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Plasticity of grasshopper vitellogenin production in response to diet is primarily a result of changes in fat body mass

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Abstract Life history plasticity is the developmental production of different phenotypes by similar genotypes in response to different environments. Plasticity is common in early post-embryonic or adult development. Later in the developmental stage, the transition from developmentally plastic to canalized (i.e., inflexible) phases is often associated with the attainment of a threshold level of storage. Thresholds are often described simply as total body mass or cumulative consumption of food. The physiological characteristics of thresholds, such as the contributions of the growth of particular organs or the production rate of proteins, are largely unstudied. To address the physiology underlying a threshold-induced developmental transition, total vitellogenin production in response to diet quality in the lubber grasshopper was studied. For individuals that differed in age or dietary protein, somatic mass, ovarian mass, fat body mass, mass-specific vitellogenin production, vitellogenin titer, and storage protein titer were measured. Age and diet strongly affected these parameters, with ovarian mass and fat body mass contributing most to the differences. During mid vitellogenesis, females were highly plastic in response to changing food quality. Only during late vitellogenesis were females unresponsive to changes in food quality. Fat body mass was a more important component of plasticity than was

mass-specific vitellogenin production. Because these two variables together make up total vitellogenin production, the greater contribution of fat body mass than mass-specific vitellogenin production suggests that growth factors may be more important than tissue stimulators in producing developmental changes in total vitellogenin production. To our knowledge, this is the first study to demonstrate that mass gain of an organ is more important to developmental plasticity than is the output of that same organ.

Keywords Phenotypic plasticity · Thresholds · Developmental canalization · Life history · Resource allocation

Introduction

Phenotypic plasticity is the production through development of different phenotypes in response to different environments, in spite of similar genotypes. In contrast to plasticity, developmental canalization is the production during development of similar phenotypes in spite of different environments (Schlichting and Pigliucci 1998; West-Eberhard 2002). Most animals exhibit developmental plasticity only during the early portion of life cycle stages that lead to post-embryonic developmental transitions, such as molting, metamorphosis, or oviposition (Boorse and Denver 2003; Denver et al. 1998; Hatle 2003; Schoech and Bowman 2003; see Shafiei et al. 2001 for an exception). Later during the life history stage, but before the developmental transition, these animals exhibit canalization, in which life history tactics (e.g., age at metamorphosis) do not change in response to environmental changes.

The transition from plastic to canalized phases of development may often involve the attainment of some threshold level of storage to fuel development (Davidowitz et al. 2004; Juliano et al. 2004; Moczek and Nijhout 2002; Nijhout and Williams 1974). Studies of life history transitions have often postulated a role of

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storage thresholds (Hentschel and Emler 2000; Juliano et al. 2004; Reznick 1990). Except for work on metamorphosis in *Manduca sexta* (D'Amico et al. 2001; Davidowitz et al. 2003, 2004), there is little information on the physiological characteristics of these thresholds. Many studies have measured storage simply as body mass, without determining the relative contributions of specific organs or physiological processes. Knowledge of physiology underlying thresholds is needed to understand how organisms adjust allocation of resources and the mechanisms leading to canalization (D'Amico et al. 2001; Davidowitz et al. 2003, 2004; Denver 1997, 1998; Hodin 2000).

To investigate physiological parameters underlying a threshold-induced transition from plastic to canalized development, reproduction in the lubber grasshopper *Romalea microptera* was studied. The focus was on total vitellogenin production in response to diet quality. Lubber grasshoppers are phytophagous and lay a few large clutches of protein-rich eggs. Well-fed lubbers lay their first clutch about day 35. Females require a threshold quantity of adult feeding to commit to egg production (Juliano et al. 2004). Experiments in which diet quantity was reduced abruptly during the oviposition cycle suggest that age at oviposition is determined at least 14 days before laying, and the number of eggs is determined at least 7 days before laying (Moehrlin and Juliano 1998). The size of individual eggs is largely unaffected by diet.

