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# Interpopulation variation in the trade-off between body-mass gain and age at oviposition in the eastern lubber grasshopper, *Romalea microptera*

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## Abstract

Life-history models predict populations under shorter growing seasons will invest earlier and more heavily in reproduction than populations under longer growing seasons. Populations of *Romalea microptera* inhabit distinctly different climates and differ in mtDNA cytochrome-b gene sequences. Previous work suggested a latitudinal trend in the trade-off among body-mass gain, age at first oviposition, and clutch mass, in populations from Miami FL, Lydia LA, and Athens GA: but this study was confounded by longitudinal variation. Hence, we compared the Miami and Athens populations to a population from Jacksonville, FL, which is equidistant from both, yet under a distinct climate. The Athens population had less body-mass gain and an earlier age at oviposition than the other two populations. When corrected for initial body mass or age at oviposition, the Athens population produced larger clutches. Developmental profiles of juvenile hormone and hemolymph lipids did not differ across populations. In comparison to the Miami population, the Jacksonville population showed a nonsignificantly greater body-mass gain and age at oviposition, in contrast to the latitudinal trend observed previously. These data suggest that reproduction in *R. microptera* involves a trade-off between body-mass gain and age at oviposition, which is consistent with a current vs future reproduction trade-off.

## Key words

interpopulation variation, terminal investment, trade-off, reproductive tactics, body size, latitudinal variation

## Introduction

Studying interpopulation variation in 'common garden-variety' experiments can provide evidence of differences due to natural selection (Futuyma 1998). Populations from localities with shorter growing seasons may exhibit earlier life-history transitions at lower body masses (Forrest 1987, Rowe & Ludwig 1991, Temte 1993, Berkenbusch & Rowden 2000, Hatle *et al.* 2002, Luker *et al.* 2002). This trade-off between development time and body mass is most critical when time constraints on growth and reproduction are imposed by seasonality (*e.g.*, onset of winter). Models of life-history evolution speculate that a decrease in lifespan will result in earlier development and an increased reproductive investment at early ages (*e.g.*, Williams 1966, Charlesworth 1980, Reznick *et al.* 1990, Roff 1992, Partridge *et al.* 1995).

*Romalea microptera* (Beavois), the eastern lubber grasshopper, inhabits a geographic range that includes distinctly different climates. *R. microptera* is flightless and disperses little ( $\sim 50$  m / lifetime, Whitman 1990). In addition there are distinct differences in the body size and color of distant populations of *R. microptera*. Sequencing of the mtDNA cytochrome-b gene showed that nearly all populations are distinguishable (Mutun & Borst 2004). Previous work

suggested a latitudinal trend in the trade-off among body-mass gain, age at oviposition, and clutch mass (Hatle *et al.* 2002). This previous study, which included populations from Miami, Fla, Lydia, La, and Athens, Ga, was confounded by longitudinal variation. To address this weakness, we compare a population from Jacksonville, Florida with Miami and Athens *R. microptera*.

The Jacksonville population of *R. microptera* is of particular interest. First, it falls directly on a latitudinal cline between previously studied Athens and Miami populations, and is almost exactly between them. Second, the Jacksonville population exists in a remarkably different ecosystem than the Miami and Athens populations. Jacksonville's climate lies moderately between the highly seasonal temperate, continental climate of Athens and the less variable, subtropical climate of Miami. Jacksonville is separated from Miami by the "frost line," which marks the transition from mangroves in the south (Myers & Ewel 1990) to pines in north Florida. Distinct from both Jacksonville and Miami, Athens is in the primarily deciduous Southern Piedmont Province (NARSAL 2007). Athens has 249 frost-free days, Jacksonville has 345 frost-free days, and Miami is frost-free year round (NCDC 2007). The distance between Athens and Miami is approximately 900 km (560 mi).

In all major insect orders except *Diptera*, juvenile hormone (JH) is a major gonadotropin: it stimulates vitellogenin synthesis and mediates potency (Nijhout 1994). In *R. microptera*, JH is required for vitellogenin-mRNA production (Fei *et al.* 2005). Further, JH levels are associated with the timing of oviposition: *R. microptera* on low feeding rates reach the maximum level of JH later and oviposit later than those on high feeding rates (Hatle *et al.* 2000).

