

## Hemolymph ecdysteroids do not affect vitellogenesis in the lubber grasshopper

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Ecdysteroids in lubber grasshopper oogenesis

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**ABSTRACT**

The role of hemolymph ecdysteroids in the reproduction of non-dipteran insects is unclear. Recent reports suggest that hemolymph ecdysteroids may stimulate vitellogenesis in locusts. In this paper, we examine the role(s) of hemolymph ecdysteroids during egg production in the lubber grasshopper, Romalea microptera. In all individuals, hemolymph ecdysteroids rose to a sharp peak with similar maxima and then fell to undetectable levels. The time from the adult molt to the maximum ecdysteroid titer ( $E_{\max}$  titer) varied in response to food availability, whereas the time from  $E_{\max}$  titer to oviposition was unrelated to food availability. Because both the timing of egg production and the timing of  $E_{\max}$  responded similarly to environmental changes, ecdysteroids may be involved in egg production. We hypothesized that this role is the stimulation of vitellogenesis, as in Locusta. Ovariectomized females had vitellogenin but no ecdysteroids, so ecdysteroids are not necessary for vitellogenin production. In addition, treatment of females with ecdysteroids did not consistently elevate Vg titers and never stimulated ovarian growth. Ovarian ecdysteroids increased at the same age in development as hemolymph ecdysteroids. In contrast to hemolymph ecdysteroids, ovarian ecdysteroids persisted until oviposition. Despite this, [ $^3\text{H}$ ]ecdysone injected into the hemolymph was detected later at very low levels in the ovary, suggesting hemolymph ecdysteroids are not sequestered by the ovary. In summary, our studies indicate that hemolymph ecdysteroids in adult females of the lubber grasshopper are associated with the timing of egg production, but they neither regulate vitellogenesis nor act as a source of the ecdysteroids for the ovary.

**Keywords:** oocyte development; phenotypic plasticity; comparative physiology

## INTRODUCTION

The role of hemolymph ecdysteroids in the reproduction of non-dipteran insects is unclear (cf. Perriere et al. 1993; Gaede et al. 1997; Tawfik et al. 1997). For orthopterans, vitellogenin synthesis appears to be controlled largely by juvenile hormone (JH; Engelmann 1983; Wyatt and Davey 1996). However, some evidence suggests that hemolymph ecdysteroids stimulate vitellogenesis and egg production (e.g., Girardie et al. 1992; 1996; 1998). Treatment with ecdysteroids stimulated vitellogenesis in both locusts (Girardie and Girardie 1996) and decapitated cockroaches (Perriere et al. 1993), and increased lifetime fecundity by 250% in crickets (Behrens and Hoffmann 1983). Likewise, treatment of crickets with compounds that could potentially inhibit ecdysteroid synthesis significantly reduced lifetime fecundity (Hoffmann et al. 1996). Hydroprene (a JH analog) and 20-hydroxyecdysone together increased fecundity in allatectomized crickets to levels similar to control crickets (Hoffmann et al. 1996).

One method of analyzing the developmental role of a hormone is to determine whether its titer (either the level or timing) is developmentally plastic. Many insects can respond to environmental changes via phenotypic plasticity, the developmental ability to produce multiple phenotypes (e.g., different ages at reproduction) from one genotype (Stearns 1992; Nijhout 1999). Such developmental responses are often controlled by hormones (Nijhout 1994; Denver 1997a;b; Hatle et al. 2000). However, not all developmental events are plastic. Endocrine systems have inherent limitations (Schlichting and Pigliucci 1998), such as a relatively slow mechanism of action (cf. neural responses) or a strictly defined response that might not be subject to alteration once begun. A possible consequence of these limitations is canalization, the production of consistent phenotypes by similar genotypes, in spite of different environments (Stearns 1992). Little is known about the physiological control of plastic and canalized phases or the physiological events that define these periods (Garland and Carter 1994).

Reproductive timing and output of the Eastern lubber grasshopper (*Romalea microptera*) show phases of plasticity and canalization. Moehrlein and Juliano (1998) showed that the lubber grasshopper was initially flexible in time to oviposition, but then entered a phase of fixed development. During the canalized phase, first the time to oviposition and then the number of eggs laid became unresponsive to changes in feeding rate. In well-fed female lubbers, vitellogenin is first present at age ~10 days, increases steadily until a peak at ~22 days, and falls to low levels a few days before oviposition at age ~35 days (Borst et al. 2000). Juvenile hormone levels are relatively low until age ~17 days, reach their highest levels at ~22 days, and return to low levels before oviposition (Hatle et al. 2000). Between the adult molt and first oviposition, maximal levels of both vitellogenin (Vg; Hatle et al. 2001) and JH (Hatle et al. 2000) occur during this canalized phase. The time from the adult molt to these maxima varies with feeding rate, whereas the time from these maxima to oviposition is fixed. The role of ecdysteroids in the egg production of this species remains uninvestigated. If both the timing of egg production and the timing of hemolymph ecdysteroid profiles respond similarly to feeding rates, then ecdysteroids may be involved in egg production.

The natural history and behavior of lubber grasshoppers is very different from that of migratory locusts (Whitman 1990; Yosef and Whitman 1992; Hatle and Faragher 1998). Lubbers are flightless, sluggish, and gregarious. Further, there is evidence that these two orthopterans respond to certain hormones differently. Lubber grasshoppers were unresponsive to adipokinetic hormones (AKHs) at developmental stages during which locusts are hyperlipemic to AKHs (Gaede and Spring 1989; Hatle and Spring 1998). Therefore, the mechanisms of egg production

of lubbers, and specifically the role of ecdysteroids in regulating these processes, may also be different from those of locusts.

In this paper, we examine the role(s) of hemolymph ecdysteroids during egg production of the lubber grasshopper. Our central hypothesis is that hemolymph ecdysteroids stimulate vitellogenesis in lubbers, as in locusts. We address four questions. First, are maximal levels of hemolymph ecdysteroids attained in female lubbers during the plastic phase or the canalized phase of egg production? Second, are hemolymph ecdysteroids necessary for vitellogenesis? Third, do ecdysteroids increase vitellogenin levels in female lubbers? Fourth, is the hemolymph a source of ecdysteroids for the ovary?

