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A cumulative feeding threshold required for vitellogenesis can be obviated with juvenile hormone treatment in lubber grasshoppers

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SUMMARY

Developmental thresholds can ensure that an adequate condition has been attained to proceed through major transitions (e.g. initiation of reproduction, metamorphosis). Nutrition is critical to attaining most thresholds, because it is needed for both growth and storage. Attaining a threshold typically stimulates the release of hormones that commit the animal to the developmental transition, yet the relationships between the nutrition needed for developmental thresholds and these endocrine signals are poorly understood. Lubber grasshoppers require a cumulative feeding threshold to initiate vitellogenesis and potentially commit to oviposition. We tested the relative roles of the nutritional threshold and the major gonadotropin (juvenile hormone; JH) in initiating vitellogenesis and committing to oviposition. The source of JH was removed from all females, and then JH analog was applied after different amounts of feeding. Threshold feeding was not required to initiate vitellogenesis, suggesting that sub-threshold grasshoppers are competent to respond to JH. Further, sub-threshold grasshoppers went on to oviposit earlier than supra-threshold grasshoppers treated with JH at the same time. Hence, threshold feeding is required only to cause the production and release of JH. At the same time, we also found that individuals that were restored with JH late in life tended to favor current reproduction, at the expense of future reproduction. Both time to oviposition and vitellogenin profiles were consistent with this developmental allocation. Taken together, our results suggest that lubber grasshoppers adjust reproductive tactics primarily in response to nutrition (which only serves to release JH) and secondarily in response to age.

Key words: developmental threshold, life history, physiology, phenotypic plasticity, reproduction, terminal investment hypothesis, trade-off.

INTRODUCTION

Major post-embryonic developmental transitions (e.g. initiation of reproduction, metamorphosis, cessation of growth) are critical stages in an organism's life. Phenotypic plasticity in timing (e.g. age at oviposition) or resource allocation (e.g. size of clutch) at these developmental transitions is common (Stearns, 1992; Morey and Reznick, 2000; Schoech et al., 2004; Davidowitz et al., 2005); this plasticity can have important effects on fitness (Denver et al., 1998). Prior to a developmental transition, a threshold describes the status needed and ensures that an adequate condition has been attained to proceed through the transition (Wilbur and Collins, 1973; Frisch, 2002; Reynolds et al., 2003; Moczek and Nijhout, 2003; Nijhout, 2003; Davidowitz et al., 2005; Nijhout et al., 2006).

Thresholds can be described: (1) morphologically as a critical body or organ size (e.g. Nijhout and Williams, 1974a; Nijhout and Williams, 1974b; Davidowitz et al., 2005; Mirth et al., 2005); (2) physiologically as a level of storage (Frisch, 2002) or (3) nutritionally as an amount ingested (Nijhout, 1994; Klowden, 1987; Juliano et al., 2004). Clearly, nutrition is vital to attaining all of these thresholds, because it is needed for both growth and storage. However, these thresholds must be translated to endocrine signals that mediate the life history transition (Nijhout and Williams, 1974a; Nijhout and Williams, 1974b; Day and Rowe, 2002; Moczek and Nijhout, 2003; Juliano et al., 2004; Davidowitz et al., 2005; Nijhout et al., 2006). The relationships between developmental thresholds and these endocrine signals are poorly understood, yet they are a critical link in how environmental conditions produce variation in life histories (Emlen and Nijhout, 1999; Zera and Harshman, 2001; Davidowitz et al., 2005; Shingleton et al., 2007).

In lubber grasshoppers (*Romalea microptera*), a cumulative feeding threshold of 4.0 g dry mass of Romaine lettuce is required to initiate the transition from somatic growth to vitellogenesis and ultimately oviposition (Juliano et al., 2004) (Fig. 1). Diet prior to adult molt has little effect on the timing of reproduction (Moehrlin and Juliano, 1998). Hence, a single developmental program produces different phenotypes simply due to its expression in different environments (e.g. nutritional levels) (Reznick, 1990). Plasticity of reproductive timing is thus dependent only on the time to attain that threshold.

In well-fed lubber grasshoppers (offered 0.77 g dry mass Romaine lettuce per day of adulthood), first oviposition occurs ~35 days after the adult molt. By contrast, in low-fed animals (0.12 g dry mass day^{-1}), first oviposition occurs ~65 days after the adult molt (Hatle et al., 2000; Hatle et al., 2003a). Mature females generally lay 2–3 clutches during their life (Hatle et al., 2006b). Lubber grasshoppers are univoltine and overwinter as eggs; therefore, reproduction is potentially time-constrained with the onset of winter (Rowe et al., 1994; Luker et al., 2002).

