

Maximum Titers of Vitellogenin and Total Hemolymph Protein Occur during the Canalized Phase of Grasshopper Egg Production

J. D. Hatle*

D. W. Borst

M. R. Eskew

S. A. Juliano

Department of Biological Sciences, Illinois State University,
Normal, Illinois 61790-4120

Accepted 8/23/01

ABSTRACT

Many organisms exhibit developmental plasticity only in sensitive phases and cannot respond to environmental perturbations at other times. However, we know little about the physiological events that define plastic and canalized phases. During egg production in insects, vitellogenin (Vg) accumulates first in the hemolymph and then in the eggs. In addition, storage proteins may be important resources for egg production. Therefore, we tested hypotheses on the relationships of Vg and TP (total hemolymph protein minus Vg) titers to the transition from flexible to inflexible development during egg production. In lubber grasshoppers, ~70% of TP is contained in three proteins that range from 68 to 83 kDa. We maintained females on food treatments that produced defined plastic and canalized periods, collected hemolymph every ~4 d, and determined the ages at which oviposition and the maximum Vg and TP titers occurred. Both Vg_{max} titer and especially TP_{max} titer were predictors of the number of eggs produced. The time from eclosion to Vg_{max} was significantly affected by diet, but the time from Vg_{max} to oviposition was not. Similarly, the time from eclosion to TP_{max} was significantly affected by diet, while the time from TP_{max} to oviposition was not. Hence, Vg_{max} and TP_{max} are physiological landmarks that occur during the canalized phase of egg production.

Introduction

Many organisms meet the challenge of unpredictable environmental conditions by remaining developmentally flexible (Cal-

lahan et al. 1997; Nijhout 1999). This is known as phenotypic plasticity (flexibility), the production of multiple phenotypes by one genotype as a function of different environments (Stearns 1992; Scheiner 1993; Schlichting and Pigliucci 1998). Plasticity is a product of the developmental or endocrine systems of the organism. These systems have inherent limitations that may constrain plasticity (Schlichting and Pigliucci 1998). Canalization (inflexibility), in contrast to plasticity, is the production of a consistent phenotype by one genotype in spite of differing environments (Stearns 1992). The genetics and possible adaptive value of plasticity have been recent topics of interest. The physiology underlying plasticity, in contrast, has received less attention, despite the fact that this level of organization may also reveal general patterns.

A wide range of organisms (e.g., plants, insects, amphibians) exhibit plasticity only in certain sensitive developmental phases, during which the phenotype of the organism responds to environmental change (Hensley 1993; Brakefield et al. 1998; Denver et al. 1998; Moehrlin and Juliano 1998; Schlichting and Pigliucci 1998). Outside these sensitive phases, the phenotype of the organism is constrained, presumably by physiological mechanisms controlling development. Mechanisms initiating canalization are of interest because they can shed light on the adaptive limits of organisms (Garland and Carter 1994). Although we know that many organisms' life histories are divided into phases of plasticity and canalization, for most systems, we know little about the control mechanisms that define these periods.

One illustration of a switch from flexible to inflexible occurs during reproductive development in insects. The control of reproductive tactics (e.g., timing, reproductive allotment, offspring number) can undergo a switch from a flexible to a fixed developmental pathway in insects (Moehrlin and Juliano 1998). The timing of this switch is an important life-history event because of the close relationship between reproductive tactics and fitness (Stearns 1992). Moehrlin and Juliano (1998) showed that the eastern lubber grasshopper *Romalea microptera* (= *guttata*) has developmental flexibility during the early stages of the oviposition cycle. Both egg number and time to oviposition varied in response to the amount of food available. However, later in the first oviposition cycle, these grasshoppers entered a phase in which egg number and time to oviposition were unresponsive to changes in food availability (Moehrlin and Juliano 1998). By implementing diet switches at several different times, they determined that canalization of time to oviposition begins sometime during the second quarter of the oviposition cycle. The

* E-mail: jhatle@ilstu.edu.

canalization of number of eggs produced begins later, during the third quarter of the cycle. In short, these two reproductive tactics in lubber grasshoppers are plastic during the first portion of the cycle but then become unresponsive to the environment (Moehrlein and Juliano 1998).

Physiological models of reproduction in most insects (Davey 1993; Wyatt and Davey 1996) posit that oogenesis is controlled by juvenile hormone (JH), which stimulates synthesis of the egg-yolk precursor protein, vitellogenin (Vg). In grasshoppers, the JH titer increases early in the egg production cycle, which results in an increase in hemolymph Vg (Engelmann 1983). This Vg circulates in the hemolymph until it is transported into the developing oocytes. Levels of Vg vary as individuals progress through the oviposition cycle. We are particularly interested in how protein titers relate to the plastic and canalized phases of egg production.

We have recently reported that the JH titer of female lubber grasshoppers is correlated with the switch from reproductive plasticity to canalization (Hatle et al. 2000). Both the time from eclosion to the maximum JH titer (age at JH_{max}) and time from eclosion to oviposition (age at oviposition) were significantly affected by the feeding regime. In contrast, feeding rate had no significant effect on the time from JH_{max} to oviposition. Thus, time from JH_{max} to oviposition is unresponsive to food availability in this grasshopper. This was the first demonstration in a phytophagous insect that a particular endocrine compound (in this case, JH) can be used to distinguish reproductive canalization (Hatle et al. 2000). In a related study, we showed that Vg titers increase monotonically in well-fed lubber grasshoppers during the first ~25 d of the oviposition cycle. Thereafter, Vg titers declined until oviposition at about 35 d (Borst et al. 2000).