## MATERIALS AND METHODS

### Experimental animals

We obtained adult females of *R. microptera* from our laboratory colony and maintained them using rearing methods described previously for Western lubbers (Whitman 1986). Briefly, juveniles were reared *en masse*, fed Romaine lettuce and dry oats *ad libitum*, and isolated on the day of adult molt. During experiments, all grasshoppers were kept on a 14L:10D photoperiod with a 32:24°C thermocycle and housed in individual 500 ml ventilated plastic containers.

### Experiment 1: Timing of hemolymph ecdysteroids

Each animal was assigned to one of four treatment groups defined by its food rations: high (H), high-low (HL), low-high (LH), and low (L). High rations were 10 g Romaine lettuce and 0.15 g oats daily, and low rations were 1.5 g lettuce and 0.02 g oats daily. We switched the diets for animals in the HL and LH groups abruptly at age 25 days from high to low or from low to high rations respectively. Sample sizes were: H = 11, HL = 5, LH = 7, L = 8. Grasshoppers fed a high ration never completely consumed their daily meal. Grasshoppers fed a low ration almost always completely consumed their daily meals. We measured femur length, which is an estimate of body size, for use as a size covariate. The JH profiles (Hatle et al. 2000) and Vg and total non-vitellogenin hemolymph protein (=TP) profiles (Hatle et al. 2001) for the individual grasshoppers used in Experiment 1 have been previously reported.

We collected hemolymph samples from each grasshopper about twice each week until oviposition. Animals that did not oviposit by 98 days were omitted from the study. At each sampling period, we collected 10 µl hemolymph and immediately placed the sample in 0.5 ml acetonitrile. Within 2 h of sampling, 1.0 ml of 0.9% NaCl was added and the sample was extracted twice using 1.0 ml hexane for each extraction. The combined hexane supernatants were used for JH analysis (reported in Hatle et al. 2000) and the acetonitrile/saline lower phase was stored at -20°C until analyzed for ecdysteroids by RIA (Borst and O'Connor 1972; 1974).

Each sample was prepared for RIA by diluting it to a total volume of 2 ml with water and then drying two 400 µl aliquots. We resuspended the dried aliquots in 200 µl borate buffer (0.05 M boric acid; 0.9% NaCl; 0.1% gelatin; 0.05% Triton X-100; 7.7 mM NaAzide; pH 8.4) with approximately 4000 dpm [<sup>3</sup>H]ecdysone (ecdysone, α - [23,24,-<sup>3</sup>H(N)]; NEN; Boston; specific activity = 1.9 TBq/mmol) and a mouse monoclonal anti-ecdysteroid antibody (diluted 1:20000). After incubating these tubes for 2 h at room temperature, we chilled them at 4°C for at least 5 min and added 0.5 ml of cold, stirred, dextran coated charcoal (2.5 mM boric acid; 0.045% NaCl; 12.5 mg dextran; 38 µM EDTA; 7.7 µM NaAzide; 0.5 g charcoal; pH 8.4). After 5 min, the samples were centrifuged at 2000g at 4°C for 5 min. The supernatant (containing [<sup>3</sup>H]ecdysone bound to the antibody) was counted.

The antibody used for these studies was prepared in this laboratory and has not been described. This antibody binds ecdysone (=  $\alpha$ -ecdysone) about twice as strongly as 20-hydroxyecdysone ( $\beta$ -ecdysone or ecdysterone). We produced a standard curve by repeating the RIA procedure with standards containing 20-hydroxyecdysone (Sigma Chemical Co., St. Louis, MO, USA). All data in this paper are reported as 20-hydroxyecdysone equivalents and are referred to simply as “ecdysteroids”. For Experiment 1 our typical detection minimum was ~10 ng/ml hemolymph (~20 pg/tube).

We determined  $E_{\max}$  titers simply by comparing all the ecdysteroid titers from adult molt until oviposition for an individual and identifying the highest observed titer. These individual maxima were distinct (Fig. 1). For statistical analysis, we first used ANCOVA to determine whether variation in female size (i.e., femur length) accounted for any variation in each data set. Female size was a significant covariate only for age at  $E_{\max}$ , so we used ANOVA for all other variables. When necessary, data were transformed to meet the assumptions of homogenous variance and normality. We tested for differences among the diet treatments in: 1) age at  $E_{\max}$  titer; 2) time from  $E_{\max}$  titer to oviposition; 3)  $E_{\max}$  titer. When treatment effects were significant, we used Ryan-Einot-Gabriel-Welsch multiple range tests (SAS Institute Inc. 1989) to determine which diet treatments differed. In the case of time from  $E_{\max}$  to oviposition, we were unable to meet the assumption of homogenous variance, so these data were also analyzed using a randomization ANOVA to verify our conclusions.

Because ecdysteroid profiles and oviposition parameters were determined for each individual (Hatle et al. 2000), we were able to test the prediction that  $E_{\max}$  titer and number of eggs produced are correlated. The size of an individual egg appears to be unrelated to food availability (Moehrli and Juliano 1998), so simply counting the number of eggs is sufficient for determining variation in reproductive output. A relationship between  $E_{\max}$  titer and egg number would be consistent with the suggestion that hemolymph ecdysteroids are associated with egg production. Such an association could be due to several mechanisms, including ecdysteroid stimulation of vitellogenesis (Girardie et al. 1996) or the production of ecdysteroids by developing oocytes.