Variation in reproductive tactics and JH titers among Athens, Jacksonville, and Miami populations existing on a latitudinal cline were examined. We predicted that age at first oviposition would vary such that Athens < Jacksonville < Miami. A cost for early oviposition should be observed in the Athens population, perhaps as a reduction in clutch mass or somatic growth. Further, we predicted that age at attaining the maximum level of JH would vary such that Athens < Jacksonville < Miami.

## Methods

Juvenile *R. microptera* were field-collected from Athens, Georgia; Jacksonville, Fla, and Miami, Fla, and shipped to the laboratory in Jacksonville. Latitudes for these locations are approximately 25° N, 30° N, and 33° N, respectively. Juveniles were reared together *ad lib.* on Romaine lettuce and oats, and under heat lamps at 24±2°C on a 14L:10D photoperiod. On the day of adult molt, females were weighed, isolated and reared individually in 500-ml ventilated containers, at a 14L:10D photoperiod and a corresponding 32:24°C

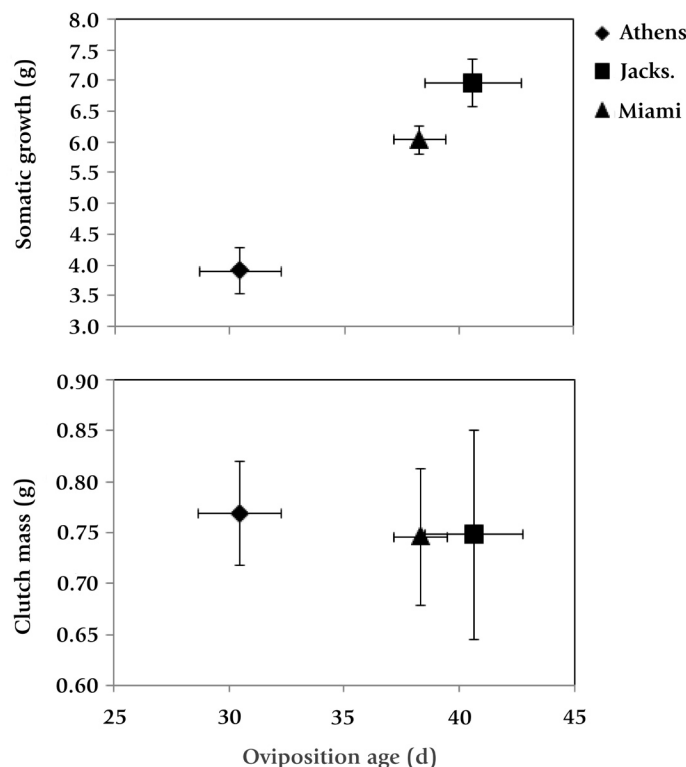


Fig. 1. Bivariate plots of reproductive tactics (means  $\pm$  s $\bar{x}$ ) in *R. microptera* grasshoppers from three populations. The Athens population differed significantly from the Jacksonville (Jacks.) and Miami populations in body mass after oviposition and oviposition age, but not clutch mass. Somatic growth represents the somatic storage retained after laying the first clutch, adjusted for mass at molt.

thermocycle. Previous research suggests that variable reproductive plasticity among interpopulations is absent; that is, Athens, Louisiana, and Miami populations all responded to low food similarly (Hatle *et al.* 2002). Therefore, individuals were offered the same relative diet adjusted for body size. To determine the amount of diet to be fed each insect, the femur length of each individual was multiplied by a constant (0.12), yielding the total mass of Romaine lettuce in grams.

Hemolymph samples were taken twice a week and stored in hexane at  $-20^{\circ}\text{C}$  for later analysis of JH titers (Hatle *et al.* 2000) and total hemolymph lipids. Beginning at day 24, females were tested for oviposition (Hatle *et al.* 2000). Once a female oviposited, she was weighed and retired from the study. Eggs from each female were counted and dried (Athens  $n = 9$ , Jacksonville  $n = 12$ , Miami  $n = 11$ ). Egg weight for each individual was obtained by averaging the weight of ten eggs. Clutch mass ( $n = 9$  for all populations) was calculated by multiplying an individual's average egg weight by the total number of eggs oviposited.