Another important class of hemolymph proteins in insects is the hexameric storage proteins (SPs; Haunerland 1996). These proteins may have roles in reproduction (e.g., Pan and Telfer 2001), particularly in long-lived insects (Wheeler 1996). For example, Wheeler and Buck (1996) provide evidence that SPs have a role in autogenous reproduction in a mosquito. Wheeler et al. (2000) demonstrated a rapid elevation of hexamerin titer in newly emerged females of a moth but not in newly emerged males. They suggest that such female-specific sequestration of amino acids in these proteins could be important in facilitating reproduction, particularly in situations where egg production is delayed (e.g., response to poor environmental conditions). Likewise, Ancsin and Wyatt (1996) describe a persistent storage protein in the hemolymph of locusts, which they hypothesize has a role in reproduction. One underlying hypothesis in these articles seems to be that SPs provide an amino acid pool that can be used for the synthesis of Vg. While Pan and Telfer (1996) found that amino acids from SPs were not specifically used for the production of Vg, these amino acids do appear to enter a general pool that can be used for the synthesis of all proteins. Therefore, SPs represent a meta-

bolic reserve that could be used during vitellogenesis. The level of such reserves may be an important factor in determining the onset of reproduction in phytophagous insects (Wheeler 1996). In the lubber grasshopper, three major proteins (putative SPs) account for most of the hemolymph protein. We have estimated their levels as total non-vitellogenin hemolymph protein (TP).

In this article, we focus on the hemolymph proteins of the animals from our article on JH (Hatle et al. 2000). Our goal is to test hypotheses about the relationships of protein titers to the transition from plasticity to canalization in reproductive tactics. These protein titers provide information about egg development beyond that which can be drawn from JH titers. Perhaps most important, TP can be used as an index of nutritional storage, whereas JH cannot. Therefore, we hypothesize that TP levels might be correlated with the switch from plastic to canalized reproduction. Vitellogenin, the product of JH action, may be a product of this nutritional storage. We hypothesize that the maximum titer of Vg may also be correlated with the switch from plastic to canalized reproduction. We define the time from eclosion to maxima for Vg and TP as age at Vg_{max} and age at TP_{max} , respectively. In contrast, we define the hemolymph concentration of Vg and TP at the maxima as Vg_{max} titer and TP_{max} titer, respectively. We test two primary predictions: (1) Vg_{max} and TP_{max} titers occur during the canalized phase of oogenesis. That is, (a) age at Vg_{max} and age at TP_{max} are significantly affected by diet (i.e., plastic), but (b) time from these maxima to oviposition are not affected by diet (i.e., canalized); (2) both Vg_{max} and TP_{max} titers are significantly and positively related to number of eggs produced.

Material and Methods

Vitellogenin and Total Non-vitellogenin Hemolymph Protein Levels through Development

The hemolymph samples analyzed in this study on Vg and TP levels came from the same individuals whose JH profiles were described by Hatle et al. (2000). We used adult females of *Romalea microptera* from our laboratory colony, which were maintained using rearing methods as described by Whitman (1986) for the western lubber grasshopper. Juveniles were reared en masse and fed ad lib. We isolated females on the day of adult ecdysis, measured their femur length (for use as a size covariate), and placed them in individual 500-mL ventilated plastic containers. Each grasshopper was assigned to one of four feeding treatments. Within these feeding regimes, a high daily ration consisted of 10 g romaine lettuce and 1.5 g oats, whereas a low daily ration consisted of 1.5 g romaine lettuce and 0.02 g oats. Thus, these diets had the same food quality but varied in the amount of food being offered. Grasshoppers in the high (H) group received high daily rations from eclosion until oviposition. Grasshoppers in the low (L) group received low daily rations from eclosion until oviposition. Grasshoppers

in the high-low (HL) group were fed high daily rations from eclosion until age 25 d and then were switched abruptly to low daily rations until oviposition. Likewise, grasshoppers in the low-high (LH) group were fed low daily rations from eclosion until age 25 d and then were switched abruptly to high daily rations until oviposition. Grasshoppers fed a high daily ration never completely consumed their daily ration; in contrast, grasshoppers fed a low daily ration almost always completely consumed their daily ration. All grasshoppers were kept on a 14L : 10D photoperiod with a 32° : 24°C thermocycle.

We collected hemolymph samples from each grasshopper once during each of the first 2 wk and then twice each week until the first oviposition. The hemolymph samples were collected before the daily feeding from punctures in the intersegmental neck membrane. Each hemolymph sample for protein analysis (1 μ L) was collected from the animal immediately after a 10- μ L hemolymph sample was collected for JH analysis (Hatle et al. 2000). Each protein sample was diluted in 50 mL of hemolymph buffer (100 mM NaCl, 1 mM ethylene diamine tetra-acetic acid, 0.1 mM dithiothreitol, 0.15% Tween 20, 10 mg/mL leupeptin, 10 mg/mL phenylmethylsulphonyl fluoride in propanol, 50 mM Tris buffer, pH 7.5) and stored at -20°C until assayed for Vg and TP. We measured Vg using an enzyme-linked immunosorbant assay (ELISA; Borst et al. 2000) and TP by the method of Bradford (1976).

Data Analysis

We compared hemolymph protein profiles among treatments (for grasshoppers that oviposited before 98 d) by comparing all the Vg titers through development for an individual and determining the maximum level of Vg for each grasshopper and the age of the individual at this maximum. Hence, although there may not appear to be distinct maxima for the means of the LH and L groups (see Fig. 1), there is a distinct maximum for Vg in each individual. We identified TP_{max} titers and ages at TP_{max} by this same method. When necessary, the data were transformed to meet the assumptions of homogenous variance and normality. We also tested whether female body size (femur length) as a covariate accounted for significant variation for each data set. Female size was never a significant covariate, so we analyzed the data using one-way ANOVA. We tested for effects of treatments on the Vg_{max} and TP_{max} titers. Next, we tested for effects of treatments on age at Vg_{max} and age at TP_{max} and on times from Vg_{max} and TP_{max} to oviposition. When treatment effects were significant, Tukey's test (SAS Institute 1989) was used to determine which diet treatments differed. Finally, we tested whether ages at Vg_{max} , TP_{max} , and JH_{max} (the latter from Hatle et al. 2000) differed using paired *t*-tests.

