorable (Kostál, 2006; Hahn & Denlinger, 2007). *Lygus hesperus* survives winter in its adult form by entering a state of diapause. For most diapausing insects, this period of dormancy is normally induced by predictable token stimuli, such as day length, and is modified by other factors such as temperature and host-plant condition (Tauber et al., 1986; Hodek, 2002; Denlinger et al., 2005). For *L. hesperus*, diapause is induced during the nymphal stages by exposure to short photophases, usually under 11 h (Beards & Strong, 1966; Leigh, 1966; Strong et al., 1970; Spurgeon & Brent, 2010). The resultant diapausing adults that emerge are found in the field from mid-fall through mid-winter, when days are short (Beards & Strong, 1966), cool, and dry. The exact dates during which diapausing *L. hesperus* are found in the field depends somewhat on geographic region and local climatic factors (Beards & Strong, 1966). For populations in central and southern Arizona, the first diapausing adults are usually seen in mid-September and are found throughout January (CS Brent, pers. obs.; Stitt, 1940). Once the photophase lengthens sufficiently and forage plants become more available, lygus bugs begin to transition out of diapause.

The exact consequences of entering diapause can vary considerably between insect species, but the resultant changes to adult behavior and physiology clearly enhance individual survivorship during a period of heightened environmental stress. These same changes might also render a species particularly susceptible to novel ecologically or culturally based management tactics (Hahn &

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Denlinger, 2007). However, development of such tactics for *L. hesperus* requires an improved understanding of their overwintering biology. Currently, we know very little about which behavioral and physiological traits might be impacted by an individual's developmental status beyond the most rudimentary differences. Similarly, little is known about sex-specific differences in the diapause response.

It has been well established that diapausers have better developed fat bodies and poorly developed reproductive organs (Bacon et al., 1964; Beards & Strong, 1966; Spurgeon & Brent, 2010). Typically, the fat body is the primary site of nutrient storage that allows an insect to survive through a period of limited food access, and can be a source of cryoprotectant molecules that shield against low temperatures (Clark & Worland, 2008; Hahn & Denlinger, 2011). Reduced investment in organ development and associated processes also conserves resources at a time when key activities, such as reproduction, have ceased (Denlinger et al., 2005). Fully diapausing insects also tend to decrease feeding and movement, although such responses are usually dependent on their species and health, and various exogenous factors, such as temperature and food availability (Tauber et al., 1986; Kostál, 2006; Hahn & Denlinger, 2007; Snodgrass et al., 2012).

In an effort to more fully characterize the diapause syndrome in *L. hesperus* and to identify components that might enhance overwintering survivorship, this research examined some of the basic behavior and physiology that might be impacted by diapause status and gender. Using laboratory-reared populations, we determined total lipid reserves, starvation resistance, and the rates of feeding, movement, and resting metabolism, in female and male, diapausing and non-diapausing plant bugs. Although the results found under such artificial circumstances may not extrapolate completely to the field, they provide a much-needed guide as to which traits warrant future investigation in field populations.