Juvenile hormone titers over the course of each individual's reproductive cycle were analyzed via radio-immunoassay (Hatle *et al.* 2000). All of the samples from a single individual were analyzed simultaneously to avoid any effects of interassay variation. Order of analysis of individuals was randomized. Maximum level of JH (Athens  $n = 9$ , Jacksonville  $n = 12$ , Miami  $n = 6$ ) was determined by comparing all samples for an individual and identifying the sample with the highest JH titer. The age at which that sample was collected was defined as the age at maximum level of JH.

The total hemolymph lipids were also measured from the same

hemolymph samples ( $n = 7$  for each population). The transportation of lipids occurs via the hemolymph, and approximately 40% of the egg is lipid (Chapman 1998). Therefore, a minimum rate of lipid transport may be required to complete vitellogenesis. If lipids play an important role in the timing of oviposition, we predict a peak of lipid transport during the period of greatest growth of oocytes. Lipids were measured as vanillin-positive material using vegetable oil standards (Hatle & Spring 1998).

All data were tested statistically for the effects of population. A MANCOVA using initial weight as a covariate was used to analyze the three-way trade-off among body-mass gain after oviposition, age at oviposition, and clutch mass (Athens  $n = 9$ , Jacksonville  $n = 10$ , Miami  $n = 12$ ). Because initial body mass was used as a covariate (Pillai's Trace = 0.337;  $F_{3,20} = 3.38$ ;  $p = 0.038$ ), body mass after oviposition estimates the somatic mass gained from adult molt to oviposition. A second MANOVA was used to analyze data on maximum level of JH, age at maximum level of JH, and time from maximum level of JH to oviposition (Athens  $n = 9$ , Jacksonville  $n = 12$ , Miami  $n = 6$ ). A one-way ANOVA was used to analyze lipid data. SAS PROC GLM was used for all analysis (SAS 1989).

## Results

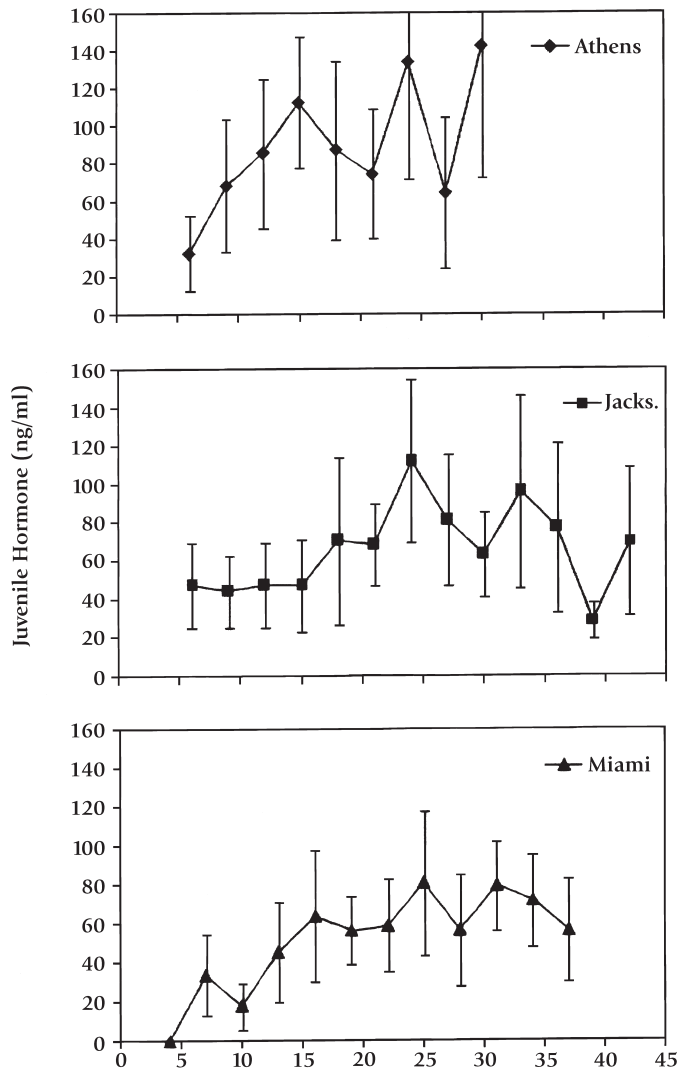
**Reproductive tactics.**—Both body mass after oviposition ( $F_{3,22} = 17.02$ ,  $p < 0.0001$ ) and age at oviposition ( $F_{3,22} = 5.81$ ,  $p = 0.004$ ) were significantly affected by population (Fig. 1, MANCOVA, Pillai's Trace = 0.598,  $F_{6,42} = 2.99$ ,  $p = 0.016$ ). Multivariate pairwise contrasts indicated that the Athens population differed significantly from both the Jacksonville ( $p = 0.0005$ ) and Miami ( $p = 0.0258$ ) populations, which did not differ from each other ( $p = 0.3196$ ).