Nutrition is needed to stimulate the production of the major gonadotropin juvenile hormone (JH) in lubber grasshoppers. But whether nutrition is needed to attain the status needed for JH response (i.e. competence) is unknown. During the oviposition cycle, JH levels start at zero, first rise to low levels (which corresponds to the initiation of vitellogenin production), then rise to a maximum around mid-vitellogenesis (which corresponds to

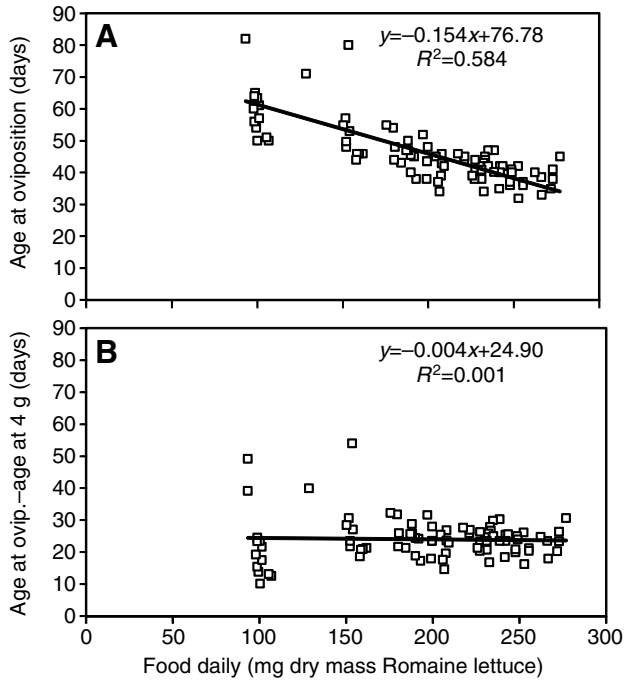

Fig. 1. (A) Time to oviposition in response to feeding rate since adulthood and (B) time to oviposition since 4.0 g dry mass of lettuce was consumed in response to feeding rate. Cumulative diet quantity strongly affects age at oviposition in female lubber grasshoppers, but this plasticity is manifested only early in adulthood. Data from (Juliano et al., 2004).

oocyte growth and patency) and finally fall before oviposition. The maximum titer of JH is the point at which JH degradation becomes favored over JH synthesis; the maximum always occurs about 12 days before oviposition (Hatle et al., 2000; Hatle et al., 2003a). These studies on the effects of nutrition on JH levels suggest that threshold feeding (see Juliano et al., 2004) (Fig. 1) is only needed to allow production of JH, and the female is competent to respond to JH prior to attaining the threshold.

By contrast, studies on vitellogenesis suggest that feeding to the threshold is required in addition to JH. Production of vitellogenin mRNA requires JH (Fei et al., 2005). In starved grasshoppers, the infusion of JH increases vitellogenin mRNA, but feeding is required for synthesis of vitellogenin protein by the fat body (Fei et al., 2005). These results suggest that JH may not be the only factor involved in the regulation of vitellogenin production, but instead some other nutrition-dependent change is needed. Similarly, Hatle et al. found that total vitellogenin production relies more on total fat body mass than on mass-specific tissue stimulation (typically by JH) (Hatle et al., 2006a). This suggests that growth factors affecting the fat body, which are likely nutrition dependent, might be a co-requirement with JH for vitellogenesis (Hatle et al., 2006a). Hence, studies on vitellogenin production suggest threshold feeding is needed both to initiate production of JH *and* to bring about competence to JH.

We manipulated both feeding and the timing of initial JH treatment to test whether JH is solely responsible for vitellogenesis or if the feeding threshold must also be met for competence to JH. We predict that the feeding threshold must be met (see Fei et al., 2005; Hatle et al., 2006a). Specifically, individuals that are sub-threshold at the start of JH analog treatment will delay vitellogenesis, and ultimately oviposition, in comparison with individuals that are supra-threshold at the start of JH analog treatment. In other words, we predict a statistically significant interaction of diet and timing of JH initiation on the onset of vitellogenesis and timing of oviposition. Alternatively, if attainment of the feeding threshold is not required along with JH, sub-threshold females treated with JH should undergo vitellogenesis in concert with supra-threshold females treated with JH.

MATERIALS AND METHODS

Experimental design

This experiment employed a 2×2 factorial design, manipulating both cumulative feeding amount and age at initial JH analog application (JHAi). New adult female grasshoppers were serially assigned into two groups: low or high diet. Within both of these two diet groups, individuals were later assigned to either early or late JHAi. The four treatment groups were: low food/early JHAi (N=14), low food/late JHAi (N=5), high food/early JHAi (N=9) and high food/late JHAi (N=2). Only individuals that ultimately oviposited were included in the study. Individuals in the late JHAi groups took longer to oviposit; therefore, their rates of survival to oviposition were lower, resulting in lower sample sizes. Fortunately, the high food/late JHAi was the least important group for addressing our hypothesis.

