

# Juvenile hormone is a marker of the onset of reproductive canalization in lubber grasshoppers

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## Abstract

To meet the challenge of unpredictable environments, many animals are initially developmentally flexible (plastic) but then may become inflexible (canalized) at major developmental events. The control of reproductive output can undergo a switch from flexible to inflexible (Moehrlin, G.S., Juliano, S.A., 1998. Plasticity of insect reproduction: testing models of flexible and fixed development in response to different growth rates. *Oecologia* 115, 492–500), and juvenile hormone (JH) may control this switch. By manipulating food availability, we tested the hypothesis that JH is involved in the reproductive canalization that appears during oogenesis in lubber grasshoppers. We used four food treatments: (1) high (H); (2) high switched to low (HL); (3) low switched to high (LH); and (4) low (L). We collected hemolymph samples approximately every 4 days and measured the ages at which maximum JH level ( $JH_{max}$ ) and oviposition occurred. Diet significantly affected both age at  $JH_{max}$  and age at oviposition. In contrast, diet had no significant effect on the time from  $JH_{max}$  to oviposition nor on the maximum JH level observed. Our data demonstrate that, after  $JH_{max}$  is reached, the time to oviposition in our grasshoppers was unresponsive to food availability. Hence, reproductive timing appears to be canalized after the  $JH_{max}$ . This is the first demonstration in a phytophagous insect that a particular factor (in this case, JH) can be used to mark the switch from reproductive plasticity to reproductive canalization. Published by Elsevier Science Ltd.

*Keywords:* Phenotypic plasticity; Radioimmunoassay; Developmental reaction norms; Reproductive timing; *Romalea microptera*; Patency

## 1. Introduction

Many organisms meet the challenge of unpredictable environmental conditions by remaining developmentally flexible when challenged with poor environmental conditions (Callahan et al., 1997; Nijhout, 1999). This is known as phenotypic plasticity (developmental flexibility), the production of multiple phenotypes by one genotype, as a function of different environments (Stearns, 1992). Plasticity may be either adaptive or non-adaptive (examples in Scheiner, 1993) and this is a product of the developmental system of the organism (Schlichting and Pigliucci, 1998). Developmental, sensory, and endocrine systems all have inherent limitations, and these limitations may constrain plasticity (Schlichting and Pigliucci, 1998). The opposite of plas-

ticity is canalization (inflexibility), the production of a consistent phenotype by one genotype, in spite of differing environments (Stearns, 1992). Organisms may exhibit plasticity only in certain developmental phases (critical periods), with canalization in other developmental phases of their life history. During the critical periods of plasticity, the phenotype of the organism is sensitive to environmental change, but during periods of canalization, the phenotype of the organism is insensitive to environmental change. The physiological and endocrine mechanisms initiating canalization are of interest because they can shed light on the adaptive limits of organisms.

Hormones may play a particularly critical role in eliminating possible developmental responses (i.e. plasticity). A good example of this has recently been demonstrated in tadpoles. Hormones released in response to an environmental change have been implicated as the mechanism that controls the developmental onset of metamorphosis in desert tadpoles. This can be seen as a switch from flexible (remaining a tadpole and permitting growth

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to a larger size) to inflexible (initiating metamorphosis at a small size). In tadpoles of the desert spadefoot toad *Scaphiopus*, the onset of metamorphosis is induced by the reduction in depth of the ephemeral pond in which the tadpole lives (Denver et al., 1998). This environmental change induces metamorphosis via corticotropin-releasing hormone (Denver, 1997a,b). Once it has begun, the process of metamorphosis cannot be halted, even if the environment changes.