### **Experiment 2: Are hemolymph ecdysteroids necessary for vitellogenesis?**

We tested whether hemolymph ecdysteroids were necessary for vitellogenesis by removing the ovary, which is the source of ecdysteroids in females of many insect species. Zero- and one-day old adult females (n=5) were ovariectomized by removing one pair of wings and making a U-shaped incision on one side of the fourth abdominal segment. The entire ovary was removed, 25  $\mu$ g of gentamycin was placed in the cavity, and the wound was sealed with Instant Krazy Glue<sup>®</sup> (Elmer's Products, Inc., Columbus, OH, USA). Sham-operated control grasshoppers (n=5) were treated identically except some of the fat body and trachea were removed instead of the ovary. All grasshoppers were fed 2 g Romaine lettuce and 0.02 g oats daily. This diet quantity is limiting and delays reproductive development, but not to the same degree as 1.5 g lettuce and 0.02 g oats daily (i.e., the low diet from Experiment 1). Twice weekly, we collected 5  $\mu$ l hemolymph samples, placed them in 250  $\mu$ l hemolymph buffer (100 mM NaCl; 1 mM EDTA; 0.1 mM DTT; 0.15% Tween 20; 10  $\mu$ g/ml leupeptin; 10  $\mu$ g/ml PMSF in propanol; 50 mM Tris buffer; pH 7.5), and stored them at -20°C. We measured Vg by enzyme-linked immunosorbent assay (ELISA; Borst et al. 2000) and ecdysteroids by RIA. We determined  $Vg_{\max}$  for each individual correspondingly to the determination of  $E_{\max}$  in Experiment 1. The mean $\pm$ SE age at oviposition for the sham-operated females was 50 $\pm$ 1.6 days,

so we terminated the experiment when the grasshoppers were 55 days old. Dissections of these grasshoppers confirmed that each ovariectomy had been successful.

### **Experiment 3: Do ecdysteroids increase vitellogenin levels?**

We tested whether ecdysteroid treatment increased vitellogenin levels. All grasshoppers in Experiment 3 were fed ad libitum. In the first trial, grasshoppers were treated daily with ecdysteroids for three consecutive days, starting at age 12 days. This age is about 5 days before ecdysteroids are detectable in the hemolymph (see Fig. 2) and about 10 days before Vg levels reach their maximum. At age 12 days, we isolated individuals and collected hemolymph samples as per Experiment 2. Immediately thereafter, each grasshopper (n=6) was treated with 500 ng ecdysone plus 500 ng 20-hydroxyecdysone (Sigma Chemical Co., St. Louis, USA) in 50  $\mu$ l dH<sub>2</sub>O. Adult female lubbers have about 2 ml of hemolymph, so this dosage would approximate maximal levels observed in these animals (see Fig. 2). Controls (n=6) were treated with water only. This procedure was repeated at 13 days and again at 14 days. At 17 days, we collected a final hemolymph sample and froze the grasshoppers for later measurement of ovarian mass. All samples were stored at  $-20^{\circ}\text{C}$  until tested for Vg by ELISA.

In a second trial, grasshoppers were treated with this same dosage at age 18 days (n=6 for ecdysteroid-treated grasshoppers and n=7 for water-treated grasshoppers). At this age, ecdysteroids are first detectable in the hemolymph, and Vg levels reach their maximum about 5 days later. We collected hemolymph and treated with ecdysteroids or water at ages 18, 19, and 20 days. Final hemolymph samples were collected and then the grasshoppers were frozen at age 23 days.

In a third trial, we treated females three times daily from age 8 days through age 17 days with 1.34  $\mu$ g ecdysone plus 2.67  $\mu$ g 20-hydroxyecdysone (n=8 for both water- and ecdysteroid-treated grasshoppers). Thus, the daily ecdysteroid doses in trial three were 8-fold and 16-fold higher (for ecdysone and 20-hydroxyecdysone, respectively) than the doses in the first two trials. Females were frozen at age 17 days. This trial was designed to reflect the dosage schedule used by Girardie and Girardie (1996) for *Locusta*. We analyzed the data from each trial independently with one-way repeated-measures MANOVAs, with each sampling day as a unique response variable, blocked by individual (SAS Institute 1989).

### **Experiment 4: Is the hemolymph a source of ecdysteroids for the ovary?**

We examined the relationship of hemolymph and ovarian levels of ecdysteroids through egg development. Synchrony between these two profiles would suggest that the ecdysteroids reflect the same developmental event. Grasshoppers were reared identically to Experiment 1, except the low-food ration was 2.0 g Romaine lettuce and 0.02 g oats daily. High-fed grasshoppers were killed at 3, 8, 13, 18, 23, 28, and 33 days after the adult molt,  $\pm 1$  day for all age classes (mean $\pm$ SE sample size per cohort =  $5.1\pm 0.5$ , range 3-8). Low-fed grasshoppers were killed at 10, 20, 30, 40, 50, 60, 70, and 80 days after eclosion,  $\pm 1$  day for all age classes (sample size per cohort =  $5.5\pm 0.5$ , range 4-8). On the final day, we collected a hemolymph sample from each individual as in Experiment 2, and then weighed the ovaries and stored them in methanol at  $-20^{\circ}\text{C}$ . Later, each ovary was homogenized in methanol using a Potter-Elvehjem tissue grinder with a teflon pestle. The homogenates were centrifuged (2000g at  $4^{\circ}\text{C}$  for 1 min) and the supernatant was transferred to a clean tube. We extracted the samples using chloroform, methanol, and water in a ratio of 2:1:0.6. After equilibration for 5 min on ice, the upper, aqueous phase was transferred to a clean tube, and the lower phase was extracted once more with 50% methanol. The combined upper layers recovered  $\sim 40\%$  of [ $^3\text{H}$ ]ecdysone added to representative samples. Finally, we analyzed the combined upper phases by RIA.

To test whether hemolymph ecdysteroids are sequestered by the ovary, we injected 19-21 day old females (fed *ad libitum*) with 80000 dpm [<sup>3</sup>H]ecdysone in 50 µl water (n=12). As a comparison, 11 grasshoppers were injected with 80000 dpm of [<sup>3</sup>H]water (NEN; Boston; specific activity = 37MBq/g) in 50 µl dH<sub>2</sub>O. The ovaries were harvested 4 days later, homogenized and extracted as above, and the combined upper layers were analyzed for radioactivity.

## RESULTS

### Experiment 1: Timing of hemolymph ecdysteroids

The ecdysteroid levels in each animal showed a sharp peak toward the end of the oviposition cycle (Fig. 1). In most individuals, ecdysteroids were only detectable in two samples (a range of 6-8 days). Group ecdysteroid profiles for H and HL grasshoppers were similar (Fig. 2). These grasshoppers typically had no detectable ecdysteroids until age 19 days, a peak of ecdysteroids at 22 days, and undetectable levels from 29 days to oviposition at about 35 days. Grasshoppers in the LH group had undetectable hemolymph ecdysteroids until 29 days, a peak at 36 days, and essentially undetectable levels from 43 days to oviposition at about 46 days. Low-fed grasshoppers had little detectable hemolymph ecdysteroids until 36 days. The L grasshoppers developed more asynchronously than other groups, resulting in an erratic group profile from 50 days to oviposition.