The timing of early JHAi was chosen to ensure that, when fed *ad libitum*, the high food/early JHAi group had consumed a supra-threshold quantity of lettuce at JHAi [i.e. greater than 4.0 g dry mass as determined by Juliano et al. (Juliano et al., 2004)]. The timing of late JHAi was chosen to ensure that the low food/late JHAi group also had consumed a supra-threshold quantity of lettuce at JHAi when fed at the same rate as the low food/early JHAi group. The low food/early JHAi group was the only sub-threshold feeding group at the start of hormone treatments (Table 1).

Table 1. Mean (± s.e.m.) feeding parameters in response to food offered (high or low) and timing of gonadotropin treatments (early or late initiation of juvenile hormone analog; JHAi)

	Low food/early JHAi (sub-threshold)	Low food/late JHAi (supra-threshold)	High food/early JHAi (supra-threshold)	High food/late JHAi (supra-threshold)
Consumption rate before JHAi (g dry mass day^{-1})	0.058±0.001	0.061±0.002	0.184±0.016	0.131±0.0325
Age at JHAi (days)	40.000±0.000	89.000±0.000	40.000±0.000	89.000±0.000
Cumulative amount eaten at JHAi (g dry mass)	2.282±0.032	5.400±0.399	7.001±0.367	11.127±2.778
Consumption rate after JHAi (g dry mass day^{-1})	0.059±0.001	0.060±0.005	0.104±0.011	0.099±0.017
Cumulative amount eaten at oviposition (g dry mass)	5.176±0.175	7.843±0.495	12.692±0.592	15.347±3.430

Female lubber grasshoppers were manipulated to create one sub-threshold feeding group at JHAi and three supra-threshold feeding groups at JHAi. By manipulating food amount and hormone timing, we tested whether JH alone was sufficient to undergo vitellogenesis or if a cumulative feeding threshold was also necessary.

Animal rearing

Lubber grasshoppers *Romalea microptera* (Beavois) (=*R. guttata* Houttuyn) were shipped from a lab colony at Illinois State University in Normal, IL, USA (gift of D. W. Whitman). The colony was founded with grasshoppers from Copeland, FL, USA. Juveniles were reared *en masse* in screen cages with a 14 h:10 h L:D photoperiod at 32°C and fed Romaine lettuce and oatmeal *ad libitum*. Newly molted females were isolated and reared individually in 500 ml ventilated containers at a 14 h:10 h L:D photoperiod and a corresponding 32:24°C thermocycle.

Allatectomy procedure

The corpora allata (the sole source of adult JH; T. O. Barry, J.D.H. and D. W. Borst, unpublished data) of all individuals were surgically removed 4–6 days after adult molt. The day before surgery, food was withheld. Grasshoppers were cold anesthetized for ⩾1 h, fastened to the dissecting dish with modeling clay, and the intersegmental neck membrane was opened with a U-shaped incision. Two air sacs were removed, both corpora allata were excised, a 25 μg dose of gentamicin sulfate (ICN Biomedicals, Irvine, CA, USA) was placed in the open wound, and the neck membrane was folded back into place.

Diet treatments and timing of juvenile hormone analog initiation

Our experimental goal was to test whether each group was competent to respond to JH at the initiation of hormone treatment. Using this design, we predict that individuals that were not competent to respond to JH at the moment of JHAi should have later times of vitellogenin onset (Vg onset; the first sampling date with detectable Vg) or oviposition.

Daily food rations were weighed fresh and all grasshoppers were always fed fresh lettuce. The previously determined threshold was described as dry mass (Juliano et al., 2004), so the dry mass ingested at each meal was determined. Daily, each individual was offered a specific amount of fresh lettuce. Several 5.0 g wet mass controls were dried at 55°C and weighed to obtain a fresh-to-dry conversion factor. Using this conversion factor, the dry mass offered was calculated. The next day, each individual's uneaten food was collected, dried and weighed. The dry mass uneaten was subtracted from the dry mass offered to determine the dry mass eaten.

Juliano et al. found cumulative feeding, and not the feeding rate, to be critical for commitment to oviposition (Juliano et al., 2004). Therefore, the important feeding variable to manipulate is the amount that has been ingested when JH is restored. From adult eclosion to the day before surgery, all individuals were fed 0.15 g dry mass of lettuce and 3–5 oatmeal flakes daily. Immediately following surgery, the grasshoppers began their assigned diets. Allatectomized females ate low amounts of food (about one-third that of unmanipulated females) and therefore took a longer time to reach the feeding threshold. The feeding schedules were designed to produce a sub-threshold feeding group (low food/early JHAi) and three supra-threshold feeding groups at JHAi.

