

Plasticity and Canalization in the Control of Reproduction in the Lubber Grasshopper¹

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SYNOPSIS. The ability to change reproductive tactics during adult development in response to environmental variation is predicted to enhance fitness. Many organisms show phenotypic plasticity early in non-embryonic development, but later exhibit phases of developmental inflexibility (=canalization). Therefore, we studied reproduction-related hormones and proteins and their relationships to plasticity in the Eastern lubber grasshopper. Diet-switching experiments demonstrated plasticity early in the egg production cycle, but a switch to canalization late in the cycle. We measured developmental titers of 4 hemolymph compounds from single individuals from adult molt until first oviposition. These 4 compounds were the egg-yolk precursor protein vitellogenin, juvenile hormone (the central regulator of insect reproduction), major hemolymph proteins, and ecdysteroids (the arthropod molting hormone that ultimately is stored in the egg). Using diet manipulations, we investigated how these developmental titers relate to the switch from plastic to canalized egg production. All 4 hemolymph compounds reached their peak levels during the canalized phase, about 12 day before oviposition. Diet switches after these peak levels did not affect the timing to oviposition. Therefore, these peak titers were physiological events that occurred after the individual committed to laying. We compared these patterns in reproduction to the development toward adult molt, another major life-history event in insects. We observed an extended canalized phase before the adult molt. This canalized phase always included a peak of ecdysteroids. The similar patterns in the physiology of these life-history events suggested that common limitations may exist in major developmental processes of insects that are directed by hormones.

INTRODUCTION

The ability to change life-history tactics during development in response to environmental variation may enhance fitness, especially when there are reliable cues of environmental change (Lively, 1986). This ability to adjust a developmental process to changing environmental conditions is called phenotypic plasticity (Schlichting and Pigliucci, 1998). Plasticity in some life-history tactic is an almost universal biological phenomenon (Gilbert and Bolker, 2003; Higgins and Rankin, 1996; West-Eberhard, 2003). Plasticity can be adaptive when it allows an individual to adjust physiologically to increase its fitness in its particular environment (*e.g.*, Boorse and Denver, 2003; Denver, 1998; Denver *et al.*, 1998; Hodin, 2000; Hodin and Riddiford, 2000; Shafiei *et al.*, 2001). Plasticity can also be non-adaptive when it is a simple expression of how environmental inputs modify development because of physiological constraints (Reznick, 1990). Because life-history plasticity is both taxonomically widespread and occurs in many life-history events, it is likely that cases of both adaptive plasticity and non-adaptive plasticity exist.

Many organisms appear to be plastic early in non-embryonic development, but later in development are inflexible (=canalized) (*e.g.*, Leips and Travis, 1994; Moehrlein and Juliano, 1998). The lack of flexibility

during canalized phases could be a result of developmental constraints, and it could limit the number of phenotypes that can be produced. First, canalized phases may exist because there are physiological limitations in how rapidly an organism can respond developmentally to environmental changes (Schlichting and Pigliucci, 1998). For example, many hormones that are involved in non-embryonic development work by activating gene transcription. Hormone synthesis, transcription, translation, and post-translational modification all require time, so it may be several days before there is a change in the physiology of the whole organism. In addition, once these processes are initiated, they may not be readily altered. Cellular mechanisms such as gene activation and translation are conserved across phyla, and the time required for these mechanisms to operate also is conserved, to some degree, across phyla. Second, canalized phases may exist because there are physiological limitations in the level to which an organism can respond developmentally to environmental changes. For example, cells have a finite rate of protein production and secretion, so the synthesis and accumulation of sufficient protein might require a minimum time. In short, the limits in hormonal responses and cellular functions may be responsible, at least in part, for the canalization of developmental progression toward a life-history event. Hence, canalized phases are excellent targets for examining the physiological processes that underlie life-histories, to contribute to the goal of bridging the gap between cellular and life-history mechanisms.

Canalized phases could be important with respect to evolution. Natural selection acts on final phenotypes, not directly on the developmental processes that pro-

¹ From the Symposium *Physiology Underlying Phenotypic Plasticity and Polyphenisms* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 4–8 January 2003, at Toronto, Canada.

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duce those phenotypes. A limit on the possible number of phenotypes because of canalized phases (Schlichting and Pigliucci, 1998) could set a boundary on the phenotypic variation on which selection can act. In other words, developmental canalization could limit adaptation. Knowledge of the physiological events that underlie canalization will enrich our understanding of the possibilities and limits in making beneficial adjustments of important life-history tactics through development.

Reproductive tactics (*e.g.*, timing of reproduction, number and size of offspring) are strongly correlated with fitness (Stearns, 1992) and can be plastic early but canalized later in development. Reproductive plasticity may be regulated by endocrine mechanisms (Hatle *et al.*, 2000; Ketterson and Nolan, 1999; Schoech, 1998), similar to plasticity in other developmental events (Nijhout, 2003). However, the physiological processes that determine the transition from reproductive plasticity to canalization are poorly understood, and the physiological events underlying reproductive canalization are almost completely unknown.

Model system

We have studied the relationships among plasticity in reproduction, the hormones that control reproduction, and the proteins that are the material resources for reproduction, in Eastern lubber grasshoppers (*Romalea microptera*). These animals are an excellent model system for this work for at least 3 reasons. First, lubbers are univoltine, gregarious, and have low vagility (individuals are flightless and disperse only about 50 m during their lifetime; Whitman, 1990). This results in a large number of grasshoppers at one time and place. In a species with high local densities and little capability for dispersal, plasticity in response to local food shortages would be more likely to require a physiologic response, rather than a behavioral or phenological response. Second, lubber grasshoppers are phytophagous and lay large clutches of protein-rich eggs. Egg production in phytophagous insects generally is protein limited (Nijhout, 1994), and this appears to be true for lubbers (Waskey *et al.*, 2002). Third, lubbers are large (4–8 g), allowing collection of serial hemolymph samples sufficient for determination of both hormone and protein titers, without greatly altering the reproductive tactics of the individual (Hatle *et al.*, 2002; J.D. Hatle and S.A. Juliano, unpublished data). Using this approach, we have been able to acquire complete developmental titers of 4 hemolymph compounds for single individuals, from adult molt to oviposition. These *individual* responses (or lack of responses) to environmental changes allow us to test directly the relationships between hemolymph titers and plasticity.