Materials and methods

Insects

Animals were taken from a stock *L. hesperus* colony that has been under continuous culture for >10 years, with regular introductions of field-collected adults to maintain vigor. All insects introduced into the colony have been collected from fields in Maricopa, AZ, USA. The stock insects were held at 28.0 ± 1 °C under a 14-h photophase and provisioned with artificial diet (Debolt, 1982) packaged in Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA) (Patana, 1982). These temperatures are well within the ambient conditions (<0 to >40 °C) to which local field populations are exposed over the course of a year. The range has also been shown to be near optimal for egg hatch rates, nymphal development, and adult reproductive maturation (Cooper & Spurgeon, 2012, 2013; Spurgeon & Cooper, 2012). Eggs were obtained by placing oviposition packets (agarose gel packaged with Parafilm M) on the screened tops of rearing cages for 6–8 h. The egg-filled packets were placed in 1 890-ml waxed chipboard cups (Huhtamaki, De Soto, KS, USA), which were then held in an environmental chamber maintained with a L10:D14 photoperiod at the same temperature as the stock colony. Photoperiod has been shown to be the primary inducer of diapause with temperature having a limited impact (Beards & Strong, 1966). Emerging nymphs were held in groups and provided an ad libitum diet of green bean pods [*Phaseolus vulgaris* L. (Fabaceae)] and raw sunflower seeds [*Helianthus annuus* L. (Asteraceae)]. Beans and seeds were replenished three times weekly or more often if their quality deteriorated. Shredded paper was provided as a refuge. The number of individuals in each container was kept below 150 to ensure normal development (Brent, 2010). Beginning when fifth instars were first observed, nymphs were monitored daily to detect adult eclosion. Adults emerging on the same day were separated by gender, based on the presence or absence of an ovipositor, and placed into separate rearing containers. These adults were then reared under the same conditions as the nymphs. The L10:D14 photoperiod used has been shown to induce diapause in some, but not all, exposed individuals in laboratory-cultured populations (Spurgeon & Brent, 2010). Adults were selected for experiments at 10 days post-emergence. Although individuals begin their developmental response to light conditions during the nymph stage (Beards & Strong, 1966; Leigh, 1966; Spurgeon & Brent, 2010), the response is not physiologically evident until a few days after adult emergence, when the final stages of reproductive maturation occur. By 10 days, depending on diapause status, individuals have either fully mature and active reproductive organs (non-diapause; Strong et al., 1970; Brent, 2010) or immature and inactive gonads and very substantial fat bodies (diapause; Beards & Strong, 1966; Spurgeon & Brent, 2010). Diapause status was given an initial classification in our 10-day-old adults based on abdomen color, with yellow indicative of diapause and the presence of a substantial fat body, and dark green indicative of non-diapause (Brent, 2012). Individuals of intermediate color were not used in the experiments to avoid misclassification and to ensure that the individuals were in full diapause. All diapause classifications were later confirmed by dissection after tests were completed using the criteria detailed in Spurgeon & Brent (2010) and Brent & Spurgeon (2011). Subjects were used only once.

Starvation resistance

Diapause and non-diapause adults of both genders aged 10 days post-eclosion were sorted by color and then placed individually in mesh-covered, translucent 2.5 × 7.0-cm plastic tubes. Samples sizes ranged from 36 to 66. Tubes were stored in the same environmental chambers in which the insects had been reared. Tubes were censused twice daily for mortality, so that longevity was calculated to within a half day increment. Developmental status, as assigned by initial color assessment, was confirmed by dissection immediately after death using previously established criteria (Spurgeon & Brent, 2010; Brent & Spurgeon, 2011). To be classified as diapausing, an individual would normally have to have a clearly hypertrophied fat body. However, these animals were starved and their fat bodies were greatly diminished or absent at death. This necessitated reliance on the other criteria for verifying diapause, which was that females could not exhibit signs of vitellogenesis and males had to have undeveloped accessory glands.

Lipid content

Total lipid content was determined using the approach of Couvillon et al. (2011). All adult *L. hesperus* were reared under short-day conditions, producing a mix of diapausers and non-diapausers from the same cohort. After 10 days post-eclosion, individual diapause status was determined by dissection. Individuals were cut into several pieces using a clean razor to facilitate the lipid extraction process. The collective parts of five bugs were pooled for each sample. For each of the four groups tested (female and male diapausers and non-diapausers) there were 11–15 samples. The insect parts constituting each sample were placed on a pre-weighed section of foil and dried for 72 h in an oven set to 60 °C. The dry mass of each sample was determined on a Mettler UMT 2 microbalance (resolution 0.001 mg; Mettler Toledo, Columbus, OH, USA) prior to being transferred to a 1.5-ml microcentrifuge tube. A 500-µl aliquot of 2:1 DCM-MeOH (dichloromethane-methanol) was added to each tube. A disposable pestle was used to grind the bugs for 60 s. Tubes were capped and agitated for 15 min at 250 rpm in a refrigerated shaker (Excella E24; New Brunswick Scientific, Edison, NJ, USA) set to 20 °C. The tubes were then centrifuged at 17 000 ***g*** for 5 min, and allowed to sit for an additional 5 min. A glass pipette was used to transfer the supernatant into a second pre-weighed microcentrifuge tube. The pellet in the first tube was resuspended in a second 500 µl of DCM-MeOH and the separation process repeated. After combining both supernatant fractions in the second tube, 400 µl of 0.9% (wt/vol) NaCl was added to precipitate phase separation into aqueous and organic layers. Tubes were vortexed for 30 s at high speed, then centrifuged for 5 min at 1 000 ***g***. At that time, two layers were clearly visible. The upper aqueous phase was carefully removed with a pipette and discarded. The bottom organic layer was left in the uncapped microcentrifuge tube under a fume hood for 96 h to allow the liquid solvent to evaporate, leaving behind the lipid residue. The tubes were reweighed to determine the total lipid mass.