The control of reproductive tactics (e.g. timing, output) can also undergo a switch from a flexible to a fixed developmental pathway (Moehrlin and Juliano, 1998). This is arguably the most important developmental shift in an animal's life history because of the close relationship between reproductive tactics and fitness. We have recently shown that reproduction in insects can be highly, but not completely, flexible (Moehrlin and Juliano, 1998). Grasshoppers (*Romalea microptera*) that were fed limiting diets oviposited later and oviposited fewer eggs than did grasshoppers that were fed *ad libitum* diets, demonstrating flexibility. In contrast, grasshoppers that were fed *ad libitum* for the first 70% of the oviposition cycle, but then were switched to a limiting diet, oviposited at the same time and oviposited the same number of eggs as grasshoppers fed *ad libitum* throughout the oviposition cycle. By implementing the diet switch at several different times, Moehrlin and Juliano (1998) determined that the canalization of time to oviposition begins some time during the second quarter of the oviposition cycle. The canalization of the number of eggs to be oviposited begins some time during the third quarter of the oviposition cycle. Hence, the grasshopper's reproductive tactics are plastic during the first portion of the cycle but then become canalized and unresponsive to the environment.

Juvenile hormone (JH) is a likely candidate for controlling the switch from flexible to inflexible oogenesis in grasshoppers observed in previous studies (Moehrlin and Juliano, 1998). First, JH is known to initiate vitellogenesis and/or patency in many insects (Wheeler, 1996; Gäde et al., 1997; Wyatt, 1997). Second, corpus allatum activity (i.e. the rate of synthesis of JH) and JH titer are known to respond to feeding levels in orthopterans (Tobe and Chapman, 1979; Glinka et al., 1995). This suggests that variation in growth can produce variation in JH titers, which may in turn control reproductive timing and output.

To ascertain whether JH is involved in reproductive canalization, it is important to determine the relationship between JH and reproductive tactics (see Zera et al., 1998). Reproductive tactics, such as time until oviposition, number of eggs, and number of resorbed oocytes, are the primary measures of whether or not the individual's reproductive output was altered by a changing environment. Specifically, to implicate JH in the canalization of reproduction, we must have information

on how JH titer and the individual's reproductive tactics jointly respond to a changing environment.

We used the Eastern lubber grasshopper, *Romalea microptera* (= *guttata*), as a model to study the role of JH in reproductive canalization. Lubbers are an excellent model for these studies for several reasons. First, they are large insects (gravid females weigh 4–8 g), allowing us to bleed serially adult females without halting their oviposition cycle. Second, their first oviposition cycle (35–40 d) appears to be composed of two periods, with somatic growth first and then reproductive growth. Thus, during the first ~15 d there is no growth in primary oocyte length despite a ~50% increase in body mass (D. Otto and D.W. Whitman, unpublished data). Hence, we suspect that few food reserves from the larval stages are used for reproductive output, but instead resources devoted to reproduction are acquired during adult life. This eases the manipulation of food resources allocated to reproduction. Third, around 20 d, JH titers increase dramatically, and then drop rapidly shortly before oviposition (Eskew et al., 1997), suggesting that reproductive processes begin at this time. Finally, lubbers are univoltine; in the wild they typically lay 2–4 clutches, each of which contains up to 50 eggs (D.W. Whitman, personal communication). Hence, both the age at oviposition and the number of eggs laid can have profound effects on realized fecundity in nature.

We tested the hypothesis that JH is involved in the reproductive canalization that occurs during the oviposition cycle in lubber grasshoppers. We manipulated food availability and measured oviposition parameters and JH titers.

## 2. Methods

### 2.1. Experimental animals

We used newly eclosed adult female *R. microptera* from our laboratory colony which originated with individuals collected near Copeland, FL, USA. We isolated females on the day of adult ecdysis, housed them in individual 500-ml ventilated plastic containers, and then assigned each to one of four food treatment groups (Table 1). Grasshoppers fed a high food meal never completely consumed their meals; in contrast, grasshoppers fed a low food meal almost always completely consumed their meals. We measured femur length for use as a size covariate. All grasshoppers were kept on a 14L:10D photoperiod with a 32:24°C thermocycle.

### 2.2. JH profiles

We collected hemolymph samples from each grasshopper once during each of the first 2 weeks of the cycle and then twice each week until oviposition. At each sam-

Table 1.