Plasticity and canalization in reproductive tactics

We define a canalized phase operationally as a period during which an individual is unable to respond developmentally to an environmental change that is

large enough to produce a response during other phases of development. In our experiments, grasshoppers are raised individually, and each receives a daily ration of Romaine lettuce of either high quantity (H) or low quantity (L). The H diet in effect is an *ad lib.* diet, whereas the L diet is about 1/3 of the quantity that animals on a H diet typically consume. While some individuals remain on H or L diets until oviposition, others are given an abrupt switch in food availability (*e.g.*, H switched to L) at a defined age. For example, "HL14" designates grasshoppers offered H rations from adult molt through age 13 day and then offered L rations from age 14 day until oviposition. These diet-switch treatments are designed to place an individual in a specific developmental trajectory and then to reveal whether that trajectory changes in a new environment.

Both plasticity and canalization of reproductive tactics in the lubber grasshopper were demonstrated by a diet-switching experiment (Moehrli and Juliano, 1998). In this experiment, the first egg production cycle (from adult molt to oviposition) for H grasshoppers lasted about 30 day (14L:10D photoperiod and a corresponding 32:24°C thermocycle). Grasshopper reproduction was initially plastic; L grasshoppers laid fewer eggs later than H grasshoppers (compare H vs. L in Fig. 1). Likewise, LH7 grasshoppers responded to increased food availability; they laid earlier and laid more eggs than grasshoppers continued on the L diet. Also revealing plasticity, HL7 grasshoppers laid later and laid fewer eggs than grasshoppers continued on the H diet. Hence, the grasshoppers' reproductive timing and output were plastic during the first portion of the cycle.

In contrast, reproduction in these grasshoppers was canalized later in the oviposition cycle. For example, HL14 grasshoppers oviposited at a time that was statistically indistinguishable from grasshoppers fed H diets throughout the cycle, but laid fewer eggs. HL21 grasshoppers had oviposition times and numbers of eggs that were indistinguishable from H grasshoppers (Fig. 1). In short, these experiments showed that reproductive timing and output of these grasshoppers became inflexible during the later phases of the cycle, although at different times (Moehrli and Juliano, 1998).

This long, unresponsive phase may be in part a product of the clutch-laying tactic in concert with the phytophagy of lubbers. Females lay about 3 clutches per lifetime, and each clutch can weigh ~20% of the mass of the female (Hatle *et al.*, 2002). Producing each clutch is a major developmental event that requires large amounts of the limiting nutrient (*i.e.*, protein). This type of developmental phenomenon seems particularly likely to be plastic in response to nutrition early, because a large amount of resources must be acquired and stored before development can continue. This may require meeting a threshold of storage. Further, this type of developmental phenomenon may be canalized late, because it requires a period during which stored

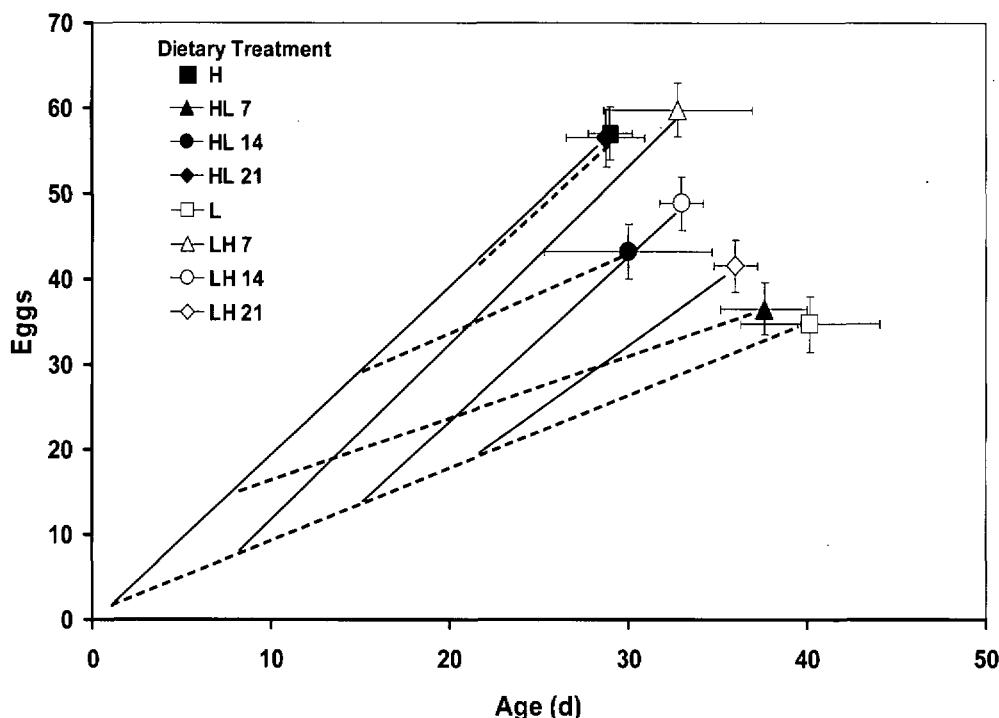


FIG. 1. Reproduction in lubber grasshoppers has plastic and canalized phases. Female lubber grasshoppers were isolated at the adult molt and were fed either a high (H) or a low (L) quantity diet. The diets of some individuals were switched after 7, 14 or 21 days. Solid lines and dashed lined indicate periods when animals received the H and the L diets, respectively. Symbols indicate the day of oviposition (\pm SE) and the number of eggs (\pm SE). Dietary switches from the H to the L diet at 14 days (HL14) and 21 days (HL21) had no significant effect on the timing of reproduction when compared to animals fed the H diet throughout. Similarly, switching from the L to the H diet at 21 days (LH21) had no significant effect on timing when compared to animals fed the L diet throughout. In addition, the dietary treatments HL21 and LH21 had no effect on the number of eggs produced compared to H and L animals, respectively. The lack of an effect from the dietary switch indicated that animals were in a canalized phase of reproduction, while a significant difference between groups indicated a plastic phase of reproduction. N = 4–5/diet. Data from Moehrlin and Juliano (1998).