Standardized canonical coefficients (age at oviposition = 0.819, body mass after oviposition = 1.48, clutch mass = -0.061) indicate that the greatest effect of population was due to the mass after oviposition, followed by the age at oviposition. Clutch mass had no contribution to population differences. Body mass after oviposition (adjusted for body mass at molt as a MANCOVA covariate) for Athens was significantly less than both the Jacksonville ( $p = 0.0003$ ) and the Miami ( $p = 0.0243$ ) populations, which did not differ from each other ( $p = 0.0847$ ). Similarly, age at oviposition for the Athens population was significantly less than both the Jacksonville (pairwise contrast statement  $p = 0.0113$ ) and the Miami ( $p = 0.0446$ ) populations, which did not differ from each other ( $p = 0.6009$ ).

The ratio of clutch size to body size (as femur length) was significantly affected by population (ANOVA,  $F_{2,25} = 8.932$ ,  $p = 0.001$ ). Pairwise comparisons indicate that the Athens population produced a larger clutch with respect to body size than both the Jacksonville ( $p = 0.001$ ) and Miami ( $p = 0.015$ ) populations, which did not differ from each other ( $p = 0.497$ ).

The ratio of clutch size to oviposition age was significantly affected by population (ANOVA,  $F_{2,18} = 10.744$ ,  $p = 0.001$ ). Pairwise comparisons indicate that the Athens population produced a larger clutch, with respect to the duration of their clutch, than both the Jacksonville ( $p = 0.001$ ) and the Miami ( $p = 0.011$ ) populations, which did not differ from each other ( $p = 1.000$ ).

**Juvenile Hormone attributes.**—There was no significant population effect on JH attributes (Fig. 2; MANOVA, Pillai's Trace = 0.338,  $F_{6,46} = 1.56$ ,  $p = 0.180$ ). Populations did not differ statistically in their age at maximum level of JH ( $F_{2,24} = 1.51$ ,  $p = 0.242$ ), time from maximum level of JH to oviposition ( $F_{2,24} = 1.60$ ,  $p = 0.222$ ), or maximum titer of JH ( $F_{2,24} = 0.22$ ,  $p = 0.807$ ).