Feeding regimes of adult female *Romalea microptera* (*guttata*) during their first oviposition cycle. Each day, each grasshopper was fed the indicated amount of fresh Romaine lettuce and oatmeal.

Treatment group	Before switch	Switch	After switch
High (H)	10 g+0.15 g	Never	
High–low (HL)	10 g+0.15 g	Day 25	1.5 g+0.02 g
Low–high (LH)	1.5 g+0.02 g	Day 25	10 g+0.15 g
Low (L)	1.5 g+0.02 g	Never	

pling period, we collected 10  $\mu$ l with microcapillary tubes from punctures in the intersegmental neck membrane. Hemolymph samples were immediately added to 0.5 ml acetonitrile. Within 2 h of sampling, 1.0 ml of 0.9% NaCl was added and the sample was extracted twice with 1.0 ml hexane. The pooled hexane fractions were stored at  $-20^{\circ}\text{C}$  until analysis by the radioimmunoassay (RIA) for JH III (Hunnicuttt et al., 1989; Huang et al., 1994).

At the time of analysis, we dried each hexane extract and resuspended it in 30  $\mu$ l methanol. We first added about 4500 dpm [ $^3\text{H}$ ] JH-III (specific activity=20.6 TBq/mmol) to each RIA tube in 100  $\mu$ l gel-PBST (137 mM NaCl; 2.7 mM KCl; 4.3 mM  $\text{Na}_2\text{HPO}_4$ ; 1.4 mM  $\text{KH}_2\text{PO}_4$ ; 0.1% gelatin; 0.01% Triton X-100; pH 7.2). Then, 2  $\mu$ l of the methanol resuspension of an unknown sample was added to one of the assay tubes. Next, 100  $\mu$ l of a rabbit anti-10R-JH III serum (1:25 000) diluted in gel-PBST were added to each assay tube. The reaction mixtures were vortexed and then incubated for 2 h at room temperature. After the incubation period, the samples were chilled on ice for 5 min and then 0.5 ml of cold, stirred dextran-coated charcoal (5 mg dextran; 5.6 mg EDTA; 0.2 mg sodium azide; 1.0 ml PBS; 100 mg charcoal; 24 ml  $\text{dH}_2\text{O}$ ) was added to each tube. The samples were incubated for 2 min and then centrifuged at 2000g at  $4^{\circ}\text{C}$  for 5 min. The supernatant (containing  $^3\text{H}$  JH III bound to the antibody but not free  $^3\text{H}$  JH) was poured into a scintillation vial and counted in a liquid scintillation counter. The radioactivity in each unknown sample was compared to a standard curve to determine the amount of JH III in the sample.

For our standard curve, we used a series of methanol dilutions of racemic JH III (Sigma Chemical Co., St Louis, MO, USA). The final concentrations of these dilutions ranged from 0 (=total bound) to 3000 pg. To calculate the amount of JH III in each unknown sample from this standard curve, the radioactivity from each sample was expressed as a percent of the radioactivity remaining in total bound tubes (= %TB). The standard curve was plotted as log JH versus %TB. This curve was fit to a quartic equation, and this equation was used to estimate the JH levels in each sample. Because this assay is chiral selective, we have expressed the results as ng 10R-JH III/ml hemolymph. We estimated that our detection limit was 50 ng 10R-JH III/ml hemolymph.

### 2.3. Oviposition parameters

After 30 d, we placed females in 1-liter plastic containers with sand containing 7% water for at least 1 h. We repeated this procedure at least every other day (usually every day) until the individual oviposited. Gravid *R. microptera* readily oviposit under these conditions (Stauffer et al., 1998). Upon oviposition, we weighed the grasshopper, recorded the age of the female, and counted the number of eggs laid. Occasionally, these grasshoppers lay only a portion of their eggs in one batch and then lay the remainder of the eggs the following day. To control for this, we froze the grasshoppers within 24 h after oviposition and later examined the carcass, especially the calyx, for full-sized eggs that had not been laid. In this paper we report total eggs (=eggs laid+full size eggs remaining in the carcass). We also counted the ovarioles and resorbed oocytes. Resorbed oocytes are distinguishable by their orange resorption body (Moehrlin and Juliano, 1998; Stauffer and Whitman, 1998).