Egg production in *R. microptera* involves accumulation of storage, production of vitellogenin (the egg-yolk precursor), transport of vitellogenin into the growing oocytes, and oviposition. Juvenile hormone (JH) is necessary for vitellogenin production, to increase vitellogenin-mRNA in the fat body (Fei et al. 2005), and in other orthopterans stimulates transport of vitellogenin into the oocytes (Chapman 1998). The hemolymph of lubber grasshoppers contains three members of the insect family of storage proteins: a hexamerin (522 KDa), a methionine-rich protein (275 KDa), and an arylphorin (92 KDa; J.D. Hatle and D.W. Borst, unpublished data). These proteins are produced by the fat body, increase during vitellogenesis, and decrease before oviposition. Both JH and vitellogenin first appear in the hemolymph about day 8. The highest levels of all three factors occur at days 20–25; these peaks occur during the canalized phase of reproduction (Hatle et al. 2000, 2001, 2003a).

In this paper we focused on the plasticity of a physiological event of oogenesis that must begin prior to the peak level of hemolymph vitellogenin, namely the production of vitellogenin by the fat body. Because vitellogenin is only produced in the fat body, total vitellogenin production is the product of fat body mass and mass-specific vitellogenin production rate. We test for ages at which total vitellogenin production is plastic, and for the relative roles of the two determinants of vitellogenin production in producing plasticity.

Methods

Experimental animals

New adult females were obtained from the colony at Illinois State University (which is descended from individuals collected near Copeland, FL, USA: Hatle et al. 2000) and isolated in 500 cm³ plastic, ventilated containers. Grasshoppers were reared on a 14L:10D photoperiod and a corresponding 32:24°C thermocycle. Individuals were assigned serially to ten feeding-treatment groups.

Feeding treatments

Each day, females were fed either a high-protein meal of ~3.0 g 5% protein artificial diet (Yang and Joern 1994a, b) and 0.5 g Romaine lettuce (H), or a low-protein meal of ~3.0 g 1% protein artificial diet and 0.5 g Romaine lettuce (L). Grasshoppers fed the same diet every day were given treatment names that described the diet and the age of dissection. For example, L10 grasshoppers were fed the low diet from adult molt until day 10 when they were dissected. These six groups were: L10, L21, L32, H10, H21, and H32.

Four additional groups were given diet switches to probe the pre-oviposition phase of development for times when individuals were able to respond to a change in dietary protein (i.e., were developmentally plastic) or were unable to respond (i.e., were developmentally canalized). Switches were made at day 13 or 24. Data on developmental titers of vitellogenin (Hatle et al. 2001) suggested that well-fed lubbers might be reproductively plastic at day 13 but reproductively canalized at day 24, and that poorly fed lubbers might be reproductively plastic at day 24. Each of these treatment groups was assigned a name describing its initial diet, the age at diet switch, the new diet, and the age at dissection. For example, H13L21 grasshoppers were fed the high-protein diet from adult molt through day 12, fed the low-protein diet from day 13 until day 20, and dissected at day 21. These groups were: H13L21, H13L32, H24L32, and L24H32. Sample sizes were 7–10 for all groups.

Data collection

On the day of dissection, the body mass of each female was measured. Next, the rate of vitellogenin production per milligram of fat body (hereafter, mass-specific vitellogenin production) was measured by the method of Fei et al. (2005), with the exception that thin sheets of fat body from the anterior abdomen instead of minced fat body from the entire thorax was used. Vitellogenin in the incubation medium was measured by enzyme-linked immunosorbent assay (ELISA; Borst et al. 2000). During incubation, wet ovarian masses were measured and

all the remaining fat body was excised. Later, the total fat body was dried at 60°C for several days and masses were determined; the amount of fat body needed for determination of vitellogenin production was a small fraction of total fat body, so both production and total mass could be determined accurately for a single female. Hemolymph samples were collected from each female at dissection. Vitellogenin titers were determined by ELISA, and storage protein titers were estimated by measuring total hemolymph protein (Bradford 1976) and subtracting vitellogenin (Hatle et al. 2001). Somatic mass was determined as body mass minus ovarian mass.