Hormone analog treatments and hemolymph sampling

Hormone replacement was achieved by applying methoprene (Sigma Chemical, St Louis, MO, USA), an analog of JH (Nijhout, 1994; Flatt and Kawecki, 2007). Once the designated age was reached, a 5 μl hemolymph sample was collected from each grasshopper and a topical application of 500 μg of methoprene in 10 μl of 95% ethanol was applied to the neck membrane [as per Chinzei and Wyatt (Chinzei and Wyatt, 1985) for locusts]. Hemolymph samples were acquired once a day for the first 5 days after methoprene treatment initiation and twice a week thereafter. All hemolymph samples were placed in 250 μl of hemolymph buffer (Hatle et al., 2001) and stored at –20°C for later analysis of Vg and total protein. Twice a week until oviposition, methoprene was applied immediately before hemolymph sampling. We repeatedly dosed grasshoppers with 500 μg methoprene to force all individuals into the same hormonal status once hormone replacement was begun. This design has low power to separate the requirements of JH for vitellogenesis from the requirements for oviposition. However, it is excellent for testing our primary experimental goal, namely determining the ability of the animals to respond to restored JH. By artificially maintaining high levels of JH in all groups regardless of past or current diet, effects from the individual's status at the time of JHAi (i.e. sub- or supra-threshold) could be identified.

Oviposition

Females were allowed to oviposit in their cages as virgins. Lubber grasshoppers will lay eggs without mating; if oviposition substrate is not available, egg laying is delayed by ~7 days but still occurs (Mefferd et al., 2005). At oviposition, the individual's age was recorded and it was removed from the study. Laid eggs were counted; because egg size is largely fixed, number of laid eggs is a good estimate of clutch mass (Moerhlin and Juliano, 1998; Hatle et al., 2000). Grasshoppers were dissected to measure the number of retained eggs, the number of secondary oocytes and the size of secondary oocytes. Secondary oocytes represent the current commitment to the second clutch. Lubber grasshoppers can alter the number of eggs per clutch by resorbing oocytes, which would then not contribute to clutch size. Laid eggs and fully developed retained eggs were combined as the total number of developed eggs.

Together, secondary oocyte size and number indicate the investment in future reproduction. The number of secondary oocytes implies the potential for the mass of the ensuing clutch. The size of secondary oocytes implies the probable timing of the ensuing clutch, because oocytes need to grow to 1.0 cm to be ready for oviposition.

Hemolymph vitellogenin

Vitellogenin was measured by ELISA [modified from Borst et al. (Borst et al., 2000)]. All samples from an individual were analyzed concurrently, and groups were analyzed alternately. The time of Vg onset for each individual was determined by identifying the age at the first sample in which Vg was detectable. The time of maximum Vg titer was determined by identifying the age at the sample with the highest amount of Vg for each individual, throughout the oviposition cycle. The maximum Vg titer is the point at which sequestering Vg into the oocytes becomes favored over synthesizing Vg and exporting it into the hemolymph (Hatle et al., 2001). Individuals that showed detectable Vg prior to JHAi (likely due to failed allatectomy) were removed from the study (N=5).

Hemolymph storage proteins

Total hemolymph protein was measured using the Bradford assay (Bradford, 1976) with bovine serum albumin standards. The amount of Vg in the same sample was subtracted from this measure of total protein. Total non-Vg hemolymph protein is an estimate of storage proteins, because ~80% of non-Vg hemolymph protein exists as three hexamerin storage proteins throughout the first

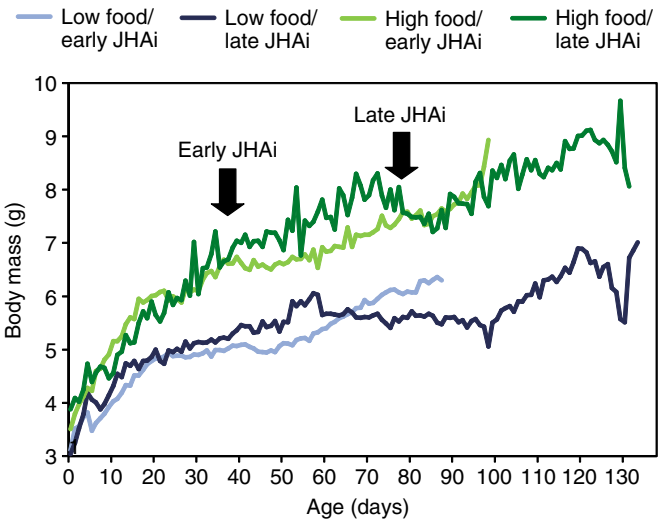

Fig. 2. Body mass profiles of lubber grasshoppers in response to diets (low or high) and timing of gonadotropin treatments (early or late initiation of juvenile hormone analog; JHAi). The body mass profiles imply enhanced weight gain with higher feeding and after hormone treatment.

oviposition cycle (Hatle et al., 2001). Hexamerins are a conserved family of storage proteins in insects (Haunerland, 1996). The time to storage protein maximum and the storage protein maximum were calculated in the same way as in the Vg analysis.