resources are converted into egg protein and that protein is sequestered into the oocytes. Such canalized phases can limit the number of phenotypes that can be produced through development (Schlichting and Pigliucci, 1998). Hence, we hypothesize that once a strategy exists that requires large amounts of a limiting resource, evolution toward more flexible development at the end of the cycle is likely to be constrained.

Trade-off theory would suggest that the fixed development of HL21 grasshoppers while on the low diet would have some cost. We have not yet identified this putative cost. The size of eggs was never affected by diet, so there is no apparent cost in egg size associated with the insensitivity of reproductive timing to diet changes at the end of the clutch (Moehrlin and Juliano, 1998). All of our subsequent studies have focused on the first clutch to allow in depth examination of physiological processes, so we have not yet studied potential costs. Nonetheless, we hypothesize that HL21 grasshoppers pay the cost of a low diet after 21 day during the 2nd clutch, by either delaying oocyte development or producing fewer eggs.

Physiological compounds involved in grasshopper egg production

Our study of the physiological mechanisms that underlie plastic and canalized phases of oocyte devel-

opment focused on the developmental titers of 4 hemolymph compounds throughout this process (Fig. 2). These are vitellogenin (Vg), juvenile hormone (JH), major hemolymph proteins (MHP), and ecdysteroids.

Vitellogenin (Vg) is the egg-yolk precursor protein that is produced by the fat body, secreted into the hemolymph, and sequestered by the oocytes (Engelmann, 1983). It is defined as a hemolymph protein that is immunologically related to egg-yolk protein.

Juvenile hormone (JH) is the central regulator of egg production in many insects, including Orthoptera. JH stimulates fat body production of Vg by increasing Vg-mRNA production and facilitates the movement of Vg from the hemolymph into the developing oocytes (Wyatt and Davey, 1996). The parallel increases and decreases of hemolymph levels of Vg, Vg-mRNA, and JH during the oviposition cycle in well-fed lubber grasshoppers (Borst *et al.*, 2000) are consistent with suggestions that JH increases Vg-mRNA levels, which in turn leads to an increase in Vg production and its release into the hemolymph. We have recently succeeded in manipulating JH levels in our grasshoppers and showed that it is necessary for oocyte development in general (Barry *et al.*, 2002) and Vg-mRNA production in particular (T.O. Barry and D.W. Borst, unpublished data).