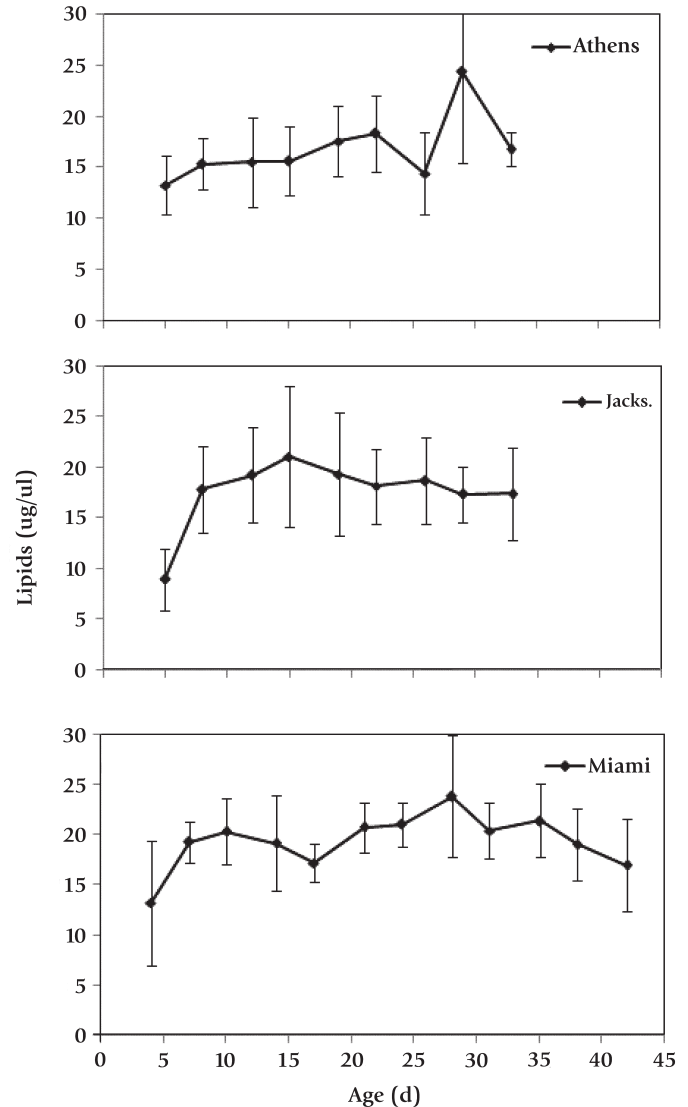


**Fig. 2.** Hemolymph juvenile hormone profiles (mean  $\pm$  s $\bar{x}$ ) for adult female *R. microptera* grasshoppers from three populations during the 1<sup>st</sup> oviposition cycle. Hemolymph samples were collected biweekly. Profiles end at the median age of oviposition for each population. Populations showed no differences in JH profiles.

**Hemolymph Lipids.**—No distinct peaks among the hemolymph lipid profiles existed for any of the populations (Fig. 3). The grand means of each population were compared by ANOVA. There was no significant population effect on mean hemolymph lipid concentration ( $F_{2,14} = 0.44$ ,  $p = 0.653$ ).

## Discussion

In this study, we examined interpopulation variation in a three-way trade-off among body-mass gain, age at oviposition, and clutch mass. We also tested interpopulation variation in JH titers and lipid transport. Populations significantly differed in the three-way trade-off among body-mass gain, age at oviposition, and clutch mass. Body-mass gain and age at oviposition were the only variables that contributed significantly to interpopulation variation (Fig. 1).



**Fig. 3.** Hemolymph lipid profiles (mean  $\pm$  s $\bar{x}$ ) for adult female *R. microptera* grasshoppers from three populations. Hemolymph samples were collected biweekly. Profiles end at the median age of oviposition for each population. Populations showed no differences in lipid profiles, suggesting no differences in the rates of lipid transport.

Across populations, early oviposition correlated with less somatic growth during the adult phase. It has been hypothesized that somatic mass gained during egg production is directed toward reproduction of subsequent clutches (Hatle *et al.* 2002). This implies that the cost for early reproduction does not appear in the current reproductive event, but rather at the cost of future reproduction.

The current reproduction over future reproduction trade-off, known as the terminal-investment hypothesis, has been observed previously in various organisms faced with the threat of a reduced lifespan (Pianka & Parker 1975). Most previous research on terminal-investment involves *individual* plasticity in responding to life-reducing events, such as exposure to bacteria, parasites, or viruses (Adamo 1999, Bonneaud *et al.* 2004), injury (Javois & Tamaru 2004), or senescence (Tatar & Carey 1995, Fronstin & Hatle 2008). Our research involves adaptive responses due to *population* variation. Further studies would be needed to determine if the terminal-invest-

ment theory might apply to populations as an adaptive response to environments that shorten lifespan.

Our results indicate that, biologically, Jacksonville populations are more similar to those of Miami than to those of Athens, despite being about equidistant from them both. The similarities between the Jacksonville and Miami populations are consistent with a linear relationship among populations, yet are not consistent with the predicted latitudinal cline. This suggests that among these three populations, only Athens has undergone evolutionary divergence in reproductive tactics. The absence of a distinction between Jacksonville and Miami populations could be due to a balance between the costs and benefits that early reproduction yields upon fitness. In this case, it might suggest that the Jacksonville population does not sustain a sufficient reduction in lifespan to warrant the cost of reduction in somatic growth during oviposition.

Investment into each reproductive event can be measured via clutch mass. When taking body size and oviposition timing into consideration, these populations differ in investment. All three populations differ significantly in body size (femur length, ANOVA,  $F_{2,30} = 39.370$ ,  $p < 0.001$ ), such that Athens < Miami < Jacksonville. Despite being smaller and ovipositing in significantly less time, Athens grasshoppers produced similar-sized clutches as Miami grasshoppers. This indicates that the Athens population invests more resources to produce larger clutches with respect to body size and age at oviposition. This accounts for the minimal body-mass gain of Athens and is consistent with the current over future reproduction strategy.