Statistical analysis

All data were tested for the effects of food, JHAi and the interaction of food and JHAi. Data were analyzed primarily by multivariate analysis of variance (MANOVA). We used three MANOVAs: (1) number and size of secondary oocytes; (2) time of Vg onset, time of maximum Vg, time from Vg maximum to oviposition and Vg maximum titer; and (3) initial storage protein titer and storage protein maximum. Data were transformed to meet assumptions of normality and homogeneity of variances as needed. Due to the inability to transform multiple variables to meet the assumptions of the test, data on oviposition timing and numbers of eggs were analyzed using separate analyses of variance (ANOVAs).

RESULTS

Diet treatment

The treatments were successful at producing groups that differed in cumulative feeding but not timing of JHAi (see Table 1). At JHAi, the low food/early JHAi group (57% of the 4.0 g dry mass threshold) was well below the threshold, whereas all other groups were above the threshold (low food/late JHAi=135% of threshold; high food/early JHAi=175% of threshold; high food/late JHAi=278% of threshold). Body mass gains through adulthood supported the efficacy of the feeding treatments (Fig. 2).

Oviposition

The time from JHAi to oviposition was significantly affected by timing of JHAi (ANOVA; F_1=7.184; P=0.013) but not by diet (F_1=2.311; P=0.141) or the interaction of JHAi and diet (F_1=2.468; P=0.129). Early JHAi groups had a longer period from JHAi to oviposition than did late JHAi groups (Fig. 3). Notably, the low food/early JHAi (sub-threshold) group did not have a longer period from JHAi to oviposition than all three other groups.

Egg and oocyte production

The number of eggs was significantly affected by the timing of JHAi (ANOVA; F_1=5.488; P=0.027) but not by diet (F_1=1.081; P=0.308) or interaction (F_1=0.064; P=0.802). Early JHAi groups produced fewer eggs than the late JHAi groups (Fig. 4).

Secondary oocyte characteristics (i.e. number and size of secondary oocytes) were significantly affected by diet (MANOVA; Pillai's trace $F_{2,25}$=7.454; P=0.003) and the timing of JHAi ($F_{2,25}$=9.417; P=0.001) but not by their interaction ($F_{1,27}$=1.020; P=0.375). Canonical coefficients (number of secondary oocytes=1.095; secondary oocyte length=0.437) suggested that the main effect was due mostly to the number of secondary oocytes, with the size of the oocytes being less important.

Upon dissection immediately following oviposition, the low food groups had fewer secondary oocytes than the high food groups (Fig. 5) (P=0.001). This was the only significant effect of diet in the entire experiment. The number of secondary oocytes was not affected by the timing of JHAi (P=0.732) or the interaction of diet and JHAi timing (P=0.173).

By contrast, early JHAi groups had larger secondary oocytes than the late JHAi groups (Fig. 5) (P<0.001). Yet, secondary

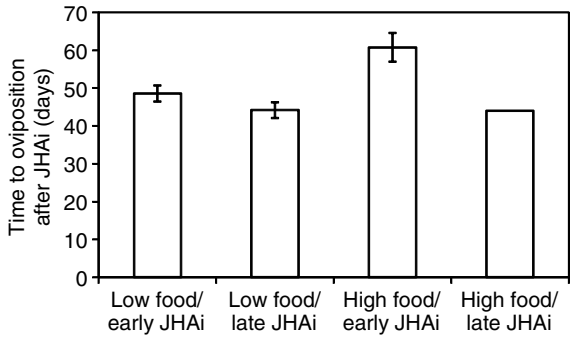

Fig. 3. Mean (± s.e.m.) times from gonadotropin treatments (initiation of juvenile hormone analog; JHAi) to oviposition in response to diets (low or high) and timing of JHAi. Regardless of whether the feeding threshold for vitellogenesis has been attained, early JHAi increases the time remaining until oviposition in lubber grasshoppers (P=0.013).

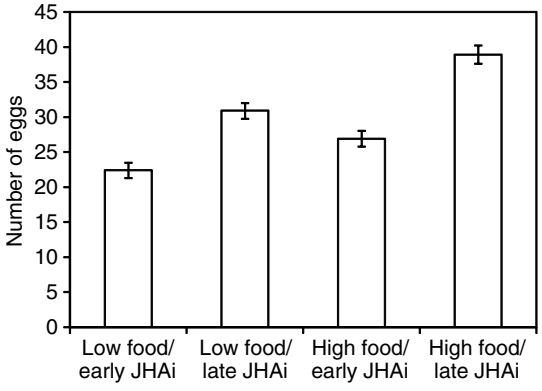

Fig. 4. Mean (± s.e.m.) number of eggs laid in response to diets (low or high) and timing of gonadotropin treatments (early or late initiation of juvenile hormone analog; JHAi). The number of eggs laid by lubber grasshoppers was significantly decreased by early JHAi (P=0.027) but was not affected by diet.