Resting metabolism

Individual metabolic rates were monitored in female and male diapausing and non-diapausing *L. hesperus* at 10 days after adult eclosion. All insects were reared under the same conditions, and all individuals tested on any given day were from the same cohort. The CO_2 flow-through respirometry system was set up using Sable Systems International (SSI) (Las Vegas, NV, USA) instruments, and it included two LI-COR CO_2 infrared gas analyzers (LI-7000, LI-6262) to allow two animals to be processed simultaneously. Compressed air was scrubbed of CO_2 and water vapor with a Balston Purge Gas generator (Cleveland, OH, USA), passed through an Ascarite column and then a DrieRite column to remove residual CO_2 and water vapor. The air stream was split and directed into two 50 SLMP Brooks mass flow valves (Hatfield, PA, USA) with the two flow rates controlled to 50 ml per min. Each air stream was directed through a reference cell of an LI-COR analyzer for measuring zero air and then, into a respirometry chamber, and then into the sampling cell of the LI-COR analyzer. The CO_2 and water vapor data outlets of the two LI-COR gas analyzers were connected to an SSI UI-2 interface that relayed data to a computer, where it was analyzed using SSI ExpeData software version 1.4.x.

Each recording was started with two empty respirometry chambers. The first 5 min recorded zero CO_2 and water vapor readings that were used as starting baseline data. Prior to monitoring, lygus bugs were weighed on a Mettler MX 5 microbalance (resolution 0.001 mg; Mettler Toledo). Two bugs were run simultaneously, one in each respirometry chamber. Test individuals were selected in such a way that each of the 16 different possible combinations of gender, diapause status, and test chamber was assessed on the same day. For each day, the order of pairings was changed to avoid any time-of-day effects on behavior. For each of the four groups tested (female and male diapausers and non-diapausers), total sample sizes ranged from 13 to 29. After being introduced to the respirometry chambers, animals were allowed to equilibrate to the

experimental temperature (25 ± 0.5 °C) and air flow for ca. 15 min. This was followed by 30 min of data recording to determine general resting metabolic rates. Afterward, individuals were removed from the chamber, reweighed, and diapause status verified by dissection. Absolute CO_2 production rates (VCO_2) in μl per min were calculated from CO_2 and air flow rate recordings, and average masses (mg) of combined before and after measures were used to calculate mass-specific rates. Once animals were removed, the two empty chambers were reconnected to the airstream, allowed to equilibrate, and a final 5 min recording of zero CO_2 and water vapor air was made to generate end baseline data. Prior to analyses, the recorded data was adjusted to correct for possible baseline drift in CO_2 and water vapor in the two LI-COR analyzers that might have occurred during the insect measurement period.

Resting and feeding rates

Adults aged 10 days post-eclosion were placed individually in mesh-covered, translucent 2.5 × 7.0-cm plastic tubes with a 5-cm section of green bean pod for food. After placement in the tube, individuals were allowed to acclimate for 15 min prior to data collection. Observations were conducted in a room maintained at 25 ± 2.0 °C and 30 ± 10% r.h. The duration and frequency of all behaviors for a given individual was recorded for 30 min using Observer v.5.0 (Noldus Information Technology, Wageningen, The Netherlands). Data collection started 1 h after lights came on and was ended at least 2 h prior to lights out. On each collection day, equivalent number of animals were observed from each test group (female and male diapausers and non-diapausers) and the starting order was rotated to prevent any timing biases. The behaviors were classified into three categories for analysis: active (fly, groom, probe, walk, etc.), feeding, and resting. Probing consisted of any insertion of the proboscis into the bean pod that lasted less than 5 s, otherwise it was considered feeding. Because feeding behavior comprised such a minor component of the time monitored, the durations of active and rest periods were essentially reciprocal values. As a consequence, only the time spent resting and feeding were analyzed. For each test group, total sample sizes ranged from 48 to 69.

Flight activity

Flight assays of adult *L. hesperus* were conducted using a computer-interfaced flight mill system modified from a design described by Beerwinkle et al. (1995). Individual insects were tethered to the end of a lightweight metal arm constructed from a hypodermic tube (30.5 cm in length). The balanced arm was levitated by opposing magnets and rotated via a Teflon bearing to minimize friction. A small magnet attached to the base of the Teflon bearing below the arm activated a Hall effect sensor as the arm rotated by action of the insect flying. The sensor was connected to a computer through a digital I/O board (National Instruments, Austin, TX, USA) and a custom-built Labview (National Instruments) program that continuously monitored the activity of the Hall effect sensor and logged the number and clock time of each rotation of the flight arm. This information was used to calculate speed, distance, and periodicity of flight. Additional system details can be found at http://entomology.tfrec.wsu.edu/VPJ_Lab/Flight-Mill.html.