Maternal environments have the ability to influence the expression of traits in their offspring (Mousseau & Fox 1998). Therefore, due to potential environmental maternal effects, the differences found between these populations may not be genetic. Some life-history traits of *R. microptera* suggest that any potential maternal effects on reproduction would only make a minor contribution to the observed interpopulation variation. Varying egg size is a common mechanism used by mothers in response to different environments (Parker & Begon 1986). For example, in unfavorable environments with limited food, a mother may lay fewer but larger eggs in order to provide offspring a better chance of survival. However, maternal diet in *R. microptera* has no effect on egg size (Moehrlin & Juliano 1998, Hatle *et al.* 2000). In addition, juvenile diet does not seem to affect reproductive tactics. In fact, unlike many insects, following adult molt, *R. microptera* undergo a period of further somatic growth prior to any reproductive growth (Hatle *et al.* 2004). These tactics employed by *R. microptera* make maternal effects seem less likely.

The maximum level of JH occurs during the fixed phase of oviposition, indicating the commitment to oviposition (Hatle *et al.* 2000). Vitellogenin and JH profiles have been shown to have similar developmental patterns (Borst *et al.* 2000; Hatle *et al.* 2000, 2001). Maximum vitellogenin levels among populations corresponded to oviposition timing (Hatle *et al.* 2004). Specifically, Athens oviposited earlier than the other populations and exhibited a younger age at vitellogenin maximum. Hence, we predicted that the maximum level of JH would vary across populations in concert with age at oviposition. However, the JH titers between populations did not differ. In previous studies, JH profiles exhibited distinct maxima (Hatle *et al.* 2000), but profiles from this study did not reveal distinct maxima. It is possible that we have not obtained an accurate picture of the JH profiles, perhaps by taking hemolymph samples only biweekly, or due to sample degradation.

There were no distinct peaks in the lipid profiles and populations did not vary in the magnitude of hemolymph lipids. Hemolymph

lipids appear to remain stable throughout both somatic and reproductive growth phases in *R. microptera*, suggesting that oocyte production does not alter the hemolymph lipid levels.

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## References

- Adamo S.A. 1999. Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Animal Behavior* 57: 117-124.
- Berkenbusch K., Rowden A.A. 2000. Latitudinal variation in the reproductive biology of the burrowing ghost shrimp *Callinassa filholi* (Decapoda: Thalassinidea). *Marine Biology* 136: 497-504.
- Bonneaud C., Mazuc J., Chastel O., Westerdaal H., Sorci G. 2004. Terminal investment induced by immune challenge and fitness traits associated with major histocompatibility complex in the house sparrow. *International Journal of Organic Evolution* 58: 2823-2830.
- Borst D.W., Eskew M.R., Wagner S.J., Shores K., Hunter J., Luker L., Hatle J.D., Hecht L.B. 2000. Quantification of juvenile hormone III, vitellogenin, and vitellogenin-mRNA during the oviposition cycle of the lubber grasshopper. *Insect Biochemistry and Molecular Biology* 30: 813-819.
- Chapman R.F. 1998. *The Insects: Structure and Function*. Harvard University Press, Cambridge, Massachusetts.
- Charlesworth B. 1980. *Evolution in Age-structured Populations*. Cambridge University Press, Cambridge.
- Fei H., Martin T.R., Jaskowiak K.M., Hatle J.D., Whitman D.W., Borst D.W. 2005. Starvation affects vitellogenin production but not vitellogenin mRNA levels in the lubber grasshopper, *Romalea microptera*. *Journal of Insect Physiology* 51: 435-443.
- Fronstin R.B., Hatle J.D. 2008. A cumulative feeding threshold required for vitellogenesis can be obviated with juvenile hormone treatment in lubber grasshoppers. *Journal of Experimental Biology* 211: 79-85.
- Forrest T.G. 1987. Insect size tactics and development strategies. *Oecologia* 73: 178-184.
- Futuyma D.J. 1998. *Evolutionary Biology*. Sinauer Associates, Inc., Sunderland, Massachusetts.
- Hatle J.D., Andrews A.L., Crowley M.C., Juliano S.A. 2002. Geographic variation of reproductive tactics in lubber grasshoppers. *Oecologia* 132: 517-523.
- Hatle J.D., Andrews A.L., Crowley M.C., Juliano S.A. 2004. Interpopulation variation in developmental titers of vitellogenin, but not storage proteins, in lubber grasshoppers. *Physiological and Biochemical Zoology* 77: 631-640.
- Hatle J.D., Borst D.W., Eskew M.R., Juliano S.A. 2001. Maximum titers of vitellogenin and total hemolymph protein occur during the canalized phase of grasshopper egg production. *Physiological and Biochemical Zoology* 74: 885-893.
- Hatle J.D., Juliano S.A., Borst D.W. 2000. Juvenile hormone is a marker of the onset of reproductive canalization in lubber grasshoppers. *Insect Biochemistry and Molecular Biology* 30: 821-827.
- Hatle J.D., Spring J.H. 1998. Inter-individual variation in sequestration (as measured by energy dispersive spectroscopy) predicts efficacy of defensive secretion in lubber grasshoppers. *Chemoecology* 8: 85-90.
- Javois J., Tammaru T. 2004. Reproductive decisions are sensitive to cues of life expectancy: the case of a moth. *Animal Behavior* 68: 249-255.