Grasshoppers that did not oviposit by 98 d (all of which were in the L group) were excluded from statistical comparisons with other treatment groups. We killed, froze, and later dissected these grasshoppers to determine whether their ovaries

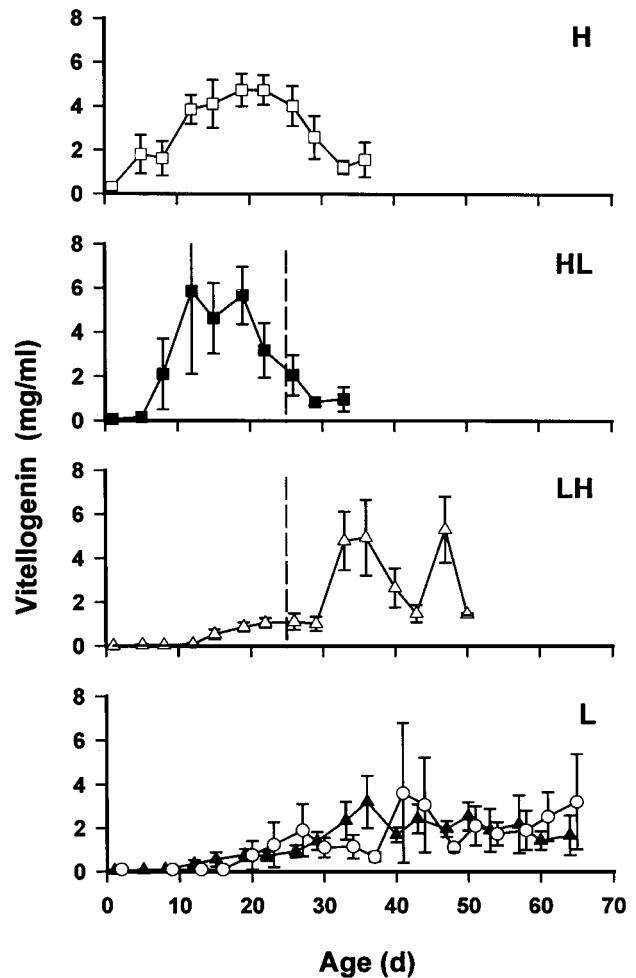


Figure 1. Vitellogenin titer profiles in adult female lubber grasshoppers, from eclosion to oviposition. See text for explanation of feeding treatments. Hemolymph samples were collected every ~4 d. The data are presented as means \pm SE for all individuals sampled during the 3- or 4-d developmental period (i.e., 1-4, 5-7, 8-11 d, etc.) for each treatment group. The dashed lines indicate the moment of the diet switches for HL and LH grasshoppers. For the L grasshoppers, the filled triangles represent females that oviposited before 98 d; the open circles represent females that did not oviposit by 98 d, when the study was terminated. The open circles (i.e., low fed that did not oviposit) are offset by +1 d to avoid overlap.

contained developing oocytes. For analysis, we compared their ages at Vg_{max} and TP_{max} and their Vg_{max} and TP_{max} titers to those from L grasshoppers that did oviposit. These grasshoppers that did not oviposit were also excluded from analysis of JH profiles by Hatle et al. (2000).

Regression of Totals Eggs versus Vg_{max} or TP_{max} Titters

Because we both collected serial hemolymph samples and determined oviposition parameters for each individual, we could

test the prediction that the maximal titers of these proteins predict egg number. We tested this prediction by regressing the number of eggs produced against the Vg_{\max} or TP_{\max} titers. We also ran a multiple regression, using both Vg_{\max} and TP_{\max} as predictors, in order to determine directly whether these variables make independent contributions to prediction of egg production. We tested these regressions without regard to the food treatments because our hypothesis predicts that if diet affects the number of eggs laid, it does so through Vg_{\max} or TP_{\max} . Hence, the relationship should hold across treatment groups.

Percentage of Total Hemolymph Protein due to Three Hemolymph Proteins

To estimate the percentage of total hemolymph proteins that are due to putative SPs, we used sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Female grasshoppers were isolated from the colony on the day of eclosion, placed in individual cages, and fed an ad lib. diet of lettuce and oats. We collected hemolymph samples every other day until oviposition. We separated hemolymph proteins by SDS-PAGE and stained the gels for 40–60 min with SYPRO-Orange protein stain (Molecular Probes, Eugene, Oreg.), diluted 1 : 1,000 in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2). We determined the fluorescence of each protein band with a Phospho-Imager Analyzer (Molecular Dynamics series 840c) and ImageQuaNT software (Molecular Dynamics 1994). The percentage of total hemolymph protein in specific bands was calculated by dividing the fluorescence of that band by the total fluorescence in the entire lane.

Results

Effects of Diet on Egg Production and Survival

Details on the survivorship and reproductive tactics of these individuals have already been published (Hatle et al. 2000). In brief, there were no significant differences in survivorship, but both the time from eclosion to oviposition and the number of eggs produced differed significantly among treatments (Table 1; statistical analyses given in Hatle et al. 2000). Thus, our diet treatments significantly affected the reproductive timing and output of the grasshoppers but not their survivorship.

Vitellogenin (Vg) Levels

Vitellogenin profiles fell into two distinct groups: grasshoppers that were started on high rations (i.e., H and HL) and grasshoppers that were started on a low rations (i.e., LH and L). H and HL grasshoppers had undetectable levels of Vg during the first 3 d of the cycle but then showed a large increase in Vg levels during the second week of the cycle (Fig. 1). Vitellogenin levels in these grasshoppers remained high until about 20–25 d, when levels dropped. Hence, H and HL grasshoppers had

similar Vg profiles despite differences in diet after 25 d. Grasshoppers on the LH and L diets had low levels of Vg before 25 d. Levels of Vg in these grasshoppers increased at about 30 d (Fig. 1). The increase was greater in LH grasshoppers, but even L grasshoppers showed a small increase in Vg levels. There was no significant difference in Vg_{\max} titers among any of the groups ($F_{3,28} = 2.78$, $P = 0.0594$, log transformed), although Vg_{\max} titers tended to be higher for H and HL grasshoppers (Table 1).