Flight mills were located in a room maintained at 25 ± 0.5 °C and 50 ± 10% r.h., with a photoperiod of L14:D10. Adult *L. hesperus* aged 10 days post-eclosion were tethered by the pronotum to the end of a small piece of quilting thread (0.35 mm in diameter) with dental wax. The other end of the thread was attached to the flight arm. Insects were anesthetized with CO_2 (exposure time <1 min) to facilitate tethering. Flight assays were conducted for a 24-h period beginning in the early afternoon. The entire system consisted of 24 simultaneously monitored mills. The flight assays were blocked by time so that six replications of the four test groups (diapausing and non-diapausing females and males) were run on a given day. The actual mills on which each group were assayed were rotated each sample day (e.g., diapausing males: mills 1–6 on day 1, 7–12 on day 2, 13–18 on day 3, 19–24 on day 4, etc.). The health of each insect was noted at the end of the collection period. Only those insects surviving the 24-h assay period were used in subsequent analyses. Sample sizes for each test group ranged from 19 to 62.

Statistical analysis

For most experiments, the data were non-normally distributed, necessitating the use of a Kruskal–Wallis ANOVA on ranks for comparison between diapausing and non-diapausing females and males. Dunn's test for multiple comparisons was used where the ANOVA indicated significance. For lipid content and mass of individuals used in the metabolism assay, the data were sufficiently normal for standard ANOVA, followed by post-hoc analysis using the Holm–Sidak method. For resting metabolic rate, insufficient resolution from the Kruskal–Wallis ANOVA necessitated an additional analysis by two-way ANOVA to determine the contributions of gender, diapause status, and any interaction effect. The associations of gender and diapause status with mortality during the flight mill assay were examined in a contingency table χ^2 analysis. All analyses were conducted using SIGMAPLOT 11.0 (Systat Software, 2008).

Results

Starvation resistance

Diapause status was significantly associated with an insect's capacity to tolerate simultaneous starvation and dehydration (Kruskal–Wallis ANOVA: $H_{3,196} = 138.7$, $P<0.05$; Figure 1). Diapausers survived roughly three times longer than non-diapausers in both females ($Q = 7.55$, $P<0.05$) and males ($Q = 8.66$, $P<0.05$). There was no discernible effect of gender on survival of diapausers ($Q = 0.42$, $P>0.05$) and non-diapausers ($Q = 2.41$, $P>0.05$).

Lipid content

Lipid content was significantly influenced by treatment (ANOVA: $F_{3,48} = 12.17$, $P<0.05$; Figure 2). Diapausers had more fat than non-diapausers in both females (Holm–Sidak: $t = 2.77$, $P<0.05$) and males ($t = 4.94$, $P<0.05$). There was no discernible effect of gender for diapausers ($t = 0.35$, $P>0.05$), but among non-diapausers females had greater lipid content ($t = 2.53$, $P<0.05$).

Resting metabolism

There was a significant difference between test groups for mass (Kruskal–Wallis ANOVA: $H_{3,80} = 4.45$, $P>0.05$; Table 1), with non-diapausing females being larger than diapausing females (Holm–Sidak: $t = 3.14$, $P<0.05$), and diapausing ($t = 4.15$, $P<0.05$) and non-diapausing males ($t = 7.71$, $P<0.05$). Among diapausers, females did not differ from males ($t = 1.54$, $P>0.05$). A comparison of individual body mass with VCO_2 across all sampled individuals indicated a significant positive relationship between the two measures (Pearson's correlation: $r = 0.31$, $P<0.05$). To account for the effects of size on metabolism, mass-specific rates were used in subsequent analyses. Although there was a statistically significant difference in mass-specific resting metabolic rate among the treatment combinations of gender and diapause status (Kruskal–Wallis ANOVA: $H_{3,80} = 8.82$, $P = 0.03$), no differences were found between diapausers and non-diapausers within gender (Figure 3). A two-way ANOVA indicated a significant gender effect ($F_{1,80} = 8.64$, $P<0.05$), with males having a resting mean metabolism 24% higher than that of females. In contrast, neither diapause status ($F_{1,80} = 0.79$, $P>0.05$) nor an interaction effect between gender and status ($F_{1,80} = 0.50$, $P>0.05$) contributed significantly to the difference in mean values.