### 2.4. Statistical analysis

We first tested whether female size (femur length) as a covariate accounted for significant variation in time from eclosion to oviposition, maximum level of JH, number of eggs, and number of resorbed oocytes. It did so only for number of eggs, hence for that variable we used analysis of covariance (ANCOVA) to test the response variables. When female size was not significantly related to the response variable, we used a one way analysis of variance (ANOVA). When treatment effects were significant, we used Bonferroni pairwise comparisons of least square means (ANCOVA) or Ryan–Einot–Gabriel–Welsh multiple range tests (ANOVA) to determine which diet treatments differed. These statistical analyses were conducted using SAS (SAS Institute Inc., 1989).

## 3. Results

### 3.1. Grasshopper survivorship

There was no significant difference in survivorship among the four diet treatment groups (Chi-square test;

$t^2=1.65$ ;  $df=3$ ;  $P=0.648$ ). The ratio of grasshoppers remaining at the end of the study to grasshoppers at the beginning of the study for each group was: H, 15/26 (58%); HL, 5/11 (45%); LH, 7/10 (70%); L, 13/26 (50%). Six grasshoppers in the L group were still alive but had not oviposited by day 98 when the study was terminated; all other grasshoppers classified as ‘finishing the study’ finished by ovipositing. We infer that our diet treatments were not drastic enough to produce differences in survivorship.

### 3.2. JH profiles

All grasshoppers had JH profiles that were low throughout the cycle except for a single peak of JH in the second half of the cycle (Fig. 1). To simplify the data analysis, we focused on two aspects of the JH profiles: the individual’s maximum level of JH ( $=JH_{max}$ ) and the time at  $JH_{max}$ .

Diet significantly affected the time from eclosion to  $JH_{max}$  in our grasshoppers (Fig. 2; ANOVA;  $F_{3,29}=43.15$ ;  $P<0.0001$ ). High ( $n=15$ ) and HL ( $n=5$ ) grasshoppers had no difference in the time to  $JH_{max}$ , with  $22\pm 0.6$  d and  $23\pm 1.2$  d respectively (mean $\pm$ SE). Low-high ( $n=7$ ) grasshoppers had a significantly later  $JH_{max}$  at  $33\pm 2.9$  d, and L grasshoppers ( $n=7$  that actually laid eggs) had  $JH_{max}$  at  $49\pm 3.1$  d, which was significantly later than all other treatments. In summary, the timing of  $JH_{max}$  followed the order  $H=HL<LH<L$ .

The  $JH_{max}$  levels were log transformed to meet assumptions of ANOVA and then tested with ANOVA. Diet did not significantly affect the level of  $JH_{max}$  (Table

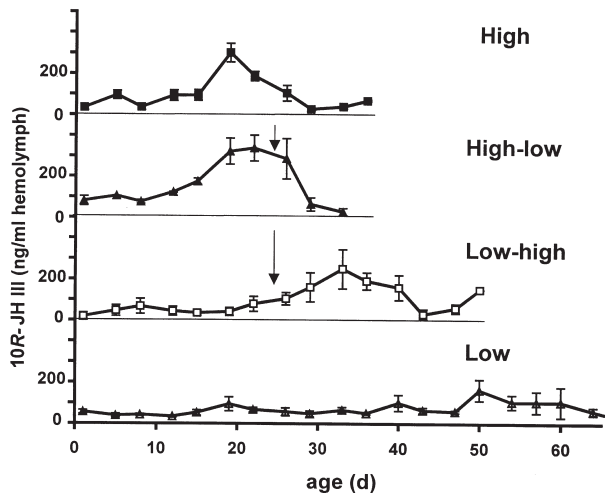


Fig. 1. Hemolymph juvenile hormone profiles (mean $\pm$ SE) for adult female lubber grasshoppers on four diet treatments. See Table 1 for explanation of diets. Hemolymph samples were collected approximately every 4 days. Each mean $\pm$ SE represents all samples from the given treatment group for a 3- or 4-day period (e.g. day 1 represents samples from days 1–4 inclusive). The vertical arrows indicate the date of diet switches for both HL and LH fed grasshoppers.