Prediction 1a: Vg_{\max} Titers Occur during the Canalized Phase of Reproduction

Age at Vg_{\max} was significantly affected by diet (Fig. 2; $F_{3,29} = 20.81$, $P < 0.0001$, square root transformed). Ages at Vg_{\max} for the H and HL groups (which did not differ from each other) were significantly less than ages at Vg_{\max} for the L and LH groups (which did not differ from each other; Fig. 2). Thus, it appears that the initial feeding rate (through 25 d) was an important factor in determining the age at Vg_{\max} . In contrast, the time from Vg_{\max} to oviposition was not significantly affected by diet ($F_{3,29} = 0.79$, $P = 0.5101$, log transformed). Thus, for grasshoppers that had sufficient nutrition to produce a clutch of eggs during the first oviposition cycle, reproductive timing was responsive to diet before the Vg_{\max} titer but unresponsive to diet after the Vg_{\max} titer.

Hemolymph Contains Non-vitellogenin Proteins That Are Putative SPs

SDS-PAGE analysis of hemolymph from adult females showed the presence of three major proteins between 68 and 83 kDa (Fig. 3; Eskew et al. 1997). These proteins were also observed in juveniles and adult males; the proteins were not related to Vg, as determined by Western blotting using an antivitellogenin serum (Eskew et al. 1997). Image analysis of SYPRO-Orange-stained gels demonstrated that the major proteins accounted for about 70% of the total hemolymph protein in adult female grasshoppers (see Fig. 3). In comparison, Vg typically accounts for 10% or less of the hemolymph protein in vitellogenic females. The abundance and size of these proteins, and their presence in juveniles, males, and females, suggest they are putative SPs (Hauerland 1996). We estimated the amount of these proteins by measuring the levels of total hemolymph protein and subtracting the amount of Vg ($TP = \text{total non-vitellogenin hemolymph protein}$).

Total Non-vitellogenin Protein Levels

Profiles of TP fell into two distinct groups: grasshoppers that were started on high rations (i.e., H and HL) and grasshoppers that were started on low rations (i.e., LH and L). H and HL grasshoppers had low levels of TP at eclosion, which then increased about fourfold during the second week of the cycle

(Fig. 4). Levels of TP in these grasshoppers remained high through 23 d, then dropped to an intermediate level. Hence, H and HL grasshoppers had similar TP profiles despite the large difference in diet between the two groups after 25 d. LH and L grasshoppers had low levels of TP throughout the experiment, despite the fact that LH grasshoppers were switched to high rations at 25 d. In fact, TP_{max} titers were significantly affected by diet (Table 1; $F_{3,29} = 16.59$, $P < 0.0001$), such that $H = HL > LH = L$.

Prediction 1b: TP_{max} Titers Occur during the Canalized Phase of Reproduction

Age at TP_{max} was significantly affected by diet (Fig. 5; $F_{3,29} = 26.07$, $P < 0.0001$, log transformed). Age at TP_{max} was smallest for the H and HL groups, which did not differ significantly from one another. Age at TP_{max} was significantly greater for the LH group, which was significantly less than the time for the L group (Fig. 5). In contrast, the time from TP_{max} titer to oviposition was not significantly affected by diet ($F_{3,29} = 0.52$, $P = 0.6706$). For grasshoppers that had adequate nutrition to produce a clutch during the first oviposition cycle, reproductive timing was responsive to diet before the TP_{max} titer but not responsive after the TP_{max} titer.

Relative Timing of Maxima

All three maximal titers (Vg_{max} , TP_{max} , and JH_{max} [the latter from Hatle et al. 2000]) occurred at statistically indistinguishable times in the cycle. Vg_{max} titer occurred 1.52 ± 1.4 d (mean \pm SE) before TP_{max} titer (paired t -test; $t_{32} = 1.11$, $P = 0.2763$). Vg_{max} titer occurred 1.94 ± 1.6 d before JH_{max} titer ($t_{31} = 1.25$, $P = 0.2207$). Finally, TP_{max} titer occurred 0.38 ± 1.3 d before JH_{max} titer ($t_{31} = 0.29$, $P = 0.7729$).

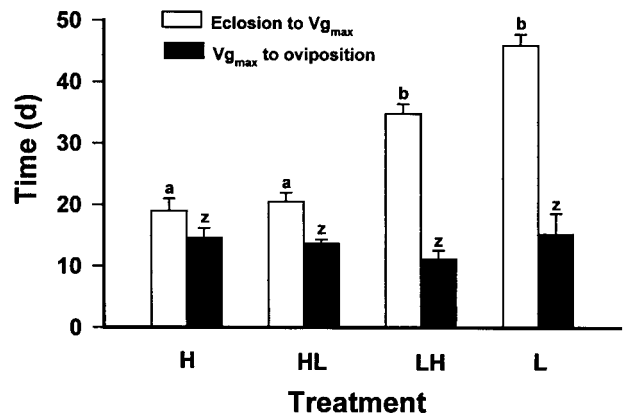


Figure 2. Times from eclosion to Vg_{max} and from Vg_{max} to oviposition in lubber grasshoppers. See text for explanation of feeding treatments. Vg_{max} titer is defined as the sampling date with the highest Vg titer for an individual grasshopper. Letters represent statistical differences within a developmental period.

Prediction 2: Vg_{max} Titer and TP_{max} Titer Are Significant Predictors of Number of Eggs

Finally, we tested the prediction that Vg_{max} and TP_{max} titers are significant predictors of the number of eggs a female will produce. When tested individually, both Vg_{max} titer (Fig. 6A; $F_{1,30} = 4.70$, $P = 0.0383$) and TP_{max} titer (Fig. 6B; $F_{1,30} = 20.93$, $P < 0.0001$) yielded significant linear regressions with the number of eggs produced. The better predictor of the number of eggs was TP_{max} titer (cf. r^2). When the two maxima were used as predictors in a multiple regression, TP_{max} titer was significant ($F_{1,29} = 15.85$, $P = 0.0004$), whereas Vg_{max} titer was not ($F_{1,29} = 1.55$, $P = 0.2225$). This last result implies that the TP_{max} and Vg_{max} titers are highly correlated and largely redundant as predictors of egg production.