The age at  $E_{max}$  was significantly affected by diet (Fig. 3;  $F_{4,26} = 36.3$ ;  $P < 0.0001$ ). Ages at  $E_{max}$  for the H and HL groups (which did not differ from each other) were significantly less than both the LH and L groups. In addition, age at  $E_{max}$  for the LH grasshoppers was significantly less than for L grasshoppers. Regardless of diet regime, the age at  $E_{max}$  for a group was similar to its ages at  $JH_{max}$  (Hatle et al. 2000),  $Vg_{max}$ , and  $TP_{max}$  (Hatle et al. 2001). In contrast to age at  $E_{max}$ , the time from  $E_{max}$  to oviposition was not significantly affected by diet (ANOVA;  $P = 0.4122$ ), and this result was confirmed using a randomization ANOVA ( $P = 0.4101$ ). Thus, the timing of reproductive events was responsive to diet before  $E_{max}$ , but unresponsive to diet after  $E_{max}$ . The plasticity in the timing of ecdysteroid profiles was similar to the plasticity in timing of egg production (Moehrlin and Juliano 1998; Hatle et al. 2000). Hence, we searched for a role(s) of ecdysteroids in egg production and specifically hypothesized that they stimulate vitellogenesis.

The  $E_{max}$  titer for an individual was not correlated with the number of eggs produced by that individual (Fig. 4;  $P = 0.093$ ;  $r = 0.307$ ;  $n = 31$ ). Although  $E_{max}$  titers were significantly affected by treatment ( $F_{3,27} = 3.37$ ;  $P = 0.0329$ ), the only pairwise comparison of  $E_{max}$  titers that yielded a significant difference was HL vs. L. The diet treatments had the following  $E_{max}$  titers (mean±SE): H=710±150; HL=1260±300; LH=1000±120; L=500±130.

### Experiment 2: Are hemolymph ecdysteroids necessary for vitellogenesis?

Hemolymph levels of ecdysteroids in ovariectomized females were below the detection limit of the RIA (10 ng/ml). In contrast, sham-operated females had ecdysteroid profiles similar to the LH females from Experiment 1. Sham-operated grasshoppers had an average  $E_{max}$  titer of 550±210 ng/ml at 35±1.9 days after the adult molt. Despite the lack of detectable ecdysteroids, ovariectomized females produced vitellogenin. In fact, ovariectomized females had a mean  $Vg_{max}$  titer (99±52 mg/ml) that was fourfold higher than that of sham-operated females (25±11 mg/ml), although there was no significant difference between the two groups (Student's t-test;  $P = 0.220$ ).

### Experiment 3: Do ecdysteroids increase vitellogenin levels?

Ecdysteroid treatment *in vivo* did not consistently affect Vg titers. Females treated with a physiological dose of ecdysteroids for three consecutive days starting at age 12 days had higher Vg levels throughout the experiment than water-treated grasshoppers (Fig. 5A; repeated measures ANOVA;  $F_{1,10} = 9.18$ ;  $P = 0.0127$ ). However, none of the individual days showed a significant difference in Vg levels due to ecdysteroid treatment (all  $P > 0.10$ ). There was no significant effect of time (MANOVA; Pillai's Trace;  $F_{3,8} = 0.33$ ;  $P = 0.8038$ ). The interaction of age and treatment, which compares the trajectories of vitellogenin levels, was not significant ( $F_{3,8} = 0.52$ ;  $P = 0.6776$ ). Ovarian masses at age 17 days also did not differ between ecdysteroid-treated ( $363 \pm 57$  mg) and water-treated ( $431 \pm 74$  mg) grasshoppers (Student's t-test;  $t_{10} = 0.725$ ;  $P = 0.485$ ).

In the second trial, females treated with this same dose of ecdysteroids starting at age 18 days had Vg levels throughout the experiment that were similar to those of water-treated grasshoppers (Fig. 5B;  $F_{1,11} = 0.87$ ;  $P = 0.3699$ ). Again, none of the individual days showed a significant difference in Vg levels due to ecdysteroid treatment (all  $P > 0.25$ ). There was no significant effect of time (MANOVA; Pillai's Trace;  $F_{3,9} = 1.48$ ;  $P = 0.2834$ ). The interaction of age and treatment was not significant ( $F_{3,9} = 0.38$ ;  $P = 0.7682$ ). Ovarian masses at age 23 days did not differ between these ecdysteroid-treated ( $1176 \pm 105$  mg) and water-treated ( $1125 \pm 202$  mg) grasshoppers (Student's t-test;  $t_{12} = 0.224$ ;  $P = 0.827$ ).

We conducted a third trial to re-examine the unclear results from the first trial (i.e., significant overall effect of ecdysteroid treatment but non-significant results on each individual day). Females treated with higher levels of ecdysteroids for nine consecutive days had Vg levels throughout the experiment nearly the same as those of water-treated grasshoppers (Fig. 5C; repeated measures ANOVA;  $F_{1,14} = 0.01$ ;  $P = 0.9233$ ). None of the individual days showed a significant difference in Vg levels due to ecdysteroid treatment (all  $P > 0.20$ ). There was a significant effect of time (MANOVA; Pillai's Trace;  $F_{8,7} = 4.03$ ;  $P = 0.0412$ ), with vitellogenin levels increasing with age in both ecdysteroid- and water-treated grasshoppers. This agrees with previous results showing that Vg titers tend to rise during this stage of the oviposition cycle (Borst et al. 2000; Hatle et al. 2001). The interaction of age and treatment was not significant ( $F_{8,7} = 1.15$ ;  $P = 0.4342$ ). Ovarian masses at age 17 days also did not differ between ecdysteroid-treated ( $303 \pm 60$  mg) and water-treated ( $321 \pm 117$  mg) grasshoppers (Student's t-test;  $t_{14} = 0.130$ ;  $P = 0.898$ ). In summary, ecdysteroid treatment of females during either early- or mid-vitellogenesis, even at very high dosages, affected neither vitellogenin levels nor ovarian development.