Statistical analysis

An analysis that allowed inferences on the relative contributions of each of the six response variables to plasticity was used. The combined responses of somatic mass, wet ovarian mass, total dry fat body mass, mass-specific vitellogenin production, vitellogenin titer, and storage protein titer were tested with a single MANOVA followed by 14 planned, pairwise multivariate contrasts (Scheiner 2001; Hatle et al. 2002). All statistical tests were done using SAS version 8.12 (SAS Inst. Inc. 1999; PROC GLM). For both MANOVA and multivariate contrasts, Pillai's trace was chosen as the test statistic. The variables that contributed most to significant intergroup differences were determined using standardized canonical coefficients (SCC). SCCs define the canonical function that is a linear combination of the original variables that maximizes the difference between groups (Scheiner 2001). SCC values of relatively large magnitude indicate a large contribution of that dependent variable to an observed significant effect. Currently, there is no consensus on the magnitude of "large" SCCs (i.e., no value comparable to 0.05, which is accepted as the critical level for *P*-values in test of hypotheses). The value take 1.0 was taken as a "large" SCC. SCC values of opposite signs indicate that two dependent variables are negatively correlated across groups (i.e., they

respond in opposite fashion to the treatment; Scheiner 2001). All variables were log transformed to meet MANOVA assumptions of normally distributed error, with homogeneous variances.

Results

Combinations of age and dietary protein strongly affected the physiological and morphological variables (Table 1). The first three canonical functions were significant and summarized >98% of the variance due to treatments. SCC values indicated that ovarian and fat body masses were the major contributors to the first two canonical functions, whereas fat body mass and mass-specific vitellogenin production rate were the major contributors to the third canonical function, which summarized only 3.7% of the variance from treatments. Somatic mass and hemolymph protein concentrations were relatively unimportant (Table 1).

Fourteen pairwise multivariate comparisons of treatment groups provided information on how groups differed. These contrasts were divided into three groups, focusing on the effects of: age (i.e., chronological age) with constant dietary protein; dietary protein with constant age; and switches of dietary protein with constant age (Table 2). In general, ovarian and fat body masses contributed most to the pairwise effects of age and dietary protein, while somatic mass and hemolymph vitellogenin and storage proteins were relatively unimportant; mass-specific vitellogenin production rate was intermediate in influence (Table 2; Figs. 1–3).

Age

All pairwise comparisons among days 10, 21, and 32 within a dietary protein regimen were highly significant (Table 2). For low-protein diet, fat body mass contributed greatly to significant differences between days 10 and 21 but contributed only little to significant

Table 1 MANOVA for treatment effects on somatic mass, ovarian mass, fat body mass, mass-specific vitellogenin production, hemolymph vitellogenin, and hemolymph storage proteins

Canonical function	Proportion variance	<i>F</i>	df (num., den.)	<i>P</i>	Standardized canonical coefficients (SCC)					
					Somatic mass	Ovary mass	Fat body mass	Vitellogenin production	Hemolymph vitellogenin	Hemolymph storage protein
Overall		5.16	(54, 450.0)	0.0001						
1	0.750	10.64	(54, 361.5)	0.0001	-0.187	2.381	1.605	0.002	0.053	-0.108
2	0.200	5.14	(40, 312.3)	0.0001	0.010	-2.111	2.172	0.326	0.053	0.153
3	0.037	2.01	(28, 261.0)	0.0027	0.377	0.299	-0.830	1.259	-0.182	0.256

Plasticity in reproduction of female lubber grasshoppers is due largely to changes in ovarian mass and fat body mass, and less to changes in the other four variables. See text for details on diet regimens. Grasshoppers were dissected at days 10, 21, and 32 and reproductive parameters were measured. The canonical functions are linear combinations of the original variates that maximally distinguish among the ten treatment groups (Scheiner 2001). The *proportion variance* column shows that the first three canonical functions together describe 98.7% of the variance in the data. The *standardized canonical coefficients* (SCC) quantify the contribution of the original variables to significant treatment effects (see text for further explanation). SCCs in bold contribute greatly to significant canonical functions

differences between days 21 and 32 (Fig. 2). In contrast, ovarian mass increased dramatically between days 21 and 32, but little between days 10 and 21 (Fig. 1).

For the high-protein diet, the difference between days 10 and 21 was due to increases in ovarian and fat body mass. The difference between days 21 and 32 involved an increase in ovarian mass and decrease in fat body mass and mass-specific vitellogenin production. Perhaps most telling, days 10 and 32 differed greatly in ovarian mass, but almost not at all in other variables.