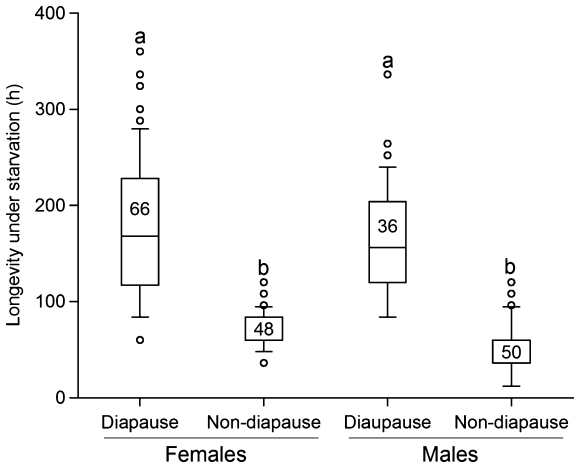

Figure 1 Longevity in female and male diapausers and non-diapausers of *Lygus hesperus* after being isolated from food and water. The medians, interquartile ranges, 90th and 10th percentiles (error bars), and outliers are shown. Bars capped with different letters are significantly different (Dunn's test: $P<0.05$). Sample sizes are indicated in the columns.

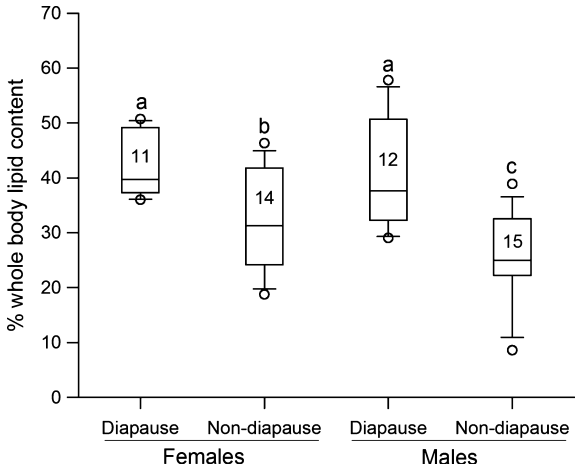

Figure 2 Whole body lipid content of diapausing and non-diapausing females and males of *Lygus hesperus*. The medians, interquartile ranges, 90th and 10th percentiles (error bars), and outliers are shown. Bars capped with different letters are significantly different (Dunn's test: $P<0.05$). Sample sizes are indicated in the columns.

Resting and feeding rates

Among test groups, there were also significant differences in rates of resting (Kruskal–Wallis ANOVA: $H_{3,227} = 28.7$, $P<0.05$; Figure 4) and feeding ($H_{3,227} = 27.9$, $P<0.05$; Figure 5). Diapausers spent roughly 20% more time at rest than non-diapausers for both females (Dunn's: $Q = 4.04$, $P<0.05$) and males ($Q = 3.52$, $P<0.05$). In addition, gender had no effect on rest rates for diapausers ($Q = 0.52$, $P>0.05$) and non-diapausers ($Q = 0.43$, $P>0.05$). Similarly, diapausers fed one-fifth as often as non-diapausers for both females ($Q = 3.78$, $P<0.05$) and males ($Q = 2.67$, $P<0.05$). Feeding rates did not differ between genders for diapausers ($Q = 0.49$, $P>0.05$) or non-diapausers ($Q = 1.30$, $P>0.05$).

Table 1 Summary statistics of animal mass and raw CO_2 release rates. Statistically different measurements (P<0.05), as determined by ANOVA for mass and Kruskal–Wallis ANOVA on ranks followed by Dunn's test for VCO_2, are indicated by different letters in the grouping term

	Mean ± SE	Minimum	Maximum	n	Grouping
Mass (mg)					
Diapause females	7.10 ± 0.19	5.05	8.64	24	a
Non-diapause females	7.97 ± 0.27	5.20	9.68	18	b
Diapause males	6.63 ± 0.18	5.20	7.42	13	ac
Non-diapause males	5.91 ± 0.14	4.74	7.39	29	c
VCO_2 (μl per min^{-1}) × 10^{-3}					
Diapause females	4.40 ± 0.32	0.008	0.680	24	a
Non-diapause females	4.93 ± 0.37	0.041	0.738	18	a
Diapause males	4.85 ± 0.40	0.243	0.718	13	a
Non-diapause males	4.75 ± 0.33	0.291	1.030	29	a