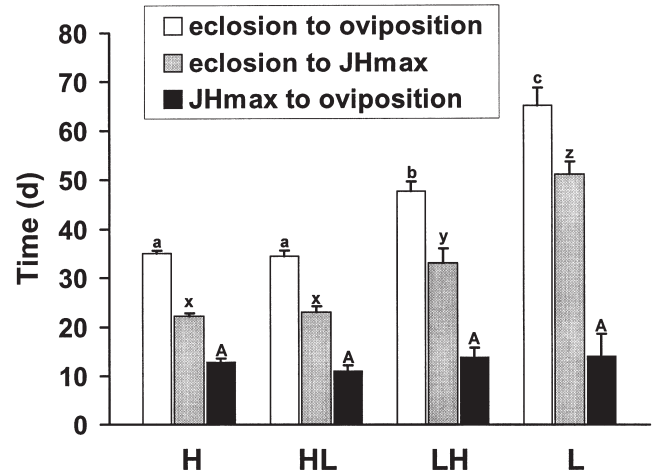


Fig. 2. Durations (mean $\pm$ SE) of developmental periods in adult female lubber grasshoppers on four diet treatments. See Table 1 for explanation of diets.  $JH_{max}$  is defined as the sampling date with the highest JH titer for an individual grasshopper. Letters represent statistical differences within a developmental period.

2;  $F_{3,29}=1.97$ ;  $P=0.139$ ). The timing of the  $JH_{max}$  within the LH and L grasshoppers was highly asynchronous; hence, the averaged data (Fig. 1) create the illusion of a flatter, broader  $JH_{max}$  in LH and L grasshoppers. In fact, each individual grasshopper had a high  $JH_{max}$  but the timing of these peaks varied considerably.

### 3.3. Time to oviposition

Diet significantly affected time from eclosion to oviposition (Fig. 2; ANOVA;  $F_{3,29}=80.59$ ;  $P<0.0001$ ). High and HL grasshoppers did not differ significantly in time to oviposition. In other words, limiting the diet during the last 30% of the oviposition cycle diet did not delay the time of oviposition. Low-high grasshoppers oviposited significantly later than H and HL, and L grasshoppers oviposited still later than LH (Fig. 2). Because six L grasshoppers did not oviposit by day 98 (and were not included in this analysis), the mean time to oviposition in L grasshoppers (65 d) may be an underestimate.

In contrast to the significant effects of diet on times from eclosion to  $JH_{max}$  and to oviposition, diet had no significant effect on the time from  $JH_{max}$  to oviposition (Fig. 2; ANOVA;  $F_{3,29}=0.21$ ;  $P>0.886$ ). Hence, after  $JH_{max}$ , time until oviposition appeared to be unresponsive to food availability.

### 3.4. Ovariole, total egg, and resorbed oocyte numbers

There were no significant differences in ovariole number among the four diet groups (Fig. 3;  $F_{3,29}=1.89$ ;  $P=0.153$ ). Number of eggs was significantly and positively affected by size ( $F_{1,28}=5.26$ ;  $P<0.0295$ ), so we tested these data using an ANCOVA with femur length