Table 1: The effect of diet regimes on reproduction and hemolymph proteins in lubber grasshoppers

	High	High-Low	Low-High	Low
Time to oviposition (d)	35.2 ^a (34.5–35.9)	34.3 ^a (33.2–35.4)	46.2 ^b (44.4–48.2)	63.6 ^c (60.1–67.4)
Number of eggs	49.4 \pm 2.5 ^a	45.0 \pm 1.8 ^{ab}	36.4 \pm 3.3 ^b	15.0 \pm 1.4 ^c
Vg_{max} titer (mg/mL)	7.38 ^a (6.70–8.31)	7.56 ^a (6.08–9.39)	4.54 ^a (3.57–5.76)	4.44 ^a (3.58–5.50)
TP_{max} titer (mg/mL)	95.4 \pm 5.4 ^a	96.0 \pm 8.5 ^a	52.6 \pm 6.5 ^b	53.6 \pm 6.5 ^b
<i>n</i>	13	5	7	8

Note. We present the means \pm SE (or SE intervals) for time to oviposition and number of eggs (both summarized from Hatle et al. 2000) and maximum levels of vitellogenin (Vg_{max}) and total hemolymph proteins (TP_{max}). See text for details of the diet treatments. Means and SE intervals for time to oviposition and Vg_{max} titers are back transformed from reciprocals and logarithms, respectively. Means followed by the same letter are not significantly different.

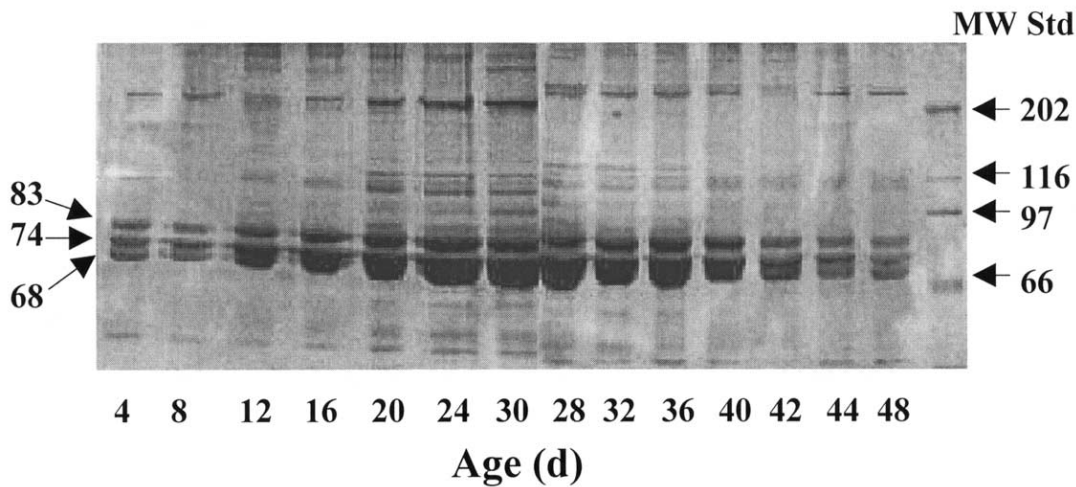


Figure 3. SDS-PAGE with SYPRO-Orange staining of hemolymph proteins from an adult female lubber grasshopper through the first oviposition cycle. The rightmost lane contains molecular weight standards (*MW Std*), and the numbers at the extreme right are the molecular weights of the proteins in this lane. Numbers on the horizontal axis indicate age (d) of the female when the respective sample was collected. The majority of non-vitellogenin proteins occur as three bands with estimated sizes of 68–83 kDa. The qualitative profiles of Vg and TP concur with our quantitative profiles determined by ELISA and Bradford's assay (see Figs. 1, 4).

Discussion

Prediction 1: Vg_{max} and TP_{max} Titers Occur during the Canalized Phase of Reproduction

We have identified Vg_{max} and TP_{max} titers as physiological landmarks that are associated with the canalized phase of oogenesis in grasshoppers. Grasshoppers that produced a clutch of eggs oviposited on average 14 d after Vg_{max} titer and 13 d after TP_{max} titer (Figs. 2, 5), regardless of diet regime. Hence, after these hemolymph proteins reached their highest levels, changes in food availability had no influence on time to oviposition. These data imply that a female is in the canalized phase of egg production when Vg_{max} and TP_{max} titers occur. In addition to this canalization, our grasshoppers showed phases of developmental plasticity (e.g., LH grasshoppers), demonstrating that egg development can respond to the environment during certain developmental phases.

We have not demonstrated causal connections between Vg_{max} and TP_{max} titers and the timing of oviposition. These maxima occur at statistically indistinguishable times, and this suggests that attaining any one of these maxima is not a trigger that induces either of the other two. If Vg and TP production all were halted during the canalized phase, this could explain how these maxima occur during the canalized phase. We hypothesize that some other factor(s) stops the increase in the titers of Vg and TP by either halting their synthesis or initiating their uptake or conversion. One candidate for such a factor is adipokinetic hormone (AKH), which has been shown to inhibit Vg synthesis in locusts late in the oviposition cycle (Moshitsky and Apple-

baum 1990). In addition, allatostatins (which inhibit JH synthesis) or allatotropins (which stimulate JH synthesis) may have a role in the transition from plastic to canalized phases of reproduction (Stay 2000).

Canalized phases represent physiological and developmental processes that require sufficient time to occur and thereby may limit adaptation by limiting the range of phenotypes that can be produced by selection (Garland and Carter 1994). For example, grasshoppers that lay clutches of eggs may need several days to produce these eggs in synchrony. The biochemical processes involved in these events (e.g., hormonal stimulation of Vg production, Vg synthesis, patency, transport of Vg into the egg, and chorionization) require time for completion. Hence, laying eggs in clutches may preclude the ability to adjust to environmental changes that occur late in the cycle. Information on the hormonal events that occur during canalized phases is of interest because it can shed light on the types of physiological processes that might limit adaptation.