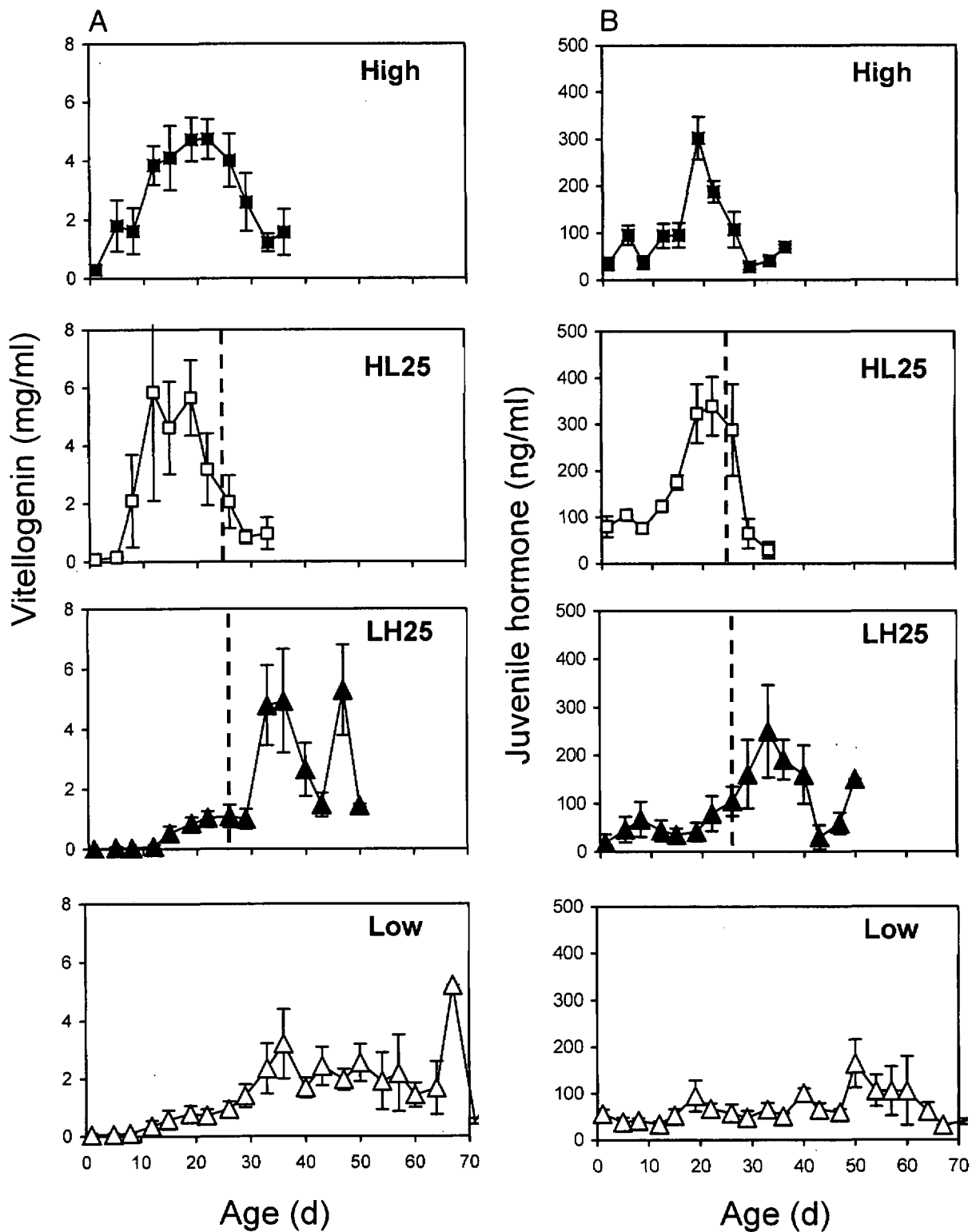


FIG. 2. Developmental titers (means \pm SE) of 4 hemolymph compounds in lubber grasshoppers on 4 diet regimes during the first oviposition cycle. Juvenile hormone (A); vitellogenin (B); major hemolymph proteins (C); and ecdysteroids (D). See text for further description of diet regimes. Levels of all 4 compounds peaked about 12 days before oviposition, regardless of diet regime. $N = 13$ for H; 5 for HL25; 8 for LH25; and 7 for L. Data from Hatle *et al.* (2000, 2001, 2003a).

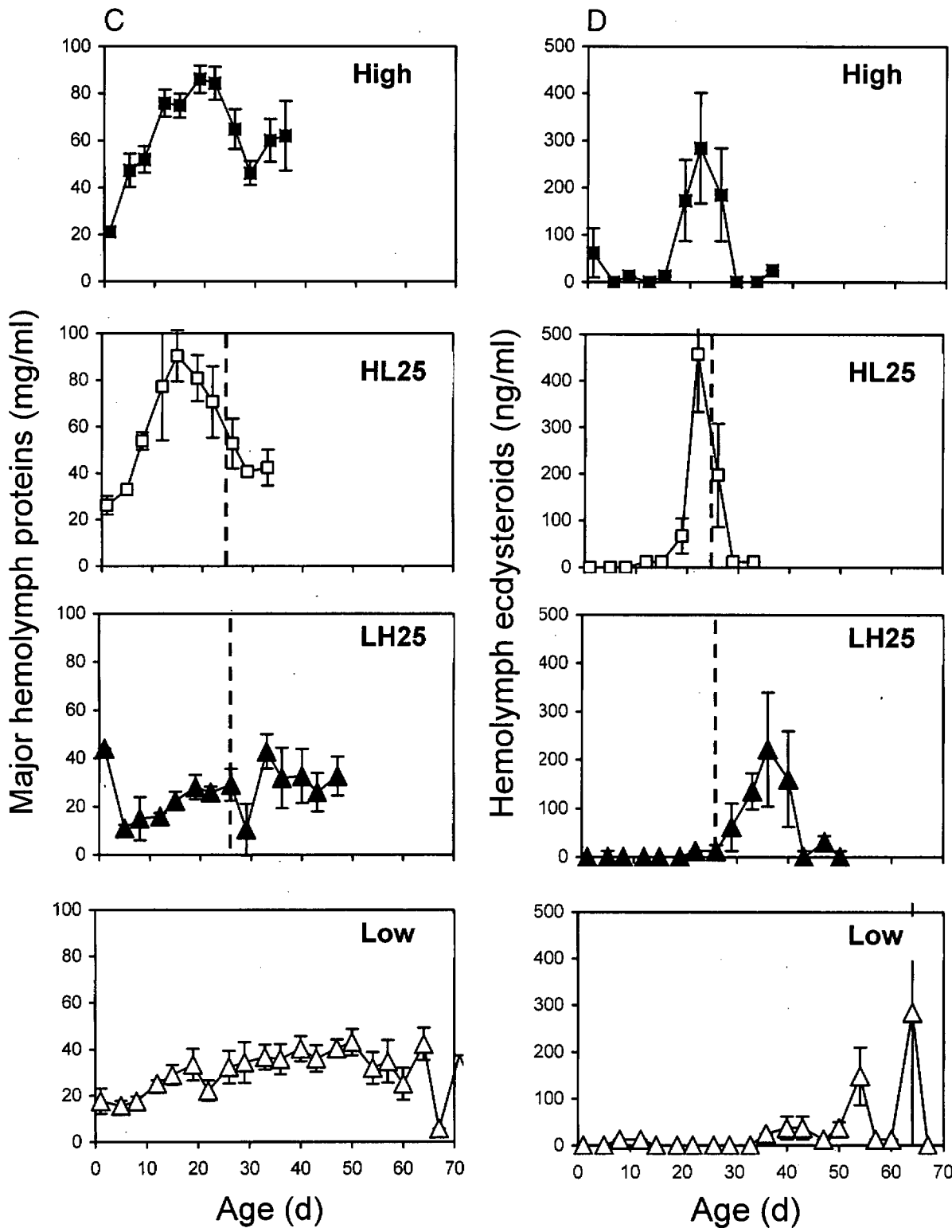


FIG. 2. Continued.