Dietary protein

For days 10, 21, and 32, low- and high-protein diets differed significantly, and greater fat body mass in the high protein group contributed substantially to those differences (Table 2, Fig. 2). For day 10, ovarian masses did not contribute to the intergroup difference (Fig. 1). At days 21 and 32, ovarian mass was an important contributor to the difference between groups, and its relative contribution increased with age. In contrast, the relative contribution of fat body mass declined during this period. No other variables contributed to differences between diets. Overall these pairwise comparisons suggest that dietary protein affects fat body mass early, and ovarian mass late during vitellogenesis.

Switches of dietary protein

Only one contrast, between H32 and H24L32 was not significant (Table 2), indicating that reducing dietary protein after day 24 in well-fed females had no effect on these reproductive parameters. Dietary switches at

earlier ages in well-fed females, and dietary switches at day 24 in poorly fed females, produced significant changes in physiological parameters (i.e., induced a plastic response). Specifically, reducing dietary protein at day 13 reduced ovarian and fat body masses at days 21 or 32 (Table 2; Figs. 1, 2). Reducing dietary protein at day 13 reduced mass-specific vitellogenin production at day 21 but increased mass-specific vitellogenin production at day 32 (because vitellogenin production at day 32 is low in well-fed females). Increasing protein at day 24 affected ovarian mass and especially fat body mass.

Discussion

Most important for the interpretation of our physiological data (follows subsequently), reproductive development in female lubber grasshoppers on high-protein diets was clearly plastic at day 13 but became canalized by day 24. In addition, reproductive development in females on low-protein diets was still plastic at day 24. It has been hypothesized (Juliano et al. 2004) that some storage threshold must be attained to initiate reproductive development. The data obtained are consistent with the existence of such a storage threshold for initiation of the developmentally canalized phase of oogenesis.

Allocation of mass

The present study suggests that females initially allocate mass to somatic growth and then switch to ovarian growth. This is consistent with the conclusion of

Table 2 Results of planned, multivariate pairwise contrasts among ten diet regimens

	Contrast	$F_{6,70}$ *** $P \leq 0.0001$	Standardized canonical coefficients					
			Somatic mass	Ovary mass	Fat body mass	Vitellogenin production	Vitellogenin titer	Storage Protein titer
Age	L10 vs L21	5.5 ***	-0.15	0.082	2.696	0.283	0.044	-0.118
	L10 vs L32	18.0 ***	-0.05	2.044	1.684	0.332	-0.074	0.039
	L21 vs L32	9.1 ***	0.05	2.917	0.227	0.252	-0.145	0.155
	H10 vs H21	17.5 ***	-0.17	2.662	0.873	0.365	-0.0004	-0.066
	H10 vs H32	32.8 ***	-0.13	3.163	-0.581	-0.118	0.017	-0.207
	H21 vs H32	13.6 ***	-0.00	-1.807	2.015	0.642	-0.027	0.252
Dietary protein	L10 vs H10	33.9 ***	-0.11	0.343	2.696	0.057	0.091	0.002
	L21 vs H21	51.8 ***	-0.15	1.870	1.946	0.173	0.063	0.002
	L32 vs H32	24.5 ***	-0.27	2.473	1.383	-0.382	0.215	-0.291
Diet switches	H21 vs H13L21	12.1 ***	-0.13	1.396	2.192	0.227	0.169	-0.123
	H32 vs H13L32	6.1 ***	-0.35	2.050	1.648	-0.652	0.134	-0.287
	H32 vs H24L32	0.8	0.50	0.389	-0.806	0.250	0.798	-0.205
	H13L32 vs H24L32	7.4 ***	-0.48	1.736	1.748	-0.670	-0.130	-0.196
	L32 vs L24H32	8.9 ***	-0.17	0.673	2.563	0.226	0.105	-0.215