Importance of Vg_{max} and TP_{max} Titers

These maxima, like the JH_{max} titer (Hatle et al. 2000), are useful physiological markers of reproduction. The maximum titer of these hemolymph components can be determined precisely and accurately from serial hemolymph samples, which makes it possible to determine when an animal's development is canalized. This should prove useful as we investigate factors that control the transition from flexible to inflexible reproduction. In addition, these maxima are interesting because a maximum

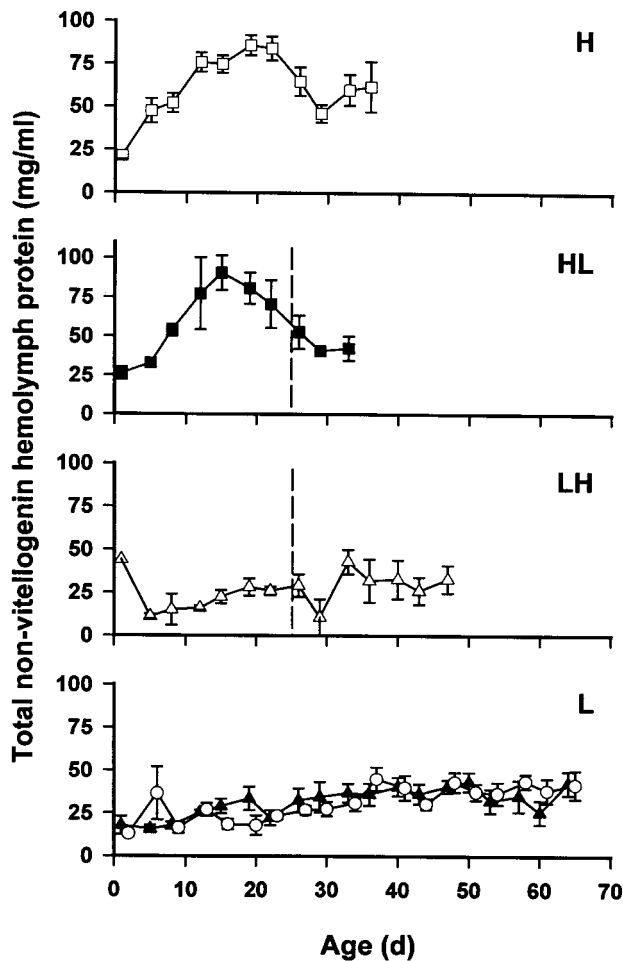


Figure 4. Total non-vitellogenin protein titer profiles in adult female lubber grasshoppers, from eclosion to oviposition. See text for explanation of feeding treatments. Hemolymph samples were collected every ~4 d. The data are presented as means \pm SE for all individuals sampled during the 3- or 4-d developmental period (i.e., 1–4, 5–8, 8–11 d, etc.) for each treatment group. The dashed lines indicate the moment of the diet switches for HL and LH grasshoppers. For the L grasshoppers, the filled triangles represent females that oviposited before 98 d; the open circles represent females that did not oviposit by 98 d, when the study was terminated. The open circles (i.e., low fed that did not oviposit) are offset by +1 d to avoid overlap.

titer indicates the point at which the rate of a compound's removal from the hemolymph has exceeded the rate of its introduction into the hemolymph.

In particular, TP may be useful as an index of nutritional storage to phytophagous, protein-limited insects. Initiation of the canalized phase of egg production is a developmental transition into a stage that likely requires large amounts of protein. For example, we have estimated that a full clutch of eggs for lubber grasshoppers contains 300 mg of vitellin (M. R. Eskew and D. W. Borst, unpublished observation). Such a develop-

mental transition might rely on a physiological cue, and the amount of protein stored seems an ideal candidate. We hypothesize that a threshold level of hemolymph proteins is the physiological cue that initiates the canalized phase (see last paragraph of "Discussion").

Developmental Changes in Allocation of Ingested Protein

Our data suggest that there may be a fundamental change in the use of ingested nutrients during the oviposition cycle. The increase in TP levels by LH grasshoppers in response to high daily rations after 25 d appears less dramatic than the concurrent increase in Vg levels in these same grasshoppers (Figs. 1, 4). In contrast, H and HL grasshoppers produced high levels of TP in response to high daily rations during the second week of the cycle. Importantly, TP_{max} titers in H and HL grasshoppers were significantly higher than TP_{max} titers in LH grasshoppers (Table 1). Hence, grasshoppers may change through development the destination of the amino acids derived from ingested protein. The hypothesis that female grasshoppers switch the allocation of ingested proteins through development can be directly tested by feeding grasshoppers radiolabeled amino acids and tracking their accumulation in specific proteins.

Variability within the Low-Fed Grasshoppers

We did not include L grasshoppers that failed to oviposit by 98 d (designated L_{no-egg}) in the above analysis because all L grasshoppers that laid (designated L_{egg}) did so by 83 d, with a mean oviposition age of 64 d (Table 1). The L_{no-egg} grasshoppers had 11.0 ± 3.2 (mean \pm SE) developing oocytes, averaging 8.8 ± 2.4 mm long (with approximately 67% of the volume of mature lubber grasshopper eggs). Clearly, L_{no-egg} grasshoppers had initiated the process of developing eggs.

Both age at Vg_{max} (two-sample $t_{12} = 1.87$, $P = 0.0857$) and age at TP_{max} ($t_{12} = 1.47$, $P = 0.1671$) did not differ significantly between L_{egg} grasshoppers and L_{no-egg} grasshoppers. There was a nonsignificant tendency for both ages to be greater in L_{no-egg} grasshoppers (means \pm SE: Vg_{max} , 63.5 ± 8.0 d; TP_{max} , 63.5 ± 9.1 d) compared with the L_{egg} grasshoppers (means \pm SE: Vg_{max} , 47.0 ± 4.7 d; TP_{max} , 50.3 ± 3.9 d). Titers of Vg_{max} ($t_{11} = 0.52$, $P = 0.6134$) and TP_{max} ($t_{12} = 0.96$, $P = 0.3545$) for these two groups of L grasshoppers also did not differ significantly. Thus, the hemolymph profiles of these two proteins do not provide a simple explanation for why L_{no-egg} animals failed to oviposit.