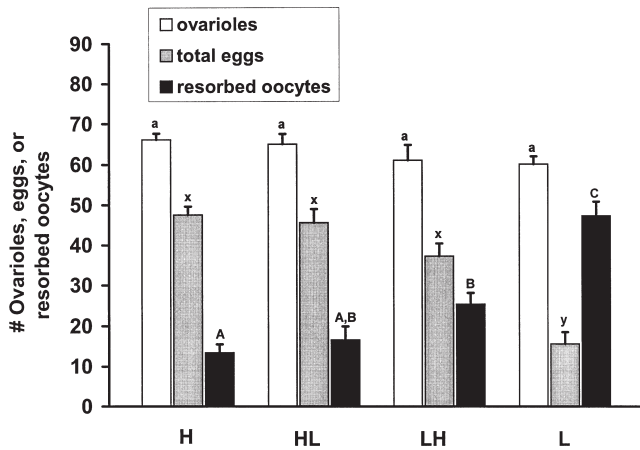


Fig. 3. Numbers (mean±SE) of ovarioles, total eggs, and resorbed oocytes in adult female lubber grasshoppers on four diet treatments. See Table 1 for explanation of diets. Letters represent statistical differences with a response variable.

as the covariate. Diet significantly affected the number of eggs produced (Fig. 3;  $F_{3,29}=27.28$ ;  $P<0.001$ ). High fed grasshoppers produced similar numbers of eggs to HL grasshoppers ( $P=0.9661$ ), marginally more eggs than LH grasshoppers ( $P=0.0739$ ), and significantly more eggs than L grasshoppers ( $P<0.0001$ ). High–low fed grasshoppers had similar eggs to LH grasshoppers ( $P=0.2721$ ) and significantly more eggs than L grasshoppers ( $P<0.0001$ ). Low–high fed grasshoppers had significantly more eggs than L grasshoppers ( $P<0.0001$ ).

Diet significantly affected the number of oocytes resorbed (Fig. 3; ANOVA;  $F_{3,29}=28.91$ ;  $P<0.0001$ ). High fed grasshoppers had similar numbers of resorbed oocytes compared to HL grasshoppers but significantly fewer resorbed oocytes than LH and L grasshoppers. High–low fed grasshoppers had similar numbers of resorbed oocytes to LH grasshoppers but significantly fewer resorbed oocytes than L grasshoppers. Low–high fed grasshoppers had significantly fewer resorbed oocytes than L grasshoppers.

#### 4. Discussion

We have identified a marker for the switch from reproductive plasticity (i.e. flexibility) to canalization (i.e. inflexibility), namely, the peak in hemolymph JH. Our results are consistent with previous research (Moehrlin and Juliano, 1998) and suggest that timing of insect reproduction is flexible during the early stages of the oviposition cycle, but is inflexible in response to food availability during the last 35% of the cycle, and perhaps sooner. The  $JH_{max}$  appears to be at least correlated with the onset of canalized events that lead up to oviposition. We believe this to be the most direct evidence for a hormonally regulated switch from plastic to canalized repro-

duction. This is the first demonstration in a phytophagous insect that a particular factor (in this case, JH) can be used to mark the switch from reproductive plasticity to reproductive canalization.

Such a role for insect hormones has been shown in canalized development toward metamorphosis (Riddiford, 1981; Riddiford, 1996) and has been incorporated into general models of insect juvenile development (Bradshaw and Johnson, 1995). That JH may play similar roles during important developmental transitions in two life-cycle phases is not surprising; it suggests that insect developmental systems may be prone to critical periods of environmental sensitivity followed by canalization (Nijhout, 1994).

#### 4.1. JH profiles

JH profiles in our grasshoppers were affected by diet, and presumably by growth rate. JH levels in H grasshoppers were initially low and then rose to  $JH_{max}$  at  $22\pm0.6$  d (Fig. 1). Low–high fed grasshoppers had low levels of JH at 22 d, but then after the increase in food at 25 d, the JH peaked at  $33\pm2.9$  d at levels similar to those in H grasshoppers. Low fed grasshoppers showed low levels of JH until 36 d, but then showed a  $JH_{max}$  at  $49\pm3.1$  d (Table 2). Hence, the ability of our grasshoppers to increase JH to maximal levels (i.e. levels attained by H grasshoppers) remained plastic at least until 36 d (as demonstrated by L grasshoppers). Both the shape of the JH profiles of our grasshoppers and the ability of JH levels to respond to diet are consistent with previous work on *Locusta migratoria* using ad libitum and ‘standard fed’ grasshoppers (Glinka et al., 1995). This plasticity prior to the  $JH_{max}$  was in direct contrast to the canalization of JH levels seen after  $JH_{max}$ . In general, JH titers in grasshoppers on all four diets dropped dramatically after  $JH_{max}$ .