The major hemolymph proteins (MHPs) are a group of related proteins in the lubber grasshoppers that make up ~80% of hemolymph protein in juveniles, males, and females throughout the egg production cy-

cle (Hatle *et al.*, 2001). In comparison, Vg makes up ~10% of hemolymph protein at its maximum. Three MHPs of lubbers appear to be members of the family of insect storage proteins. The largest of these proteins

is a hexamerin similar to the persistent storage protein (PSP1) of *Locusta migratoria* (Ancsin and Wyatt, 1996), the intermediate-sized protein may be a methionine-rich storage protein, and the smallest may be an arylphorin (J.D. Hatle and D.W. Borst, unpublished data). The amino acids contained in these MHPs seem likely to be important resources for the protein-limited process of egg production. The developmental titers of MHPs are consistent with the hypothesis of MHPs being used to fuel egg production (Hatle *et al.*, 2001).

Finally, ecdysteroids, the arthropod molting hormones, are present in high levels in the hemolymph during a short period of oocyte development (Fig. 3). High levels of ecdysteroids are also found in well-developed oocytes (Fig. 3a; Hatle *et al.*, 2003a) and eggs after oviposition (J.D. Hatle and D.W. Borst, unpublished data). The role(s) of ecdysteroids in orthopteran egg production is unclear. In locusts they may stimulate vitellogenesis (Girardie and Girardie, 1996), and in cockroaches they may inhibit vitellogenesis (Engelmann, 2002). In lubber grasshoppers, hemolymph ecdysteroids appear to have no effect on vitellogenesis (Hatle *et al.*, 2003a). Nonetheless, the precise timing of the ecdysteroid peak during oocyte development, the amount of ecdysteroids present (~300 ng/ml), and the retention of ecdysteroids in the ovary suggests that these steroids have some role in egg production (Hatle *et al.*, 2003a).

Physiological processes underlying reproductive plasticity and canalization: timing of events

The physiological mechanisms involved in reproduction reflect the plastic and canalized phases of this process. In an experiment that followed the work of Moehrli and Juliano (1998) cited above, we studied these relationships by using 4 diet treatments (H, HL25, LH25, and L) to generate identifiable phases of plastic and canalized oocyte development. In these same individuals, we measured levels of Vg, JH, MHPs, and hemolymph ecdysteroids throughout egg production. In these experiments, the mean age at oviposition was 35 day for H grasshoppers. Because this was somewhat longer than that observed by Moehrli and Juliano (1998), we changed the age at the dietary switch (25 days rather than 21 days).

The developmental titer of each hemolymph compound had a distinct peak during the egg production cycle (see H grasshoppers in Fig. 2A–D). We reasoned that the timing of the maximum titer of each hemolymph factor was an important switch in development. Before the maximum, the compound accumulated in the hemolymph (usually through synthesis). After the maximum, the compound was removed from the hemolymph (*e.g.*, degradation, sequestration by the ovary). Hence, these peaks likely represented an important shift in the physiological mechanisms regulating reproduction, one that could be monitored in a non-destructive way. The disadvantage of this method is that it is post-hoc. Yet, such non-destructive methods are critical for studying mechanisms of plasticity (see Fed-

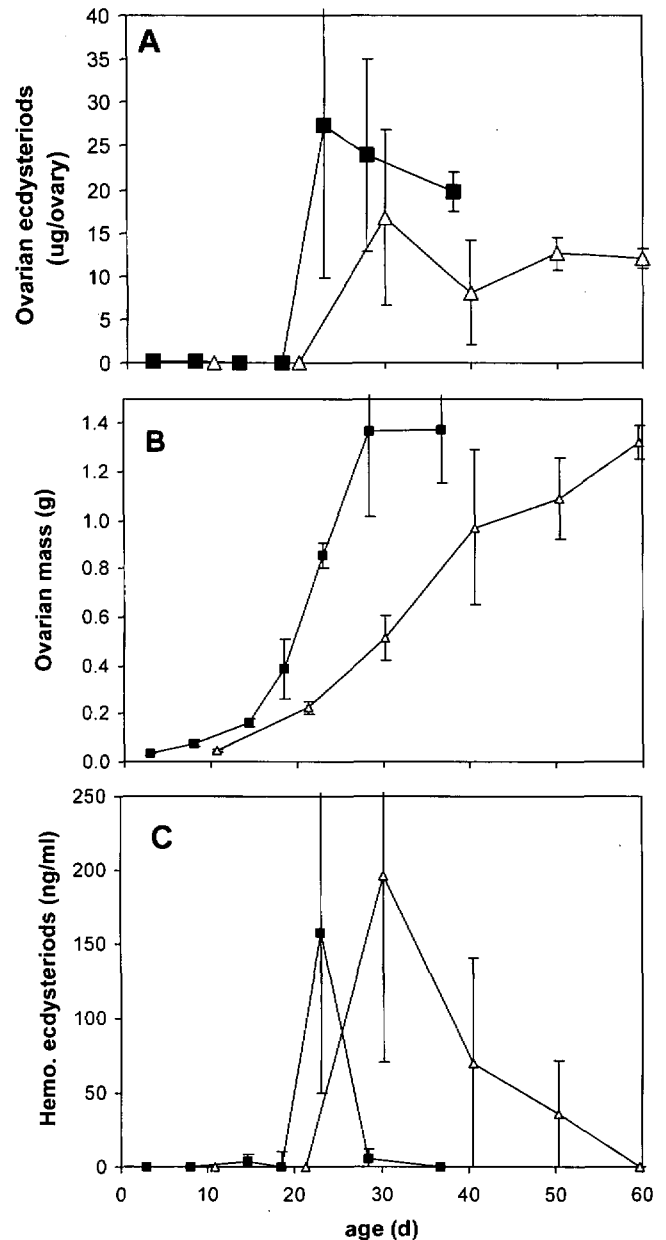


FIG. 3. Ovarian ecdysteroid levels (A), ovarian masses (B), and hemolymph ecdysteroids (C) of adult female lubber grasshoppers fed high- (filled squares) or low- (open squares) quantity diets. After reaching maximal levels, ecdysteroids remained in the ovary but disappeared from the hemolymph.

er *et al.*, 1987), because they enable measurement of physiological parameters and tracking that same individual to some developmental event.