There are several possible explanations for the differences in reproductive responses of the L_{no-egg} and L_{egg} grasshoppers despite their identical treatments and similar hemolymph protein parameters. First, there may simply have been considerable individual variation among these grasshoppers in the level of food intake necessary to complete oogenesis. Those individuals with the greatest food requirement presumably accumulated in the L_{no-egg} group. Second, the L_{no-egg} grasshoppers may not have

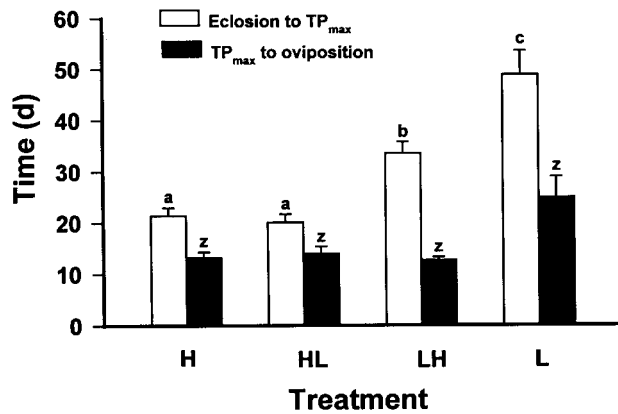


Figure 5. Times from eclosion to TP_{max} and from TP_{max} to oviposition in lubber grasshoppers. See text for explanation of feeding treatments. TP_{max} titer is defined as the sampling date with the highest titer of total non-vitellogenin hemolymph protein for an individual grasshopper. Lowercase letters represent statistical differences within a developmental period.

attained their actual Vg_{max} and TP_{max} titers when we terminated the study. Third, our hypothesis that Vg_{max} and TP_{max} indicate the canalized phase may be wrong.

Prediction 2: Vg_{max} Titer and TP_{max} Titer Are Significant Predictors of Number of Eggs

Both Vg_{max} and TP_{max} titers were significant predictors of the number of eggs a female eventually produced, but TP_{max} was the better predictor (Fig. 6). The relationship between Vg_{max} titer and egg production was weak, whereas the relationship of TP_{max} titer and egg production was stronger. The rise and fall of TP levels during the oviposition cycle of H and HL animals suggest that it is a major metabolic reserve that can be used in part for egg production (Pan and Telfer 1996, 2001; Wheeler 1996; Wheeler and Buck 1996; Wheeler et al. 2000). That TP_{max} titer was a highly significant predictor of egg production in our grasshoppers supports our hypothesis that TP is critical for determining the quantity of eggs produced.

Our results lead us to a model of oogenesis in phytophagous insects in which reproductive development is initially flexible and at least partly dependent on the accumulation of stored reserves. We postulate that reproductive development later becomes inflexible and proceeds toward oviposition independent of feeding rate. Such a model is similar to developmental models for insect juvenile development (Nijhout 1994; Bradshaw and Johnson 1995; Flanagan et al. 2000) and thus is consistent with other functions of the insect endocrine system. Furthermore, we postulate that hemolymph proteins are critical for controlling this process. Specifically, hormonal cues are triggered when the grasshopper surpasses some critical threshold

for protein in the hemolymph, thereby initiating the canalized phase of oogenesis. We postulate that during this inflexible phase of oogenesis, ingested proteins and hemolymph proteins are converted into Vg in the fat body and then released into the hemolymph for transport to the oocytes. That physiological markers such as JH_{max}, Vg_{max}, and TP_{max} titers are attained at apparently fixed times in advance of oviposition is consistent with this model. Critical testing of this model will require explicit identification of threshold values of specific hemolymph proteins, detailed experimental tracking of ingested amino acids, and experimental manipulation of hemolymph protein levels.

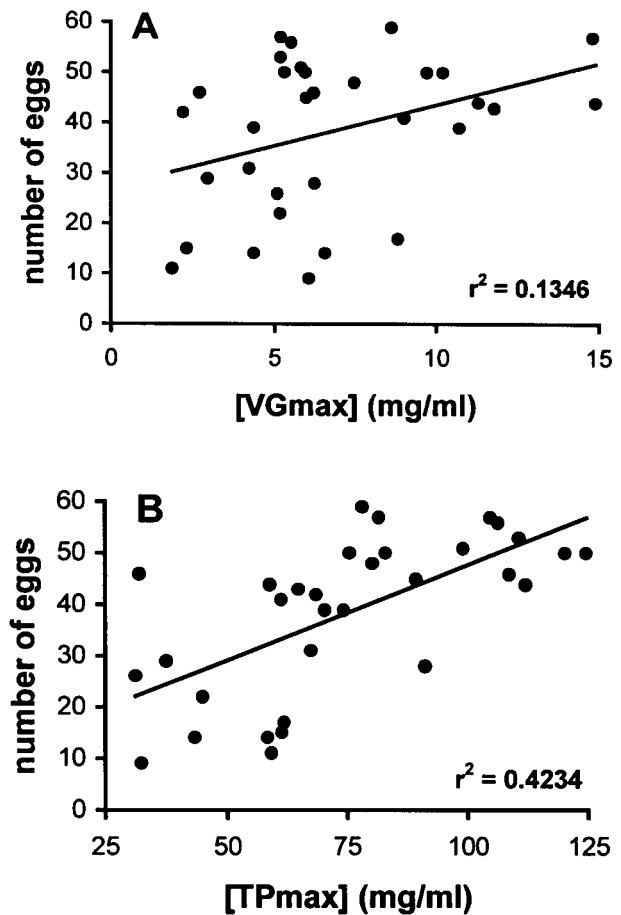


Figure 6. Regressions of titers of Vg_{max} (A) and TP_{max} (B) against total number of eggs produced by individual female lubber grasshoppers. Data are pooled from all four feeding treatments (see text for explanation). A multiple regression revealed that Vg_{max} and TP_{max} titers are highly correlated, and only TP_{max} titer was a significant predictor of number of eggs. The multiple regression yielded the relationship: number of eggs = 0.33 × TP_{max} + 0.823 × Vg_{max} + 7.123.