Plasticity in reproduction of female lubber grasshoppers mostly is due to changes in ovarian mass and fat body mass, with mass-specific vitellogenin production having only minor contributions. See text for details on diet regimens. Six contrasts test effects of age within diets, three contrasts test effects of diets within ages, and five contrasts test effects of diet switches in groups started on the same diets and dissected at the same age. For significant contrasts, *standardized canonical coefficients* (SCC) in **bold** type are deemed large, and indicate that the associated reproductive parameter makes a substantial contribution to the observed significant difference in that contrast (see Scheiner 2001 for details)

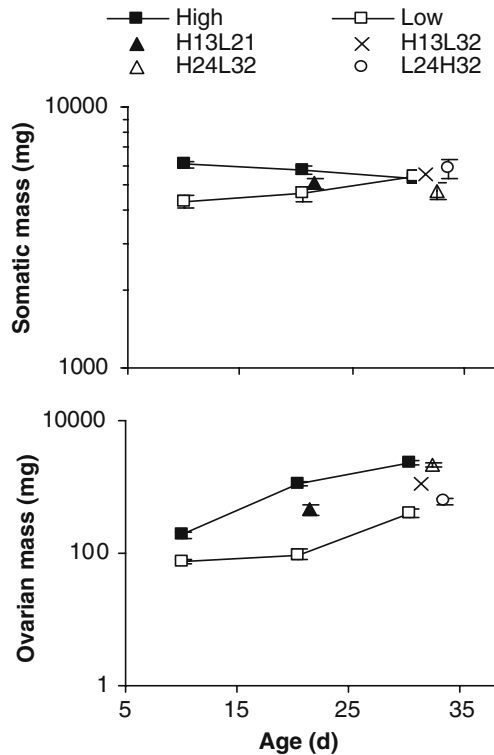


Fig. 1 Developmental plasticity in response to dietary protein of ovarian mass and somatic mass in female lubber grasshoppers. Grasshoppers were dissected at days 10, 21, and 32; data are offset for clarity. See text for descriptions of diet regimens. *Error bars* are ± 1 SE. Significant differences between critical pairwise contrasts are indicated in Table 2

Sundberg et al. (2001) that in well-fed lubber females somatic growth occurs during the first 10 days of adulthood and then reproductive growth occurs until oviposition.

Total vitellogenin production

Total vitellogenin production is the product of mass-specific vitellogenin production rate times the total fat body mass, and hence these two parameters are discussed together. Total vitellogenin production was plastic in response to changes in dietary protein early in vitellogenesis, but canalized in response to changes in dietary protein late in vitellogenesis. The synchronized peaks in both fat body mass and mass-specific vitellogenin production (see H21 in Fig. 2) suggest that both these parameters contribute to the plasticity in total vitellogenin production. Specifically, the increase in total vitellogenin production between days 13 and 21 is dependent on dietary protein. In contrast, total vitellogenin production was not sensitive to a reduction in dietary protein at day 24. Juvenile hormone does not appear to regulate the decrease in mass-specific vitellogenin production in response to a reduced diet, suggesting that some other, unidentified factor(s) regulates such declines (Fei et al. 2005). The lack of plasticity in

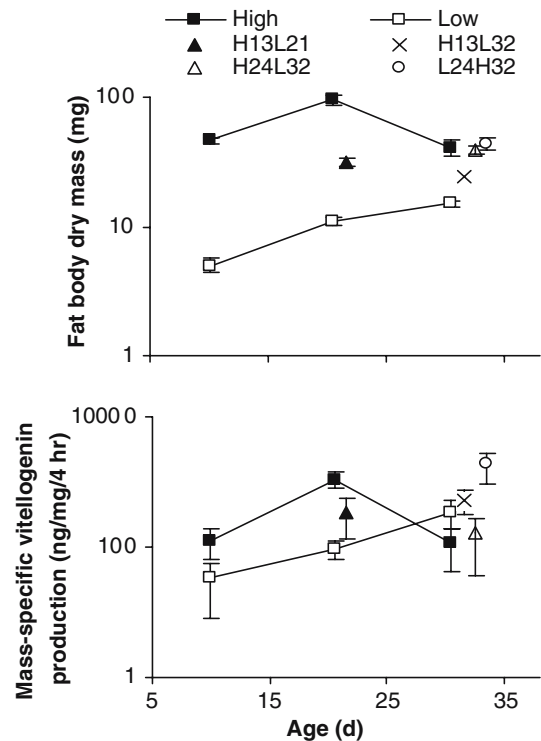


Fig. 2 Developmental plasticity in response to dietary protein of fat body mass and mass-specific vitellogenin production in female lubber grasshoppers. Grasshoppers were dissected at days 10, 21, and 32; data are offset for clarity. See text for descriptions of diet regimens. *Error bars* are ± 1 SE. Significant differences between critical pairwise contrasts are indicated in Table 2