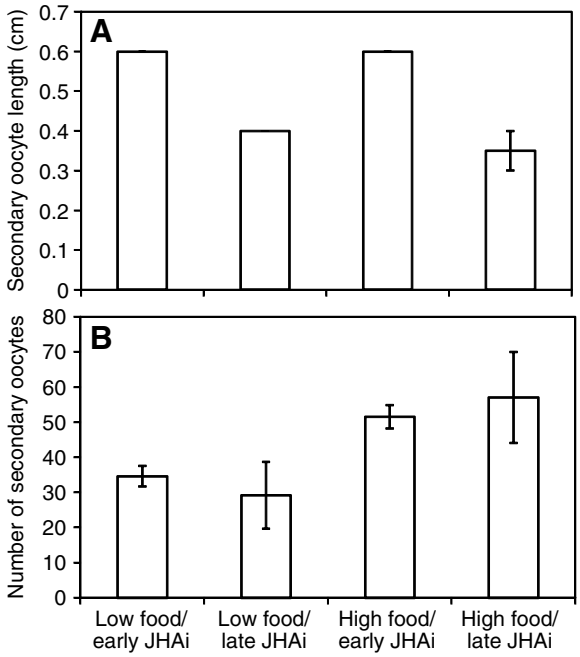

Fig. 5. Mean (± s.e.m.) length (A) and number (B) of secondary oocytes in response to diets (low or high) and timing of gonadotropin treatments (early or late initiation of juvenile hormone analog; JHAi). The number of secondary oocytes was significantly greater on high diet than on low diet in lubber grasshoppers (P=0.001) but was not affected by the timing of JHAi. By contrast, the length of secondary oocytes was greater in grasshoppers subjected to early JHAi than late JHAi (P<0.001) but was not affected by diet. Three groups have no error bars for secondary oocyte length because there was no variance in these data sets.

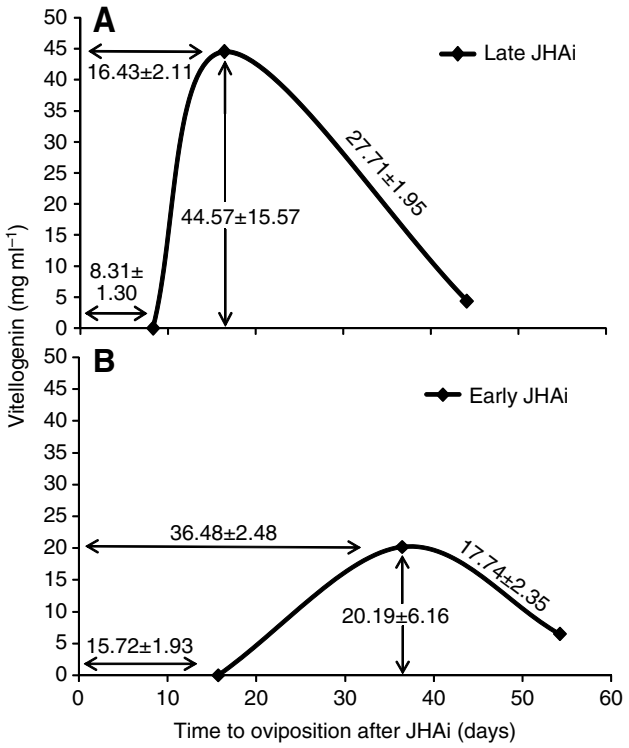

Fig. 6. Characteristics of vitellogenin profiles in response to timing of gonadotropin treatments (early or late initiation of juvenile hormone analog; JHAi). (A) Female lubber grasshoppers subjected to late JHAi had vitellogenin profiles consistent with favoring current reproduction at the expense of future reproduction, relative to females on early JHAi (P=0.001) (B). Values (mean ± s.e.m.) on the graphs show, from left to right, time from JHAi to vitellogenin onset, time from JHAi to vitellogenin maximum, maximum level of vitellogenin, and time from vitellogenin maximum to oviposition.

oocyte length was not significantly affected by diet (P=0.968) or interaction (P=0.471).

Analysis of vitellogenin

Vitellogenin profile characteristics were significantly affected by the timing of JHAi (MANOVA; Pillai's trace $F_{4,21}$=6.918; P=0.001) but not by diet ($F_{4,21}$=2.754; P=0.055) or interaction ($F_{4,21}$=0.541; P=0.708). For diet, all univariate P>0.10. Because diet did not have a significant effect on Vg parameters, we combined the Vg parameter data by diet groups for clearer graphical presentation (Fig. 6).

Canonical coefficients (maximum level of Vg=0.351; time of Vg maximum=0.219; time from Vg maximum to oviposition=1.024) suggest that the effect on Vg timing was due primarily to the time from Vg maximum to oviposition and secondarily to timing from JHAi to Vg maximum and the maximum level of Vg, and Vg onset was not significant. Compared with late JHAi groups, early JHAi groups had a longer period from JHAi to Vg maximum, a shorter time from Vg maximum to oviposition, and a lower maximum level of Vg (Fig. 6).