#### 4.2. Timing of $JH_{max}$ and oviposition

Diet significantly affected time from eclosion to oviposition and time from eclosion to  $JH_{max}$  (Fig. 2). In contrast, diet had no effect on the time from  $JH_{max}$  to oviposition. Hence, time until reproduction was unresponsive to food availability after  $JH_{max}$ . In other words, when the oviposition cycle is split into time before  $JH_{max}$  and time after  $JH_{max}$ , the time before  $JH_{max}$  appears to explain nearly all the influence of diet on time from eclosion to oviposition. The time after  $JH_{max}$  does not contribute to the explanation of variability among diet treatments. This is further, and stronger, evidence that the timing of oogenesis is flexible before  $JH_{max}$  but inflexible after  $JH_{max}$ .

Table 2.

Means ( $\pm$ SE) of the maximum levels, in ng/ml, of 10R-JH III for each food treatment group. There are no significant differences among the groups (ANOVA;  $P=0.139$ ). See Table 1 for explanation of diet regimes.

	H	HL	LH	L
JH <sub>max</sub>	272 $\pm$ 30.1	405 $\pm$ 60.3	363 $\pm$ 78.4	234 $\pm$ 50.7
log JH <sub>max</sub>	2.40 $\pm$ 0.05	2.58 $\pm$ 0.08	2.50 $\pm$ 0.10	2.28 $\pm$ 0.11

#### 4.3. Number of ovarioles and resorbed eggs

Our data on ovariole and egg numbers are consistent with Moehrlin and Juliano (1998) that reproductive allotment is plastic early in oogenesis but becomes canalized later in oogenesis. The number of ovarioles appeared to be unresponsive to diet during the adult life (Fig. 3). In contrast, number of eggs produced was highly plastic in response to diet, varying three-fold between H and L grasshoppers. The number of resorbed oocytes also appeared to be highly plastic in response to diet, varying over three-fold between H and L grasshoppers. The trend in total eggs was opposite the trend in resorbed oocytes, and the variability among diet treatments in these two response variables was similar. Hence, one mechanism contributing to the plasticity in number of eggs may be resorption of eggs by ovarioles in response to feeding rate (Moehrlin and Juliano, 1998). Late in the oviposition cycle, the lack of responsiveness of number of eggs laid to changes in feeding suggests that at a certain point, females make an irreversible commitment to resorb certain oocytes (and develop other oocytes into full eggs). The mechanism(s) of this resorption, including the relevant signals that determine the number of oocytes resorbed, and the mechanism(s) by which individual oocytes are selected for resorption, are important questions for future research.

Taken together, these data have interesting implications for the mechanism(s) that determine the number of eggs produced in an oviposition cycle. Our data suggest that, shortly before or during the time of JH<sub>max</sub>, some factor(s) acts on the ovarioles to destine some for yolking (patency) and others for resorption. This factor does not seem to be JH, because our data suggest that the level of JH<sub>max</sub> does not predict the number of eggs that are produced. Instead, JH may act as an 'on/off switch'. When we regressed the level of JH<sub>max</sub> against egg number, the relationship was weak ( $r^2 < 0.20$ ) and not significant. We hypothesize that the number of eggs laid is correlated with the maximal levels of total hemolymph protein.

We are currently examining the hypothesis that JH initiates reproductive canalization and therefore controls the time to oviposition. Our results show that JH<sub>max</sub> correlates with the canalized chain of events ending in oviposition. They do not, however, provide any evidence that the JH<sub>max</sub> initiates this canalized series of events.

We intend to test the hypothesis that JH initiates the canalized events by using precocious applications of JH and the JH analog methoprene and determining whether this manipulation induces early oviposition.

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