Plasticity in the physiology underlying egg production was shown by maximum titers of all 4 hemolymph compounds occurring later in L than in H grasshoppers (Fig. 2A–D). Further, plasticity was shown by maximum titers of JH, MHPs, and hemolymph ecdysteroids occurring earlier in LH25 grasshoppers than in L grasshoppers (Fig. 2A, B, D). These results demonstrate that, early in development, lubber grasshopper

TABLE 1. Diet effects on the timing and level of maximum titers of 4 hemolymph compounds during reproduction in lubber grasshoppers.*

	Vitellogenin	Juvenile hormone	Major hemolymph proteins	Ecdysteroids
Age at maximum titer	0.001	0.001	0.001	0.001
Time from maximum to oviposition	0.510	0.886	0.671	0.412
Level of maximum titer	0.059	0.139	0.001	0.033
Max. level predicts number of eggs?	0.038	0.956	0.001	0.093

* (Hatle *et al.*, 2000, 2001, 2003a). The Table shows the probabilities (P-values by ANOVAs or regressions) of nonsignificant diet effects on 4 physiological parameters. The ages at the maximum titer of all 4 hemolymph compounds were highly significantly affected by diet, suggesting these developmental phases are largely plastic. In contrast, the times from these maxima to oviposition were not affected by diet, suggesting these phases are largely fixed. Maximum levels of proteins tended to be affected by diet, whereas maximum levels of hormones tended not to be affected by diet. See text for a detailed discussion of levels of hemolymph compounds.

pers can respond to food availability by adjusting the timing of hemolymph compounds.

We do not assume that the observed plasticity in the timing of these maxima is adaptive. Testing the adaptive value of reproductive plasticity requires manipulating hormones that control reproduction. Such phenotypic engineering (*e.g.*, Sinervo *et al.*, 1992; Ketterson and Nolan, 1999; Schmidtt *et al.*, 1999), coupled with environmental manipulation, has proved to be an effective experimental tool for investigating the adaptive nature of phenotypes.

We have focused much of our study on the physiology underlying the canalized phase of reproduction. Canalization in the physiology underlying egg production was shown by a failure to respond to decreased food availability at the end of the cycle (*cf.* H and HL25 in Fig. 2A–D). Notably, the L diet to which HL25 grasshoppers were switched, when offered from the onset of adulthood, lengthened the time to oviposition (*cf.* H vs. L in Fig. 2A–D). Further, H and HL25 grasshoppers laid at similar ages and laid similar numbers of eggs (Hatle *et al.*, 2000), in accord with previous experiments (Moehrlin and Juliano, 1998; Fig. 1). This failure to respond to decreased food availability was not surprising, because levels of all 4 hemolymph compounds were falling before the reduction in food availability.

The canalized phase always included the maximum levels of all 4 hemolymph compounds that were observed throughout the egg production cycle (Table 1; Hatle *et al.*, 2000, 2001, 2003a). The time from adult molt until the maximum titer of all 4 compounds was significantly affected by diet, suggesting that oocyte development during this period is largely plastic. In contrast, the time from each maximum to oviposition was not affected by diet, implying that all 4 compounds peaked during a canalized (*i.e.*, inflexible) phase of oocyte development (Table 1). Thus, these maxima appear to be important physiological landmarks during egg production. It seems likely that the canalized phase consists of a series of protracted physiological steps required to prepare for egg laying. Hence, the developmental switches that regulate these 4 hemolymph compounds (Vg, JH, MHPs, and ecdysteroids) are likely to be steps during this process. Further, within diets, there was no significant difference among the ages at these maximum titers (*e.g.*, H grass-

hoppers reached the maximum titer of Vg and the maximum titer of JH at similar times). This implies that the developmental switch for each of these 4 compounds may be controlled by a single signal or simultaneous signals.

Physiological processes underlying reproductive plasticity and canalization: levels of compounds

Maximum levels of vitellogenin were marginally (but not significantly) affected by diet (Table 1; Hatle *et al.*, 2001), and H and HL25 grasshoppers tended to have higher peak Vg titers than LH25 and L grasshoppers (Fig. 2A). Perhaps more important, the maximum level of Vg was a significant predictor of the number of eggs produced by an individual (Table 1).

Maximum levels of JH were not affected by diet and did not predict the number of eggs laid (Table 1; Hatle *et al.*, 2000). The highly asynchronous development of animals within the LH25 group and within the L group creates in Figure 2 the false impression of greater maximum levels of JH in H and HL25 grasshoppers than in LH25 and L grasshoppers (*e.g.*, the peaks within the LH25 group do not match up across individuals and so do not add together in our presentation of means). Interestingly, the lack of relationship between JH and reproductive output suggested that JH may not have regulated the amount of Vg produced. Rather, JH may have regulated the synthesis of Vg-mRNA (Barry *et al.*, 2002; Borst *et al.*, 2000), but the production of Vg may have been regulated by some other, unknown factor.

Diet significantly affected the maximum level of MHPs. In turn, the maximum level of MHPs predicted the number of eggs laid (Table 1; Hatle *et al.*, 2001). The effects on levels of MHPs were stronger and clearer than the effects on levels of any of the other 3 compounds.

Diet weakly but significantly affected the maximum level of hemolymph ecdysteroids (Table 1; Hatle *et al.*, 2003a). However, only the pairwise comparison of HL25 vs. L was significant. There was no clear quantitative effect of diet on maximum ecdysteroid titers, because H grasshoppers did not have significantly higher ecdysteroid titers than L grasshoppers. Further, the maximum level of ecdysteroids did not predict the number of eggs produced. This was surprising, because if ecdysteroids are produced by the ovary, then

a larger ovary should result in more ecdysteroids. However, it may be that the production of ecdysteroids occurs before oocytes are reabsorbed and before ovary size is effected by diet.