Our primary prediction (see last paragraph of Introduction) was that vitellogenesis would be delayed in females with sub-threshold food intake that were treated with JH (i.e. low food/early JHAi). Hence, the Vg onset data are particularly relevant to our hypothesis. The time from JHAi to Vg onset was not significantly affected by JHAi (P=0.051), diet (P=0.130) or interaction (P=0.493). The mean (± s.e.m.) times of Vg onset were: low food/early JHAi= 15.5±2.1 days; low food/late JHAi=7.2±1.8 days; high food/early JHAi=16.0±3.9 days; and high food/late JHAi=10.2±2.9 days. The non-significant trend was for Vg onset to be delayed in all early JHAi groups, not only the low food/early JHAi group.

Storage proteins

Storage protein profiles were not significantly affected by the timing of JHAi (Fig. 7) (MANOVA; Pillai's trace $F_{2,26}$=1.144; P=0.334), diet ($F_{2,26}$=0.175; P=0.841) or the interaction ($F_{2,26}$=0.202; P=0.819).

DISCUSSION

Developmental thresholds are important indicators of body condition that stimulate life-history transitions, but the relative roles of diet and hormones in these transitions are not well understood. Here, we tested whether attaining a feeding threshold is needed only to cause the release of JH or if the feeding threshold is also required to create competence to JH. Our prediction that females with sub-threshold feeding would not be competent to respond to the hormone was wrong; a significant statistical interaction of JHAi and diet is needed to confirm this prediction, but there were no significant interactions in the entire study. From the results, it is clear that vitellogenesis depended only on the presence of JH. In addition, by controlling the timing of JHAi, we identified a developmental shift in the trade-off between current and future reproduction. Individuals that initiated first reproduction early in life favored future reproduction, relative to individuals that initiated first reproduction late in life, which favored current reproduction.

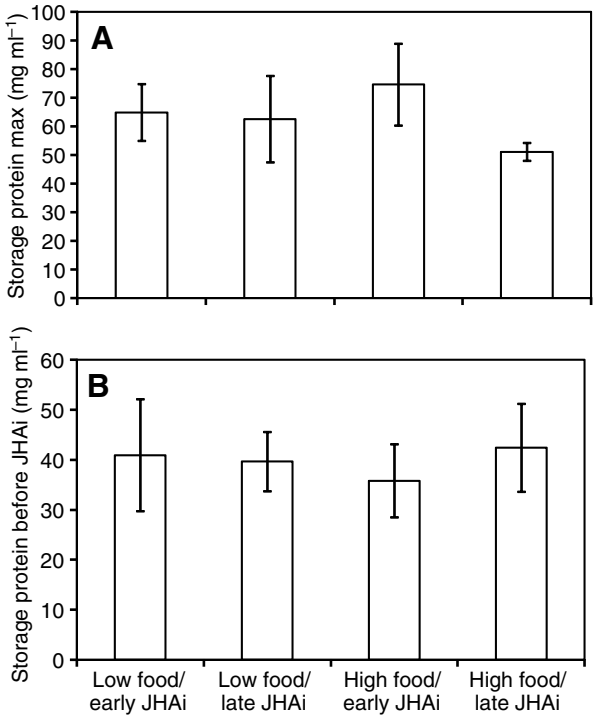

Fig. 7. Mean (± s.e.m.) storage protein maxima (A) and storage protein before initiation of gonadotropin treatment (initiation of juvenile hormone analog; JHAi) (B), in response to diets (low or high) and timing of JHAi. Hemolymph storage protein parameters were not affected by diet (P=0.841) or timing (P=0.334) of JHAi in female lubber grasshoppers.

JH was sufficient for vitellogenesis, even with sub-threshold feeding

Due to differences in the timing of JHAi, the low food/late JHAi group consumed 137% more food before JHAi than the low food/early JHAi group. Similarly, the high food/late JHAi group consumed 59% more food before JHAi than the high food/early JHAi group. Despite these large differences in cumulative consumption, when JH was controlled, the only variable affected by diet was the number of secondary oocytes. Previous work has repeatedly found strong effects of diet on the timing of first oviposition, age at Vg maximum and the number of eggs (Moehrlin and Juliano, 1998; Hatle et al., 2000; Hatle et al., 2001; Hatle et al., 2003a; Hatle et al., 2003b; Hatle et al., 2004; Juliano et al., 2004). By controlling JH levels, the present paper suggests that feeding for vitellogenesis is required only to produce adequate JH.

Our results suggest that attaining the threshold causes the release of JH. In lubber grasshoppers, JH production is positively controlled by allatotropins (Li et al., 2005). The regulation of allatotropins is somewhat unclear. It may be that attaining feeding thresholds are important in stimulating production of allatotropins.