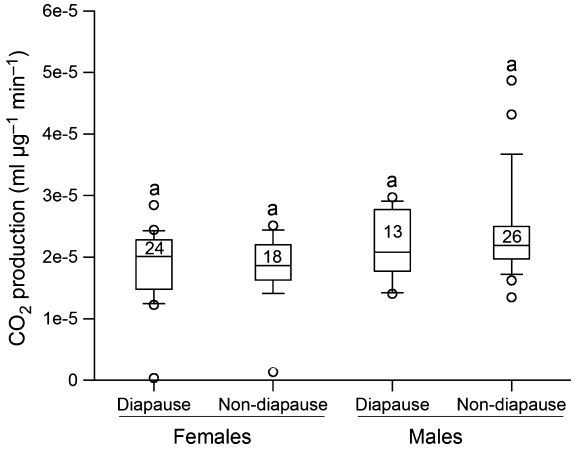

Figure 3 Mass-specific resting CO_2 production rate in female and male diapausers and non-diapausers of *Lygus hesperus*. The rate serves as an indicator of metabolic activity. The medians, interquartile ranges, 90th and 10th percentiles (error bars), and outliers (circles) are shown. Bars capped with different letters are significantly different (Dunn's test: P<0.05). Sample sizes are indicated.

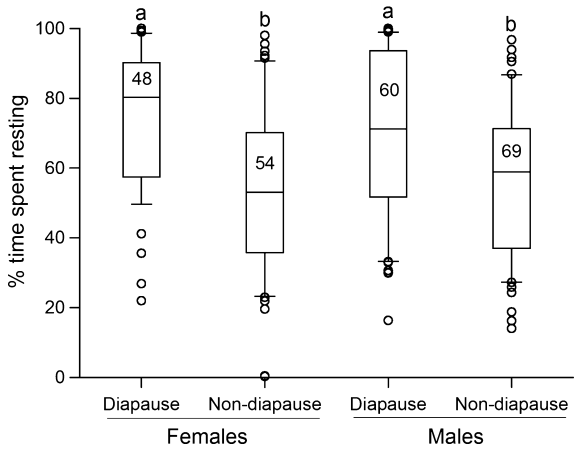

Figure 4 Percent of time spent resting by diapausing and non-diapausing females and males of *Lygus hesperus*. The medians, interquartile ranges, 90th and 10th percentiles (error bars), and outliers (circles) are shown. Bars capped with different letters are significantly different (Dunn's test: P<0.05). Sample sizes are indicated.

Flight activity

The total amount of time individuals spent flying varied greatly between groups (Kruskal–Wallis ANOVA: $H_{3,139}$ = 14.45, P<0.05; Figure 6). Non-diapausing females spent the most time flying, significantly more than diapausing (Dunn's: Q = 2.81, P<0.05) and non-diapausing males (Q = 4.06, P<0.05). Across all groups, total flight duration was positively correlated with both the number of flying bouts (average ± SE = 30.6 ± 5.8 bouts; Spearman Rank Correlation: r = 0.91, P<0.05; n = 143) and the total distance flown (average ± SE = 582.4 ± 136.5 m; r = 0.98, P<0.05; n = 143). The velocity calculated from these readings averaged 0.55 ± 0.04 m s^{-1} across the groups. Not all of the individuals survived the 24 h spent in the flight mills, and the survival rates varied between groups (χ^2 = 97.29, d.f. = 3, P<0.05; Figure 7). Non-diapausers died more frequently than diapausers, at twice the rate in females and three times the rate in males. There was also a large difference between genders, with non-diapausing males being more likely to die during the test period.

Discussion

Lygus hesperus overcomes the numerous stressors of winter by entering an adult diapause after developing as nymphs under short-day conditions (Beards & Strong, 1966; Leigh,

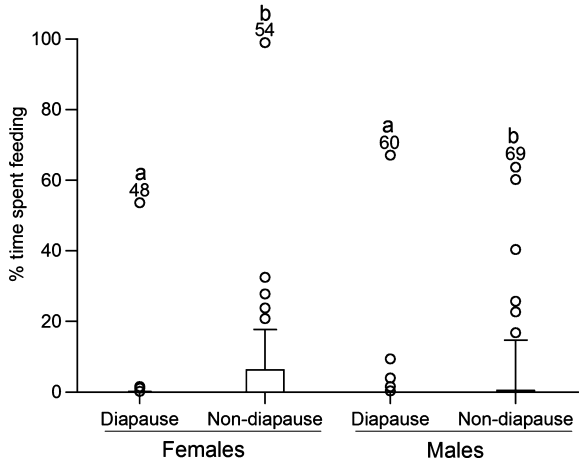

Figure 5 Percent of time spent by diapausing and non-diapausing females and males of *Lygus hesperus* in feeding on a section of green bean pod. The medians, interquartile ranges, 90th percentile (error bars), and outliers (circles) are shown, where applicable. Bars capped with different letters are significantly different (Dunn's test: $P<0.05$). Sample sizes are indicated.