In general, the maximum levels of proteins were affected by diet, whereas the maximum levels of hormones were unaffected by diet. We hypothesize that the hemolymph proteins (Vg and MHPs) quantitatively affect the protein-limited process of egg production. In contrast, the hormones (JH and ecdysteroids) act as developmental signals that regulate portions of the timing of oocyte development but do not quantitatively affect the number of eggs produced. In particular, MHPs may act as a nutritionally-dependent reservoir of amino acids that is monitored to determine both the timing of the onset of oogenesis and the number of eggs produced (Olson *et al.*, 2001; Tillman *et al.*, 2001). Thus, diet could directly affect the levels of MHPs, which will in turn affect oocyte development. For Vg, all protein in the hemolymph should accumulate in the oocytes, but changes in uptake might obscure any relationship between hemolymph levels and egg production. Experiments in which hormone and hemolymph protein levels are manipulated will be needed to test rigorously these possibilities.

Linking hemolymph and ovarian events

In a separate series of experiments, we examined ovarian ecdysteroid production and levels underlying plasticity in oocyte development (Hatle *et al.*, 2003a). This enabled us to link more directly hemolymph and organ-level dynamics. First, ovariectomized females had no detectable ecdysteroids in the hemolymph, whereas sham operated females had high levels of ecdysteroids in the hemolymph. Hence, the ovary was involved in the production of hemolymph ecdysteroids. Next, we examined putative plasticity in ecdysteroid levels. We fed grasshoppers either H or L diets, dissected individuals at regular intervals, and examined hemolymph and ovarian ecdysteroid levels. Low diets may have delayed the appearance of ecdysteroids in the hemolymph and the ovary (Fig. 3A, C). Ovarian ecdysteroids did not seem to appear at the same ovarian mass in both H and L diets, suggesting that ovary mass is not sufficient as an index of the development toward ecdysteroid production (Fig. 3B). Within diets, ovarian ecdysteroids appeared at the same point in reproductive development as hemolymph ecdysteroids. The coincidence of the appearance of hemolymph and ovarian ecdysteroids is the first direct link we have drawn between plasticity in a hemolymph factor and plasticity within an endocrine organ. Such links are critical for concatenating the life-history events of plasticity and the mechanistic control of that plasticity.

Comparison of physiology underlying canalization of reproduction and molting

The pattern in the developmental profiles of hemolymph compounds during reproduction is clear: maximum titers always occur during the canalized phase.

Is this pattern peculiar to the life-history transition of reproduction in grasshoppers? To address this question, we conducted a similar study on molting to adult, another major developmental event in insects. Specifically, we tested whether development to adult molt in lubber grasshoppers was canalized and whether the peak of molting hormone occurred during this canalized phase. If the same physiological patterns underlie both of these developmental events, it would suggest that similar temporal constraints (*e.g.*, time needed for the onset of transcription and the accumulation of translation products) may exist in many insect developmental systems.

Development before the last larval molt is canalized in several phytophagous insects (Allegret, 1964; Nijhout, 1979; Nijhout and Williams, 1974a, b; Sparks *et al.*, 1983). The endocrinological and physiological events underlying insect molting have been described in detail (see Truman, 1985; Richter, 1999 and references therein). Included in this progression of physiological events is the appearance of high levels of ecdysteroids in the hemolymph. Elevated ecdysteroid titers initiate several processes involved in molting, such as apolysis and the deposition of new epicuticle (Nijhout, 1994).

We examined the duration of the 5th (final) instar, grasshopper mass, and ecdysteroid levels (all measured at least every other day) in male lubber grasshoppers maintained on different diet treatments. The diet regimes for this experiment were analogous to those in our study on reproduction, though the sizes of the diets were adjusted for the smaller size of the nymphs. Individuals received either H rations throughout the 5th instar, L rations throughout the 5th instar, or were started on H rations and switched to L rations at ages 3 day (HL3), 8 day (HL8), or 13 day (HL13). Diet reduction at age 3 day significantly increased the duration of the 5th instar and delayed the age at maximum titer of ecdysteroids in comparison to H grasshoppers (Fig. 4). In contrast, diet reductions at ages 8 day (HL8) or 13 day (HL13) did not significantly increase the duration of the 5th instar or the age at maximum titer of ecdysteroids in comparison to H grasshoppers. In all groups, diet had no effect on the maximum levels of ecdysteroids. In addition, diet had no effect on the time from the maximum titer of ecdysteroids to the adult molt (Fig. 4). These observations indicated that the final phase of the molt cycle becomes canalized between 3 day and 8 day, and that the ecdysteroid maximum occurred within the canalized phase. These results for the physiological control that underlies molting are directly analogous to our results for the physiological control that underlies reproduction.

To further define the onset of the canalized phase, we sought a threshold ecdysteroid titer that was lower than the maximum titer and after which the time to adult molt is unresponsive to diet. Lubber grasshoppers appeared to have an ecdysteroid threshold of at least 100 ng/ml; diet treatment did not significantly affect time from 100 ng/ml to the adult molt. This titer