total vitellogenin production late in the cycle is not surprising in hindsight, because late in the cycle, the fat body declines in mass even in females maintained on a high protein diet.

Fat body growth versus stimulation of mass-specific vitellogenin production

Fat body growth was more important than mass-specific vitellogenin production in producing plasticity. Whole body mass gain has been associated with plastic developmental transitions (Nijhout and Williams 1974; Hatle et al. 2003c). However, few studies have examined the role(s) of specific organs. To our knowledge, this is the first study to demonstrate that mass change of a particular organ was more important to developmental plasticity than was the output of that organ, especially in the same individuals.

The greater contribution of fat body mass than mass-specific vitellogenin production to overall effects suggests that growth factors may be more important than tissue stimulators in producing developmental changes in total vitellogenin production. Mean mass-specific vitellogenin production increased eightfold from days 10 to 21 in females fed a high-protein diet, but the variance in this increase was so great that mass-specific vitellog-

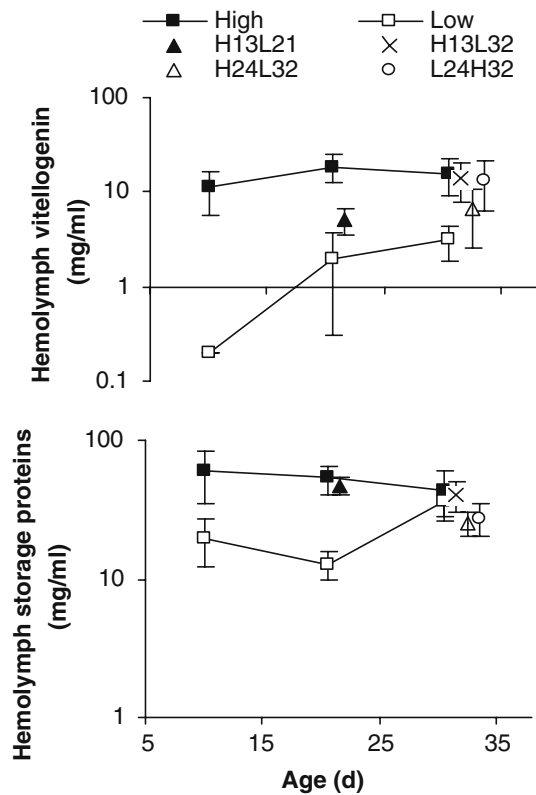


Fig. 3 Developmental plasticity in response to dietary protein of vitellogenin titers and storage proteins titers in female lubber grasshoppers. Grasshoppers were dissected at days 10, 21, and 32; data are offset for clarity. See text for descriptions of diet regimens. Error bars are ± 1 SE. Significant differences between critical pairwise contrasts are indicated in Table 2

enin production contributed little to statistical differences. Changes in fat body mass were more consistent and contributed more to overall effects. Much of the work on stimulation of vitellogenesis in insects has focused on JH and other factors (e.g., brain factor in Glinka et al. 1995) that regulate the *activity* of the fat body (i.e., mass-specific vitellogenin production), whereas growth factors and mitogens that regulate the *size* of the fat body have been somewhat neglected. We believe that studies on regulators of fat body growth may yield significant contributions to our understanding of the developmental process of insect vitellogenesis, and perhaps other developmental transitions in insects.

This tissue stimulation likely is controlled by hormones. Levels of JH and ecdysteroids, the two chief developmental hormones in insects, increase greatly from days 10 to 21 (Hatle et al. 2000, 2003a, 2003b). But neither seems likely to be involved directly in fat body tissue growth leading to increased total vitellogenin production. Instead, JH is involved in the regulation of mass-specific vitellogenin production; specifically, it is necessary for the initiation of vitellogenin mRNA transcription. Ecdysteroids are not necessary for vitellogenesis and do not augment vitellogenesis (Hatle et al. 2003b).