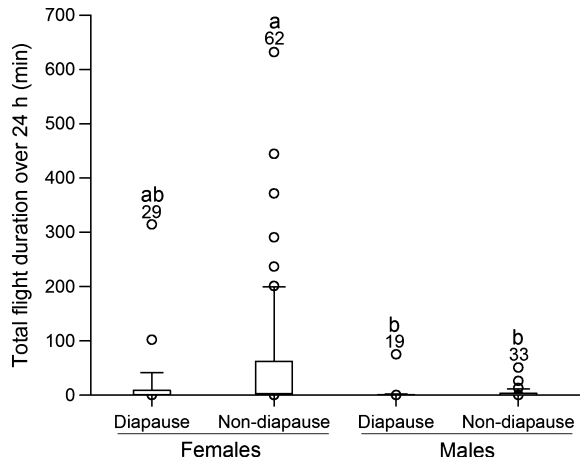

Figure 6 Total number of minutes spent flying by diapausing and non-diapausing females and males of *Lygus hesperus* observed for 24 h on a flight mill. The medians, interquartile ranges, 90th percentile (error bars), and outliers (circles) are shown, where applicable. Bars capped with different letters are significantly different (Dunn's test: $P<0.05$). Sample sizes are indicated.

1966; Spurgeon & Brent, 2010). In this experiment, we characterized some fundamental changes that accompany this transition to better understand the means by which lygus bugs might successfully overwinter. We found that individual behavior and physiology were both significantly affected by diapause status and by gender, and that these changes were associated with an enhanced survival rate under starvation and dehydration conditions.

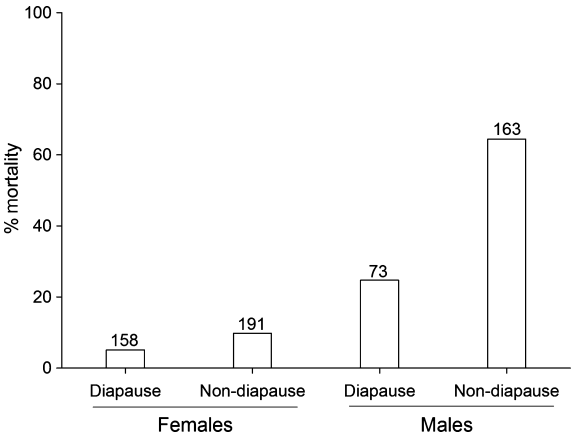

Figure 7 Percent mortality of diapausing and non-diapausing females and males of *Lygus hesperus* after 24 h tethered on a flight mill. The differences among groups were significant (χ^2 test: $P<0.001$). Sample sizes are indicated above the bars.

As would be anticipated for a condition-specific phenotype that regularly faces a scarcity of resources, diapausers denied food and water lived almost three times longer than non-diapausers under the same conditions. A substantial divergence in fat body development between the two groups, as indicated by their significantly different lipid content, was likely a key contributor to their enhanced survivorship. The insect fat body is a crucial organ for coordinating metabolic activity with the nutritive environment; during periods of resource deprivation, it serves as a sustaining source of macro- and micronutrients (Hahn & Denlinger, 2007, 2011; Arrese & Soulages, 2010). It can also serve as a source of water, released during metabolic processes. Development of the fat body in diapausing *L. hesperus* is likely enhanced by the redirection of resources away from gonadal development (Beards & Strong, 1966; Leigh, 1966; Spurgeon & Brent, 2010; Brent & Spurgeon, 2011).