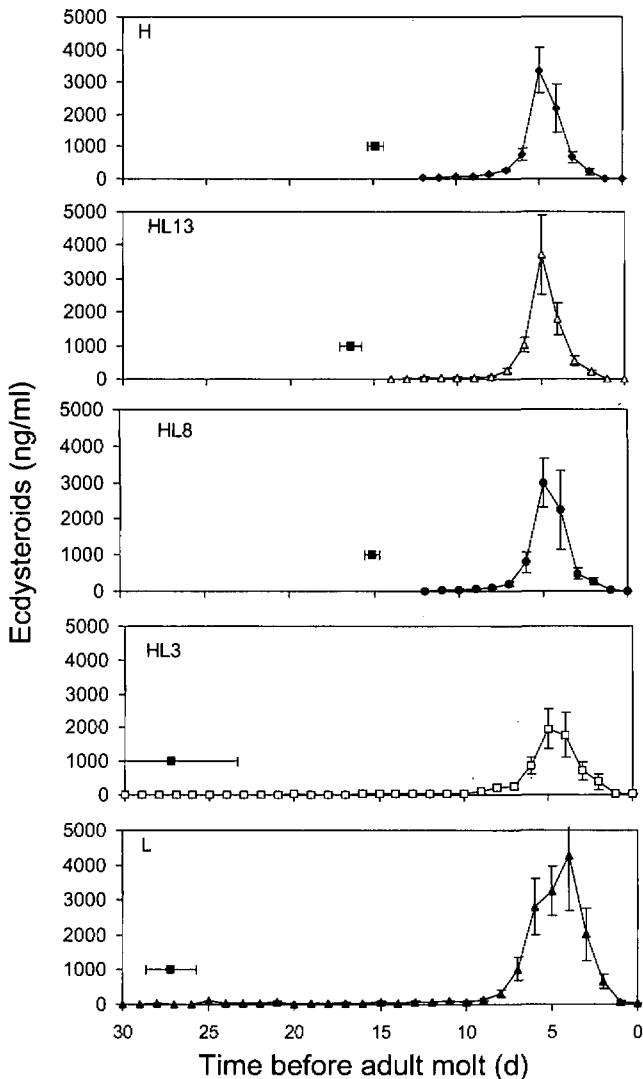


FIG. 4. The time before the adult molt at which hemolymph ecdysteroids peak is unaffected by diet treatment in lubber grasshoppers. Developmental profiles of hemolymph ecdysteroid levels in 5th instar male *Romalea microptera* fed 5 diet regimes. See text for description of diets. Black squares indicate the mean (\pm SE) time from molt to the 4th instar to the molt to the 5th instar. Therefore, the distance from each black square to the right-hand y-axis represents the duration of the 5th instar (*i.e.*, the age at adult molt). Diets varied in age at adult molt such that H = HL13 = HL8 < HL3 = L. The placement of these black squares even with 1,000 ng/ml on the y-axis was arbitrary. Data from Hatle *et al.* (2003b).

is <5% of the mean maximum titer. In contrast, 40 ng/ml (twice the detection minimum, the lowest amount that we were confident in testing) did not have this characteristic of a threshold; diet treatment significantly affected time from 40 ng/ml to the adult molt. The physiological actions of ecdysteroids in molting processes are well characterized (Truman, 1985; Richter, 1999 and references therein). Hence, our data imply that the canalized phase (from 100 ng/ml threshold to adult molt) is the time needed for these physiological processes, including gene activation, protein synthesis, and function of proteins, to proceed.

The search for general patterns in insect development

Taken together, these results suggest similar temporal limitations in insect developmental systems that are directed by hormones. Most aspects of non-embryonic development in arthropods appear to follow this pattern of plasticity early and canalization late, although few have studied the physiology associated with these switches. For example, larval development in mosquitoes (Bradshaw and Johnson, 1995), development to maturity in water fleas (Ebert, 1994), timing of metamorphosis in the copepod *Mesocyclops edax* (Twombly, 1996), and development to oviposition (Moerhlin and Juliano, 1998; Hatle *et al.*, 2000) and to metamorphosis (Flanagin *et al.*, 2000; Hatle *et al.*, 2003b) in lubber grasshoppers all are plastic early and canalized late. At least one insect does not fit this pattern of canalized physiology before a major developmental event, demonstrating that exceptions to this general pattern are possible. After reaching a threshold mass, dung beetles (*Onthophagus taurus*) actually speed their development upon starvation (Shafiei *et al.*, 2001). Hence, canalized phases before major developmental events may be common, but not universal, in arthropods.

Earlier in this paper, we hypothesized that when large amounts of a limiting resource are required for development to continue (*e.g.*, to attain a threshold), evolution toward more flexible development might be constrained. What might the role of hormones be in this putative constrained evolution (Hodin, 2000; Ketterson and Nolan, 1999)? It seems to us that the gene-level actions of the hormones (*e.g.*, gene activation, translation) may often be a component of canalized phases, because these processes require time for completion. However, a developmental phase is not necessarily canalized simply because it is directed by a gene-activating hormone. The magnitude of the developmental event might also contribute to canalization. Lubbers, which lay large clutches, have an extended canalized phase; crickets that lay eggs singly may not. For example, in egg production, converting the stored protein to egg protein, and then transporting that protein into the oocytes is a major physiological activity. In lubber grasshoppers, a female must produce and transport ~10% of her body weight in Vg. (The corresponding tasks in development to the adult molt would be the reorganization of tissues.) An additional important role of hormones in canalized phases might be to initiate the developmental event. Together, these 2 factors (*i.e.*, hormonal initiation of a developmental event and the amount of protein to be synthesized, transported, or reorganized) could produce extended inflexible phases in egg production. In turn, these canalized phases may constrain evolution by limiting the number of phenotypes that can be produced developmentally (Schlichting and Pigliucci, 1998). Rigorous evidence supporting this model would

be an important contribution linking cell and life-history mechanisms.

ACKNOWLEDGMENTS

We thank Doug Whitman for his advice throughout the course of this research, anonymous reviewers for their helpful comments, and our co-authors of studies included in this review: Amanda Andrews, Michael Crowley, Melanie Eskew, Greg Moerhlin, and Wyatt Miller. Funding was provided by National Science Foundation grants BIR-9510979 and DBI-9978810 to DWB, SAJ, and Doug Whitman.

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TITLE: Plasticity and Canalization in the Control of
Reproduction in the Lubber Grasshopper
SOURCE: Integr Comp Biol 43 no5 N 2003
WN: 0330507521005

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