#### **Experiment 4: Is the hemolymph a source of ecdysteroids for the ovary?**

High-fed grasshoppers showed their highest levels of ovarian ecdysteroids at 23 days (Fig. 6A). Ecdysteroids were undetectable before 23 days, whereas after 23 days high-fed grasshoppers typically had about 20  $\mu\text{g}/\text{ovary}$ . Low-fed grasshoppers had undetectable levels of ecdysteroids before 30 days, whereas after 30 days these grasshoppers typically had about 12  $\mu\text{g}/\text{ovary}$ .

Similar to Experiment 1, high-fed grasshoppers had a peak of hemolymph ecdysteroids at 23 days and undetectable ecdysteroids on all other days (Fig. 6B; cf. Fig. 2). Likewise, low-fed grasshoppers showed a hemolymph ecdysteroid peak at 30 days, lower ecdysteroid titers at 40 days and 50 days, and undetectable levels at 60 days. In summary, ovarian ecdysteroids appear at the same point in reproductive development as hemolymph ecdysteroids; however, ovarian ecdysteroids, but not hemolymph ecdysteroids, persist through the end of the egg production cycle.

Finally, we tested the unlikely possibility that hemolymph ecdysteroids can be sequestered by the ovary. Ovarian extracts of grasshoppers injected with [<sup>3</sup>H]ecdysone contained 1007±468 dpm (1.3% of the injected counts) whereas extracts of [<sup>3</sup>H]water-injected grasshoppers contained 122±24 dpm (0.15% of the injected counts; two-tailed t-test with Welch's correction;  $t_{10} = 1.89$ ;  $P = 0.088$ ). Water is chemically very different from ecdysteroids; hence, it is not an ideal control. Nonetheless, it does provide some benchmark of the amount of a small, polar molecule that ends up in the ovary. Most important, because <2% of the ecdysone was detected in the ovary, hemolymph ecdysteroids are taken up by the ovary weakly, if at all.

## DISCUSSION

Our data indicate that the timing of the peak of hemolymph ecdysteroids in lubber grasshoppers is plastic (Figs. 2, 3 and 6B) and that the  $E_{\max}$  titer occurs during the canalized phase of egg production (Figs. 2 and 3), when  $JH_{\max}$ ,  $Vg_{\max}$ , and  $TP_{\max}$  also occur. Despite this, ecdysteroids are not needed for vitellogenesis. Further, treatments with exogenous ecdysteroids did not consistently increase Vg titers (Fig. 5) and never augmented ovarian growth. Therefore, hemolymph ecdysteroids do not appear to augment vitellogenesis in the lubber grasshopper.

### **Hemolymph ecdysteroids peak during the canalized phase of egg production**

After  $E_{\max}$  titer, the time to oviposition was unresponsive to changes in diet (Fig. 3). Hence,  $E_{\max}$  titer occurs during the canalized phase of egg production. This is the first association of ecdysteroids with a canalized phase (i.e., a phase experimentally demonstrated to be unresponsive to food availability) of reproduction. In female *Schistocerca gregaria*, hemolymph ecdysteroids peak late in the egg production cycle (Tawfik et al. 1997; 1999), and therefore it seems likely that ecdysteroids peak during the canalized phase in this orthopteran as well. Interestingly, *S. gregaria* females oviposit immediately after ecdysteroid levels fall, whereas lubber grasshoppers oviposit about 7-10 days after hemolymph ecdysteroids become undetectable. This implies that hemolymph ecdysteroids might have distinct functions in these two insects. The earlier appearance of ecdysteroids in lubbers was one reason we tested their role in vitellogenesis.

We have now shown that  $JH_{\max}$  (Hatle et al. 2000),  $Vg_{\max}$ ,  $TP_{\max}$  (Hatle et al. 2001), and  $E_{\max}$  titers (this paper) all occur in the canalized phase of egg production. This suggests that the decrease in the levels of these four factors in the hemolymph might ultimately be controlled by a single signal, or at least simultaneous signals. Several peptides are possible regulators of this degradation or export, including allatostatins (Stay 2000), AKHs (Carlisle and Loughton 1979; Moshitsky and Applebaum 1990), and AKH precursor-related peptides (Hatle and Spring 1999).

### **Ecdysteroids do not affect vitellogenesis**

Hemolymph ecdysteroids are not necessary for vitellogenin synthesis in lubber grasshoppers. Ovariectomized grasshoppers had no detectable ecdysteroids in their hemolymph, yet these same individuals showed high levels of Vg. In comparison, sham-operated females had similar hemolymph ecdysteroid levels (cf. Experiment 1) and Vg levels (cf. Hatle et al. 2001) to non-operated females. This result is in accord with our previous studies on Vg (Borst et al. 2000); Vg is first detectable at about age 10 days, prior to the appearance of detectable ecdysteroids.

While Experiments 1 and 2 indicated that vitellogenesis can occur in the absence of ecdysteroids, it was still possible that ecdysteroids stimulate vitellogenesis so that oocytes can be provisioned more quickly. However, our data show little evidence for ecdysteroids elevating Vg titers (Fig. 5) or stimulating ovarian growth *in vivo*. Notably, grasshoppers treated with high

dosages of ecdysteroids (Fig. 5C) showed no stimulation of vitellogenesis. In addition, there was no correlation between  $E_{\max}$  titers and the number of eggs produced by an individual grasshopper (Fig. 4). These results suggest that the role (or lack thereof) of hemolymph ecdysteroids in female lubber grasshoppers differs from the role of hemolymph ecdysteroids in several other orthopterans (crickets, Behrens and Hoffmann 1983, Hoffmann et al. 1996; cockroaches, Perriere et al. 1993; locusts, Girardie and Girardie 1996).

In contrast to the stimulatory affect of ecdysteroids on vitellogenesis in locusts, ecdysteroids have been reported to inhibit vitellogenesis in other insects, notably the cockroaches Diploptera (Friedel et al. 1980) and Leucophaea (Engelmann 1971). This inhibitory affect of ecdysteroids acts by reducing JH synthesis in the corpora allata. Our data for lubber grasshoppers suggests that hemolymph ecdysteroids do not inhibit vitellogenesis. If ecdysteroids inhibited JH production, it seems likely that Vg levels in ecdysteroid treated grasshoppers would have been reduced. However, repeated injections of ecdysteroids, during early- and mid-vitellogenesis, failed to change Vg levels in comparison to controls (Fig. 5C). Hence, ecdysteroid had neither a stimulatory nor an inhibitory affect on lubber grasshopper vitellogenesis.