This demonstration of the dominance of JH over diet in vitellogenesis has been conducted in a species for which a feeding threshold has been explicitly established by two distinct approaches. Juliano et al. used constant feeding rates and mathematical modeling to estimate the threshold as 4.0 g dry mass cumulative feeding (Juliano et al., 2004). Further, Moehrlin and Juliano used abrupt switches in food availability to show that the timing of oviposition is unaffected by diet level (short of starvation) after 14 days of full feeding (Moehrlin and Juliano, 1998). Further, lubber grasshoppers are appropriate for this experiment because JH titers have been directly measured (Borst et al., 2000; Hatle et al., 2000), and the requirement of JH in Vg mRNA production is clear (Hatle et al., 2000; Fei et al., 2005). Hence, it is appropriate to use an analog of JH in experiments with lubbers (Zera, 2006).

Our experiment focused on the ability to commit to vitellogenesis, and perhaps ultimately oviposition, after certain levels of feeding. Once we restored gonadotropin (i.e. JH), we continued hormone treatments until oviposition. A weakness of this design is the low probability of identifying developmental plasticity between the initiation of vitellogenesis and oviposition. Indeed, we failed to find effects of post-JHAi diets on reproductive tactics, as could be expected with repeated methoprene applications. At least a low level of developmental plasticity between the initiation of vitellogenesis and oviposition seems likely. Indeed, complete starvation starting at 20 days (i.e. after Vg onset but 2 weeks before oviposition in well-fed grasshoppers) halts oocyte growth (Fei et al., 2005). Nonetheless, our experiment demonstrates that even sub-threshold females, maintained on a low diet throughout adulthood, have the hormonal competence and resources needed to initiate vitellogenesis and commit to oviposition if JH is provided and maintained.

Undergoing vitellogenesis in the absence of adequate nutrition (as done by the low food/early JHAi females in this experiment) implies that some cost would be incurred. In grasshoppers, the investment presently allocated for future reproduction can be observed at any point by measuring the size and number of secondary oocytes (Sundberg et al., 2001). However, both low food/early JHAi and low food/late JHAi had fewer secondary oocytes, and low food/late JHAi grasshoppers had supra-threshold feeding. Hence, the reduced number of secondary oocytes in low food/early JHAi females likely does not represent a cost of reproduction in response to insufficient nutrition.

The time from JHAi to Vg onset was statistically indistinguishable across groups; however, the low probability (P=0.051) suggests that a trend might exist. This trend was for Vg onset to occur later in both early JHAi groups, not only in the low food/early JHAi group as we predicted. These data on Vg onset are inconsistent with the notion that threshold feeding is needed for competence to JH.

Current reproduction was favored by late JHAi groups

The terminal investment hypothesis suggests that as life expectancy decreases, favoring of current reproductive investment increases at the cost of future reproduction (Williams, 1966; Hirshfield and Tinkle, 1975; Clutton-Brock, 1984). Our results are consistent with the terminal investment hypothesis. We observed a trade-off between the timing of the first clutch and the timing of the second clutch (as estimated by the length of secondary oocytes). At the expense of delaying the production of their second clutch, late JHAi individuals allocated more resources in less time to their first clutch. By manipulating JH, we have demonstrated a developmental shift that was previously undetected in experiments manipulating only diet (e.g. Juliano et al., 2004; Hatle et al., 2006a). Vitellogenin profiles also tended to fit the predictions of the terminal investment hypothesis. Taken together, our results suggest that lubber grasshoppers can adjust reproductive tactics depending on their age, but this control is secondary to JH, which is in turn subject to nutrition.

It was previously hypothesized that a threshold level of hemolymph storage protein would serve as a physiological manifestation of the feeding threshold (Hatle et al., 2003b; Juliano et al., 2004). Inconsistent with this hypothesis, sub-threshold

feeding did not affect initial storage protein titers [similar to Hatle et al. (Hatle et al., 2006b)] or response to JH. In fact, Hatle et al. (Hatle et al., 2006a) found that reproductive plasticity was affected more by the mass of the fat body (which produces storage proteins and Vg) than by changes in storage protein titers. In *Drosophila*, the fat body serves as a nutrient sensor, regulating body growth (Colombani et al., 2003). The fat body of grasshoppers may be playing a nutrient sensing role in reproduction. We hypothesize that the fat body is more critical to pre-reproductive development than are hemolymph storage proteins.

Hormonal cue exceeded nutritional threshold

In other animals that exhibit growth-dependent thresholds for development, the nutritional state or critical size induces development *via* endocrine cues (e.g. Emlen and Nijhout, 1999; Davidowitz et al., 2005; Truman et al., 2006). This suggests that endocrine-producing tissues would respond to some signal to make the hormonal signal and stimulate the commitment to the next developmental stage (e.g. Mirth et al., 2005). However, it does not yield insight into whether or not the subsequent events will be followed through without the actual nutritional state or critical size. The present experiment suggests that hormones are more important than growth or size thresholds, and individuals early in development are competent to respond to developmental hormones, but simply have not yet attained sufficient levels of these hormones. Further studies on other experimental systems are needed to test the generality of this conclusion.

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