Among females, the difference in fat body development was probably even more substantial than indicated by our measure of lipid content. Our whole-body analytical technique quantified all fats, including those stored in the yolk of developing and mature oocytes. Diapausers do not produce eggs, but by 10 days post-emergence non-diapausing females have invested heavily in egg production (Strong et al., 1970; Brent, 2010). The lipids sequestered in these eggs may account for much of the difference in content between female and male non-diapausers. However, although the yolk would have contributed to the overall lipid content, it might not have been a readily accessible nutrient resource for the females. Although many other insects that continuously produce eggs as adults can resorb

their oocytes during times of metabolic stress (Bell & Bohm, 1975), the process has not been documented in *L. hesperus*. In addition, at the insect ages used in this experiment, many of the females' eggs were already chorionated, limiting, if not wholly preventing nutrient resorption. Even in species in which oosorption can occur, it is usually not the first response to resource deprivation (Papaj, 2000). The similar survivorships of female and male non-diapausers support the conclusion that potential access to egg nutrients had little impact on dehydration/starvation resistance.

Other common resource-conserving responses among food- and water-deprived insects are reductions in metabolic rate and overall activity level (Hahn & Denlinger, 2011). These changes minimize the expenditure of an individual's limited nutrient stores until conditions improve. For *L. hesperus* the resting metabolic rate did not differ between diapausers and non-diapausers of either gender. While metabolic depression is common in species in which the overwintering stage is dormant (Tauber et al., 1986; Grodzicki & Walentynowicz, 2011), in other species in which the diapausers remain active (e.g., *Danaus plexippus* L.; Chaplin & Wells, 1982), changes in metabolism tend to be more limited (Grodzicki & Walentynowicz, 2011). Diapausing *L. hesperus* adults do not become completely quiescent, and instead retain their ability to dynamically interact with their environment. They do achieve some energetic savings by not allocating resources toward gonad development and activity.

Rather than changing metabolic rate to conserve resources, *L. hesperus* may rely upon a suppressed activity level. With dormant gonads they of course cease all reproductive behaviors. In addition, we found that there is a small but significant increase in the amount of time that the diapausers spent resting relative to non-diapausers, at least under laboratory conditions. This decline in activity was also reflected in the reduced feeding by diapausers. It is common for diapausing insects to limit or entirely cease food consumption when conditions are inhospitable, reducing both the risk of predation and the energy expended while foraging for scarce and stochastically distributed resources (Tauber et al., 1986; Hahn & Denlinger, 2007).

One activity that did not appear affected by diapause status was flight. There was no difference between diapausers and non-diapausers of either gender for the amount of time spent in flight. In contrast, there were distinct gender differences, with females, particularly non-diapausers, flying longer than males. They also took flight more frequently during the observation period. Frequent locomotion may enhance the probability that females come into contact with a potential mate, which may be crucial in a species that has no known ranged sex-specific attractant pheromone (Millar et al., 2000; Brent & Byers, 2011). Such behavior would also enhance contact rate with appropriate sites for oviposition. Endogenous sensory stimuli, such as stretch receptors activated by a full load of mature eggs, might amplify female mobility.

The flight mill assay, like the starvation assay, is a good indicator of resource reserves, as it couples an energy intensive activity with a 24-h period of starvation. Because the fat body is usually responsible for replenishing the energy stores needed for sustained flight activity (Arrese & Soulages, 2010) and can act as a source of metabolic water, diapausers should be better capable of surviving the test conditions. This is borne out by the finding that non-diapausers were more prone to dying during this assay in both genders, and the effect was most pronounced in non-diapausing males which had the least lipid content. One contributing factor to this heightened mortality is the higher resting metabolic rate of males, but this is not likely to be the only determinant. It is possible that females maintain greater stores of glycogen in their muscle tissue than males, a carbohydrate that would be expended during flights prior to fat stores being utilized (Arrese & Soulages, 2010). If *L. hesperus* females did maintain greater glycogen reserves, it could provide a crucial buffer, diminishing the acute stress effects observed in males.

Collectively the results indicate that the diapause response of *L. hesperus* extends well beyond the cessation of reproduction and accumulation of lipids. Many of the behavioral changes are consistent with what is known to occur in other insects, resulting in reduced energy expenditure while simultaneously maintaining their capacity to respond to unpredictable shifts in their environment (Hahn & Denlinger, 2007, 2011). Of course, field studies will be needed to confirm that these observed behavioral shifts do occur under more natural circumstances. Whereas undeveloped gonads, reduced activity levels, and hypertrophic fat bodies ensure the bugs have the resources necessary to sustain themselves for 1 or 2 weeks of extreme deprivation, other adaptations are also likely to contribute significantly to their chances of survival and will need to be examined before we can begin successfully targeting overwintering populations.

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