#### **Ecdysteroids are produced by but not sequestered by the ovary**

Because ovariectomized females lacked detectable hemolymph ecdysteroids, the ovary appears to be the primary source of hemolymph ecdysteroids in lubber grasshopper females. This is not surprising, because the gonads are the source of ecdysteroids in most adult insects (Nijhout 1994; Gaede et al. 1997). The first appearance of ovarian ecdysteroids was coincident with the hemolymph  $E_{\max}$  titer (Fig. 6), similar to locusts (Lagueux et al. 1977; Tawfik et al. 1999) and cockroaches (Pascual et al. 1992). The brief period during which ecdysteroids are present in the hemolymph (Figs. 1, 2, and 6B) implies that ecdysteroid synthesis occurs during only a short period of the egg production cycle. This hypothesis is supported further by the ecdysteroid profile of the ovary, which rises abruptly and then remains constant until oviposition. At the time of this peak, oocytes have attained approximately 50% their final length and 13% their final volume (Sundberg et al. 2001). Taken together, these data indicate that ecdysteroids are produced at a specific time early in the egg production cycle and are stored in the ovary until oviposition.

The simultaneous appearance of ecdysteroids in the hemolymph and the ovary, and the persistence of ecdysteroids in the ovary but not the hemolymph, implies that hemolymph ecdysteroids might be sequestered into the ovary. The sequestration of ecdysteroids likely would require active transport, because of the high concentration of ecdysteroids in the ovary. Although we thought this explanation unlikely, we tested this alternative hypothesis on the role of hemolymph ecdysteroids. Our data indicate that hemolymph ecdysteroids are not sequestered into the ovary. Less than 2% of the ecdysone injected into the hemolymph was recovered in the ovary.

Overall, these data suggest that hemolymph ecdysteroids are produced in the ovary and not an extra-ovarian site, and most of the ecdysteroids remain in the ovary. While a small fraction of these ecdysteroids are released into the hemolymph, they are not reabsorbed by the ovary. Because we failed to identify a function of hemolymph ecdysteroids, these data are consistent with the hypothesis that hemolymph ecdysteroids are present simply because they leaked out of the ovary during their synthesis. Tests of several alternative functions of ecdysteroids will be necessary before this conclusion can be accepted.

**Number of eggs produced is not correlated with the maximum ecdysteroid titer**

The number of eggs produced was not significantly associated with either  $E_{\max}$  (Fig. 4) or  $JH_{\max}$  titers (data from Hatle et al. 2000). This suggests that a hormone titer is less important than the time at which the maximal level of that hormone occurs (Nijhout 1999; Gilbert et al. 2000). If ecdysteroids do serve a function in egg production in lubber grasshoppers, the lack of association of number of eggs and  $E_{\max}$  titer suggests that the maximal level reached is less important than the timing of that maximal level. In contrast to hormone levels, the number of eggs was significantly predicted by both  $Vg_{\max}$  and  $TP_{\max}$  titers (Hatle et al. 2001), perhaps because the major constituents of eggs are proteins.

Inasmuch as hemolymph ecdysteroids are produced by the ovaries (probably the follicles around each oocyte), it is surprising that  $E_{\max}$  is poorly correlated with egg number (Fig. 4). If a small percentage of the ecdysteroids produced by each follicle is released into the hemolymph, then it would be anticipated that egg number would be correlated with  $E_{\max}$ . There are two possible explanations for the lack of correlation we observed. First, the production of ecdysteroids may occur before oocytes are reabsorbed. In other words, all individuals in the study may have the same number of developing oocytes at the time of  $E_{\max}$ , and the actual (smaller) number of laid eggs was determined later. This interpretation would be consistent with the demonstration that the number of eggs produced does not become determined until about 7 days before oviposition (Moehrlin and Juliano 1998), well after  $E_{\max}$ . Second, in Experiment 1, hemolymph samples were taken every 3 or 4 days. Because the ecdysteroid peak is quite abrupt (see Fig. 1), it is likely we failed to collect a hemolymph sample on the day each individual had its highest level of hemolymph ecdysteroids. This could result in an underestimation of  $E_{\max}$  titer, decreasing the veracity of the relationship between egg number and  $E_{\max}$  (Fig. 4).

This paper demonstrates a second physiological parameter by which this atypical grasshopper differs from other Orthoptera. Lubber grasshoppers also do not respond to AKHs in the same way as locusts (Gaede and Spring 1989; Hatle and Spring 1999). It may be that both the natural history and physiology of this flightless, sluggish-moving grasshopper is distinct from locusts.<sup>1</sup>

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**FIGURE LEGENDS**

Fig. 1. Hemolymph ecdysteroid profiles of individual female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments. Ecdysteroids were measured by RIA and are reported as 20-hydroxyecdysone equivalents.

Fig. 2. Hemolymph ecdysteroid profiles of female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments; the vertical dashed lines indicate the age of the diet switch for HL and LH grasshoppers. The end of each profile is the mean age at oviposition for the grasshoppers in that treatment group. Ecdysteroids were measured by RIA and are reported as 20-hydroxyecdysone equivalents.