Fat body tissue growth may be stimulated by a mitogen(s) that increases the rate of cell division. For

example, in *Drosophila* dietary amino acids activate quiescent cells to progress through the cell cycle (Britton and Edgar 1998). Tissue growth of the fat body could also be stimulated by a growth factor(s), enhancing cell size. Larval fat body of *Drosophila* tends to grow by endoreplication, an unusual means of tissue growth by increasing cell size. Indeed, the fat body appears to be important in regulating body size (Hoshizaki 2005; Nijhout 2004; Oldham and Hafen 2003; Stern 2003) and has been shown to be a nutrient sensor that regulates body growth in *Drosophila* (Colombani et al. 2003). Our data suggest that the regulation of the size of the fat body is also important for oocyte growth. An insulin-like peptide is a candidate for such a fat body growth factor or mitogen (Chan and Steiner 2000). In invertebrates, insulins are secreted from the brain and control cellular and organismal growth (Brogiolo et al. 2001; Chan and Steiner 2000; Ebberink et al. 1989; Nijhout and Grunert 2002). The expression of an insulin-like peptide is dependent on nutrition in *Drosophila* (Ikeya et al. 2002), and an insulin-like peptide has been sequenced in another orthopteran (Clynen et al. 2003; Hetru et al. 1991; Larocque 2000).

Similarly, mechanistic studies of the regulation of reduction in fat body size may be important in understanding the canalized phase of development at the end of oogenesis (see Hatle et al. 2003a). The removal of larval fat body in young adult *Drosophila* is thought to involve apoptosis (Hoshizaki 2005). This mechanism of programmed cell death could serve as a model for the reduction of fat body size in female lubber grasshoppers during the developmentally canalized phase of oogenesis.

Hemolymph dynamics

Neither vitellogenin nor storage protein titer contributed greatly to the overall effects of diet regimens. In previous work peaks in the levels of both vitellogenin and storage protein in the hemolymph at about day 22 have been demonstrated (Hatle et al. 2001). This pattern is clear when individuals are tracked throughout oogenesis. But, maximum levels of these proteins vary greatly among individuals, up to tenfold for vitellogenin (data from Hatle et al. 2001). When individuals are sampled only once (as in this study), the variation in maximum titers across individuals may obscure the peaks in protein titers.

Threshold of protein storage for reproduction

During oogenesis, female lubbers make a commitment to oviposit at least 12 days before actually laying (Hatle et al. 2003a; Moehrlin and Juliano 1998). Juliano et al. (2004), and data in the present paper, demonstrate that a threshold of feeding exists for initiating this developmentally canalized phase. In fact,

female lubbers starved beginning at day 25 went on to oviposit an apparently normal clutch at about day 35 (D.W. Whitman and J. Bergmann, unpublished data). Juliano et al. (2004) described the threshold only as a cumulative amount of food (i.e., lettuce) consumed. Because similar developmental patterns are seen in this experiment, while manipulating only dietary protein, it seems likely that the threshold could be described as a cumulative amount of protein consumed. However, the physiological nature of this threshold (i.e., what the “currency” for stored reserves is) and information about how those reserves influence development remain unknown. This information is important because thresholds with different properties can produce different trade-offs in life history models (Day and Rowe 2002).

This study implicates the fat body in the threshold. It has been hypothesized that the threshold for commitment to oviposition in lubber grasshoppers is the amount of storage proteins in the fat body or hemolymph. The transcripts for at least three related storage proteins exist in the fat body of lubbers. The accumulation of the translated proteins to a threshold level may signal the female to initiate a cascade of endocrine events that end in oviposition.

This study suggests that diet effects on fat body growth are more important in the regulation of vitellogenesis than are diet effects on mass-specific vitellogenin production. This finding was only possible by examining this developmental process during the whole course of egg production and by examining multiple factors simultaneously in a single, large experiment. This underscores the value of such experiments in informing the direction of experiments at the cellular and biochemical levels.

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