Acknowledgments

We thank L. Luker for performing some of the ELISAs, H. Lewis and three anonymous reviewers for critically reading the manuscript, and members of the Illinois State University Collaborative-Research at Undergraduate Institutions program for suggestions on the experiments. This work was funded by National Science Foundation grants BIR-9510979A000 and DBI-9978810 to S.A.J. and D.W.B.

Literature Cited

- Ancsin J.B. and G.R. Wyatt. 1996. Purification and characterization of two storage proteins from *Locusta migratoria* showing distinct developmental and hormonal regulation. *Insect Biochem Mol Biol* 26:501–510.
- Borst D.W., M.R. Eskew, S.J. Wagner, K. Shores, J. Hunter, L. Luker, J.D. Hatle, and L.B. Hecht. 2000. Quantification of juvenile hormone III, vitellogenin, and vitellogenin-mRNA during the oviposition cycle of the lubber grasshopper. *Insect Biochem Mol Biol* 30:813–819.
- Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle protein-dye binding. *Anal Biochem* 72:248–254.
- Bradshaw W.E. and K. Johnson. 1995. Initiation of metamorphosis in the pitcher-plant mosquito: effects of larval growth history. *Ecology* 76:2055–2065.
- Brakefield P.M., F. Kesbeke, and P.B. Koch. 1998. The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *Am Nat* 152:853–860.
- Callahan H.S., M. Pigliucci, and C.D. Schlichting. 1997. Developmental phenotypic plasticity: where ecology and evolution meet molecular biology. *BioEssays* 19:519–525.
- Davey K.G. 1993. Hormonal integration of egg production in *Rhodnius prolixus*. *Am Zool* 33:397–402.
- Denver R.J., N. Mirhadi, and M. Phillips. 1998. Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondi* tadpoles to habitat desiccation. *Ecology* 79:1859–1872.
- Engelmann F. 1983. Vitellogenesis controlled by juvenile hormone. Pp. 259–270 in R.G.H. Downer and H. Laufer, eds. *Endocrinology of Insects*. Liss, New York.
- Eskew M.R., J.P. Hunter, and D.W. Borst. 1997. Vitellogenin levels during the oviposition cycle of the lubber grasshopper, *Romalea guttata*. *Am Zool* 37:159. (Abstr.)
- Flanagin V.L., S.P. Haase, and S.A. Juliano. 2000. Effects of growth rates on development to metamorphosis in the lubber grasshopper, *Romalea microptera*. *Oecologia* 125:162–169.
- Garland T., Jr., and P.A. Carter. 1994. Evolutionary physiology. *Annu Rev Physiol* 56:579–621.
- Hatle J.D., S.A. Juliano, and D.W. Borst. 2000. Juvenile hormone is a marker of the onset of reproductive canalization in lubber grasshoppers. *Insect Biochem Mol Biol* 30:821–827.
- Hauerland N.A. 1996. Insect storage proteins: gene families and receptors. *Insect Biochem Mol Biol* 26:755–765.
- Hensley F.R. 1993. Ontogenetic loss of phenotypic plasticity of age at metamorphosis in tadpoles. *Ecology* 74:2405–2412.
- Moehrlin G.S. and S.A. Juliano. 1998. Plasticity of insect reproduction: testing models of flexible and fixed development in response to different growth rates. *Oecologia* 115:492–500.
- Molecular Dynamics. 1994. ImageQuANT User's Guide. Version 4.1. Molecular Dynamics, Sunnyvale, Calif.
- Moshitsky P. and S.W. Applebaum. 1990. The role of adipokinetic hormone in the control of vitellogenesis in locusts. *Insect Biochem* 20:319–323.
- Nijhout H.F. 1994. *Insect Hormones*. Princeton University Press, Princeton, N.J.
- . 1999. Control mechanisms of polyphenic development in insects. *BioScience* 49:181–192.
- Pan M.L. and W.H. Telfer. 1996. Methionine-rich hexamerin and arylphorin as precursor reservoirs for reproduction and metamorphosis in female luna moths. *Arch Insect Biochem Physiol* 39:149–162.
- . 2001. Storage hexamer utilization in two lepidopterans: differences correlated with the timing of egg formation. *J Insect Sci* (online), vol. 1, art. 2; <http://www.insectscience.org/1.2>.
- SAS Institute. 1989. *SAS/STAT User's Guide*. Version 6, vol. 2. SAS Institute, Cary, N.C.
- Scheiner S.M. 1993. Genetics and evolution of phenotypic plasticity. *Annu Rev Ecol Syst* 24:35–68.
- Schlichting C.D. and M. Pigliucci. 1998. *Phenotypic Evolution: A Reaction Norm Perspective*. Sinauer, Sunderland, Mass.
- Stay B. 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. *Insect Biochem Mol Biol* 30:653–670.
- Stearns S.C. 1992. *The Evolution of Life Histories*. Oxford University Press, Oxford.
- Wheeler D.E. 1996. The role of nourishment in oogenesis. *Annu Rev Entomol* 41:407–431.
- Wheeler D.E. and N.A. Buck. 1996. A role for storage proteins in autogenous reproduction in *Aedes atropalpus*. *J Insect Physiol* 42:961–966.
- Wheeler D.E., I. Tuchinskaya, N.A. Buck, and B.E. Tabashnik. 2000. Hexameric storage proteins during metamorphosis and egg production in the diamondback moth, *Plutella xylostella* (Lepidoptera). *J Insect Physiol* 46:951–958.
- Whitman D.W. 1986. Laboratory biology of *Taeniopoda eques* (Orthoptera: Acrididae). *J Entomol Sci* 21:87–93.
- Wyatt G.R. and K.G. Davey. 1996. Cellular and molecular action of juvenile hormone. II. Roles of juvenile hormone in adult insect. *Adv Insect Physiol* 26:1–155.