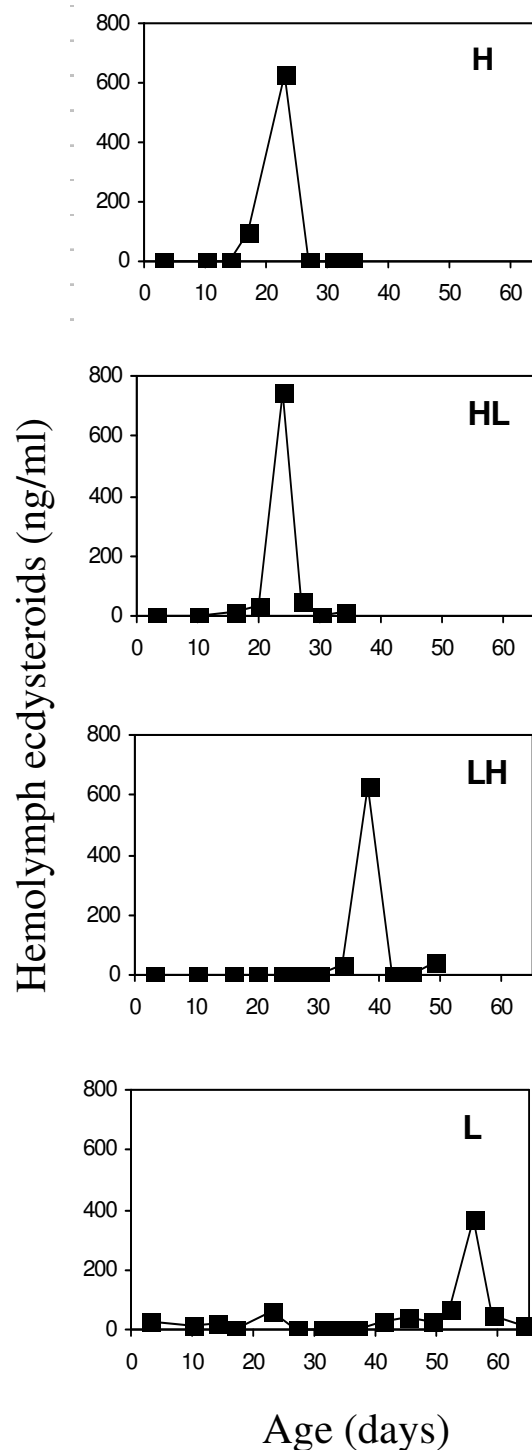
Fig. 3. Durations (mean $\pm$ SE) of two developmental periods for female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments. Letters represent statistical differences within a response variable. The times from the adult molt to  $E_{\max}$  mirror the times from the adult molt to oviposition (see Hatle et al. 2000).

Fig. 4. Correlation of  $E_{\max}$  titers with total number of eggs produced by individual female lubber grasshoppers. Data are pooled from all four feeding treatments (see text for explanation). The  $E_{\max}$  titer of an individual was only weakly correlated with the number of eggs she laid. Ecdysteroids were measured by RIA and are reported as 20-hydroxyecdysone equivalents.

Fig. 5. Hemolymph vitellogenin titers for female lubber grasshoppers injected with either a cocktail of ecdysone and 20-hydroxyecdysone (filled symbols) or water (open symbols). Data for ecdysteroid treated grasshoppers are offset for clarity. In the first trial, grasshoppers were treated with 500 ng of ecdysone and 20 ng of 20-hydroxyecdysone at ages 12, 13, and 14 days (A). In the second trial, grasshoppers were treated with this same dosage at ages 18, 19, and 20 days (B). In the third trial, grasshoppers were injected three times daily with a total daily dose of 4  $\mu$ g ecdysone and 8  $\mu$ g 20-hydroxyecdysone on ages 8 through 17 days (C). The axes of Figure C have different scales than the axes of Figures A and B.

Fig. 6. Levels of ecdysteroids in (A) ovarian extracts and (B) hemolymph of adult female lubber grasshoppers fed high- (filled squares) or low- (open squares) diets. See text for details of diet treatment. Ecdysteroids were measured by RIA as 20-hydroxyecdysone equivalents. After reaching maximal levels, ecdysteroids remained in the ovary but rapidly disappeared from the hemolymph.