- Luker L.A., Hatle J.D., Juliano S.A. 2002. Reproductive responses to photoperiod by a south Florida population of the grasshopper *Romalea microptera* (Orthoptera: Romaleidae). *Environmental Entomology* 31: 702-707.
- Moehrlin G.S., Juliano S.A. 1998. Plasticity of insect reproduction: testing models of flexible and fixed development in response to different growth rates. *Oecologia* 115: 294-500.
- Mousseau T.A., Fox C.W. 1998. *Maternal Effects as Adaptations*. Oxford University Press, Oxford.
- Mutun S., Borst D.W. 2004. Intraspecific mitochondrial DNA variation and historical biogeography of the lubber grasshopper, *Romalea microptera*. *Annals of the Entomological Society of America* 97: 681-696.
- Myers R.L., Ewel J.J. 1990. *Ecosystems of Florida*. University of Central Florida Press, Orlando, Florida.
- National Climatic Data Center (NCDC). 2007. Local Climatological Data, Jacksonville, Florida, 2000-2006 [Internet]. Florida Climate Center, [cited 15 July 2007]. Available: >[http://www.coaps.fsu.edu/climate\\_center/nav.php?a=go&s=data&p=lcd](http://www.coaps.fsu.edu/climate_center/nav.php?a=go&s=data&p=lcd)<
- Natural Resources Spatial Analysis Laboratory (NARSAL) [Internet]. 2007. University of Georgia [cited 15 July 2007]. Available><http://narsal.ecology.uga.edu/gap/Georgia.html><.
- Nijhout H.F. 1994. *Insect Hormones*. Princeton University Press, Princeton, N.J.
- Parker G.A., Begon M. 1986. Optimal egg size and clutch size: effects of environmental and maternal phenotype. *American Naturalist* 128: 573-592.
- Partridge L., Barrier B., Barton N.H., Fowler K., French V. 1995. Rapid evolution of adult life-history traits in *Drosophila melanogaster* in response to temperature. *Evolution* 49: 538-544.
- Pianka E.R., Parker W.S. 1975. Age-specific reproductive tactics. *American Naturalist* 109: 453-464.
- Reznick D.N. 1990. Plasticity in age and size at maturity in male guppies (*Poecilia reticulata*): an experimental evaluation of alternative models of development. *Journal of Evolutionary Biology* 3: 185-203.
- Roff D.A. 1992. *The Evolution of Life Histories*. Chapman and Hall, New York.
- Rowe L., Ludwig 1991. Size and time of metamorphosis in complex life cycles: time constraints and variation. *Ecology* 72: 413-427.
- Tatar M., Carey J.R. 1995. Nutrition mediates reproductive trade-offs with age-specific mortality in the beetle, *Calosobruchus maculatus*. *Ecology* 76: 2066-2073.
- SAS Institute. 1989. *SAS/STAT User's Guide*. Version 6. Vol. 2. SAS Institute, Cary, N.C.
- Temte J.L. 1993. Latitudinal variation in the birth timing of captive California sea lions and other captive North Pacific pinnipeds. *Fisheries Bulletin* 91: 710-17.
- Whitman D.W. 1990. Grasshopper chemical communication, pp. 357-391. In: Chapman R., Joern A., (Eds) *Biology of Grasshoppers*. John Wiley & Sons, New York.
- Williams G.C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's principle. *American Naturalist* 100: 687-690.