Figure 1



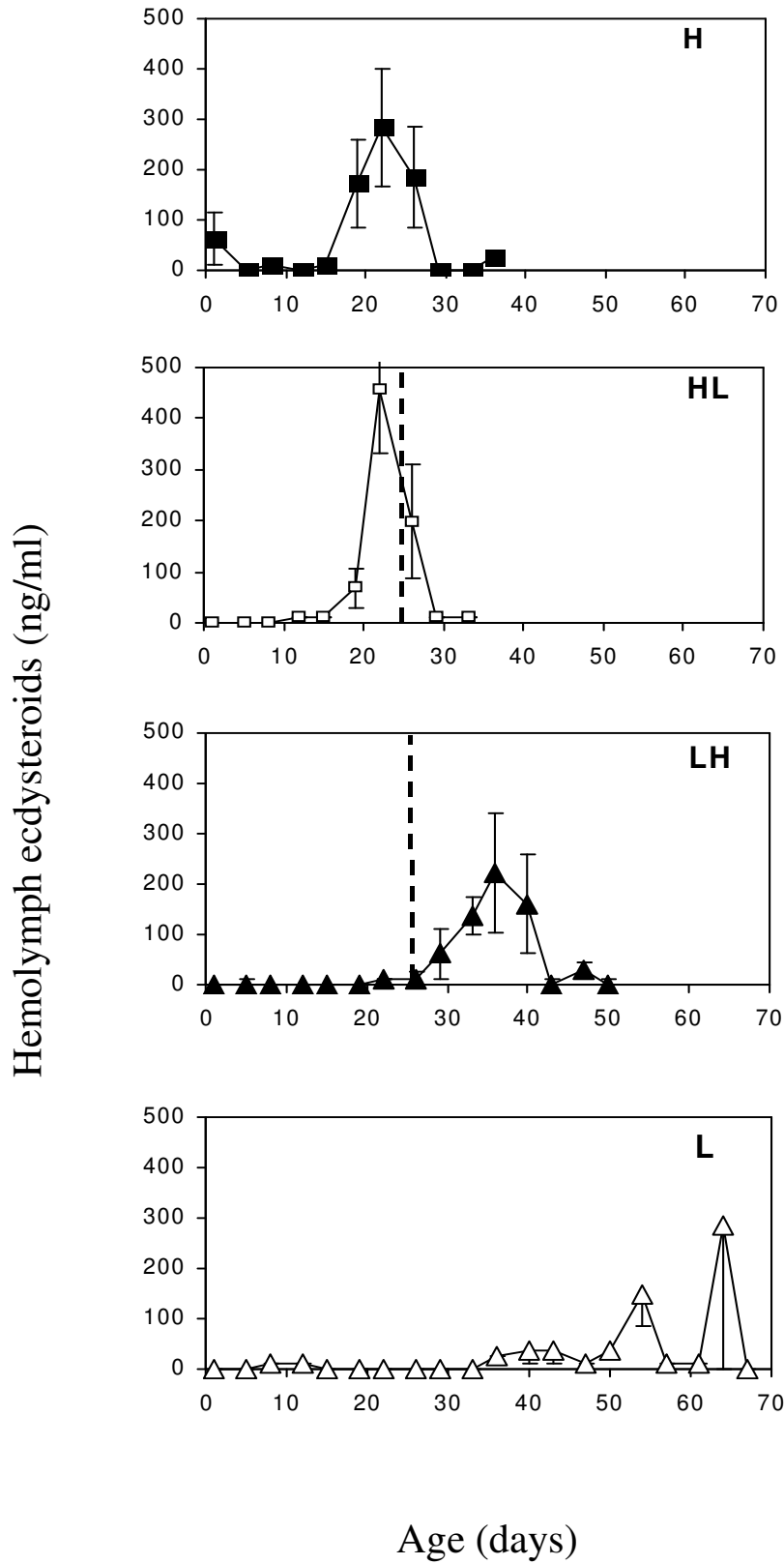


Figure 3

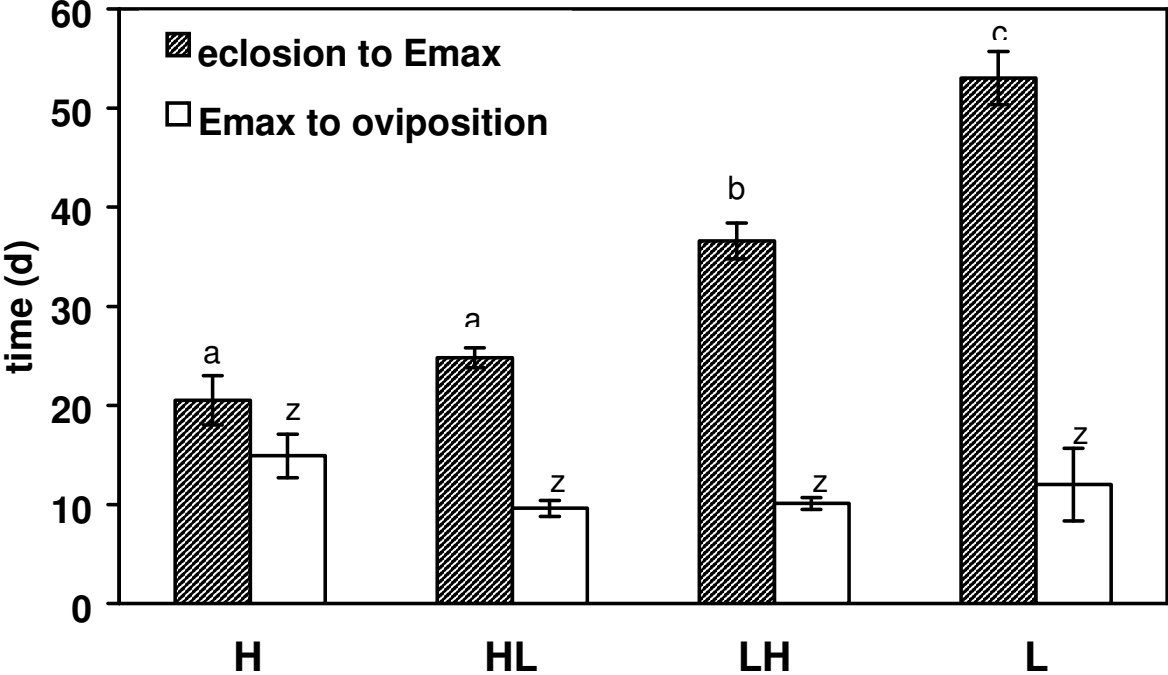


Figure 4

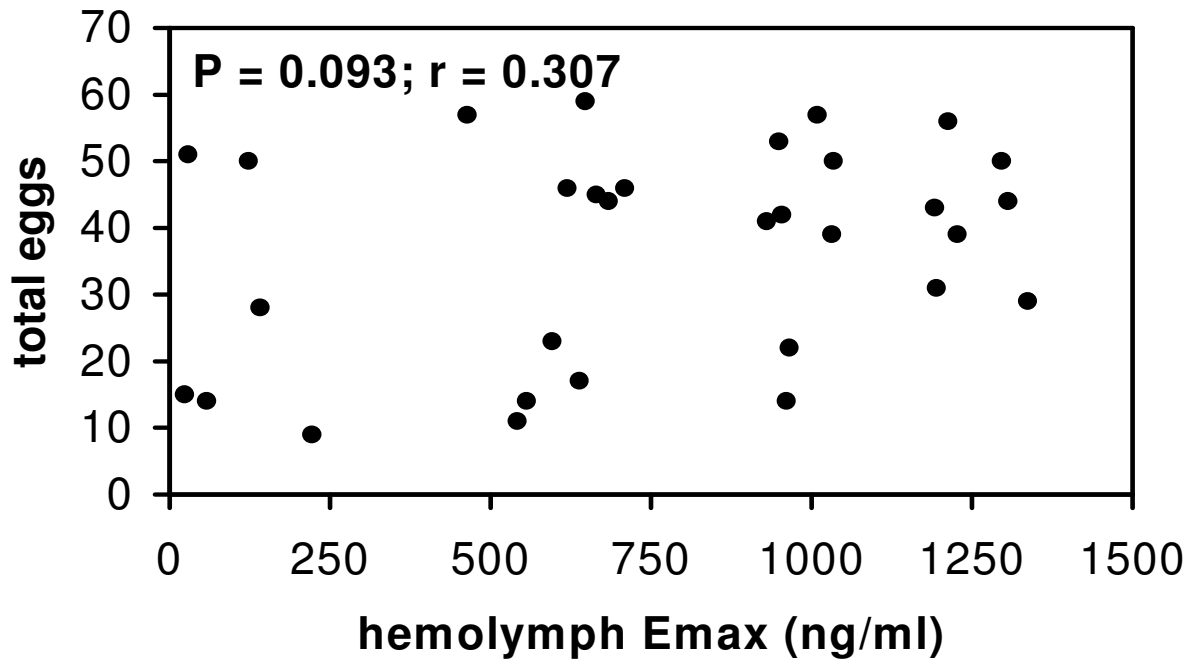


Figure 5

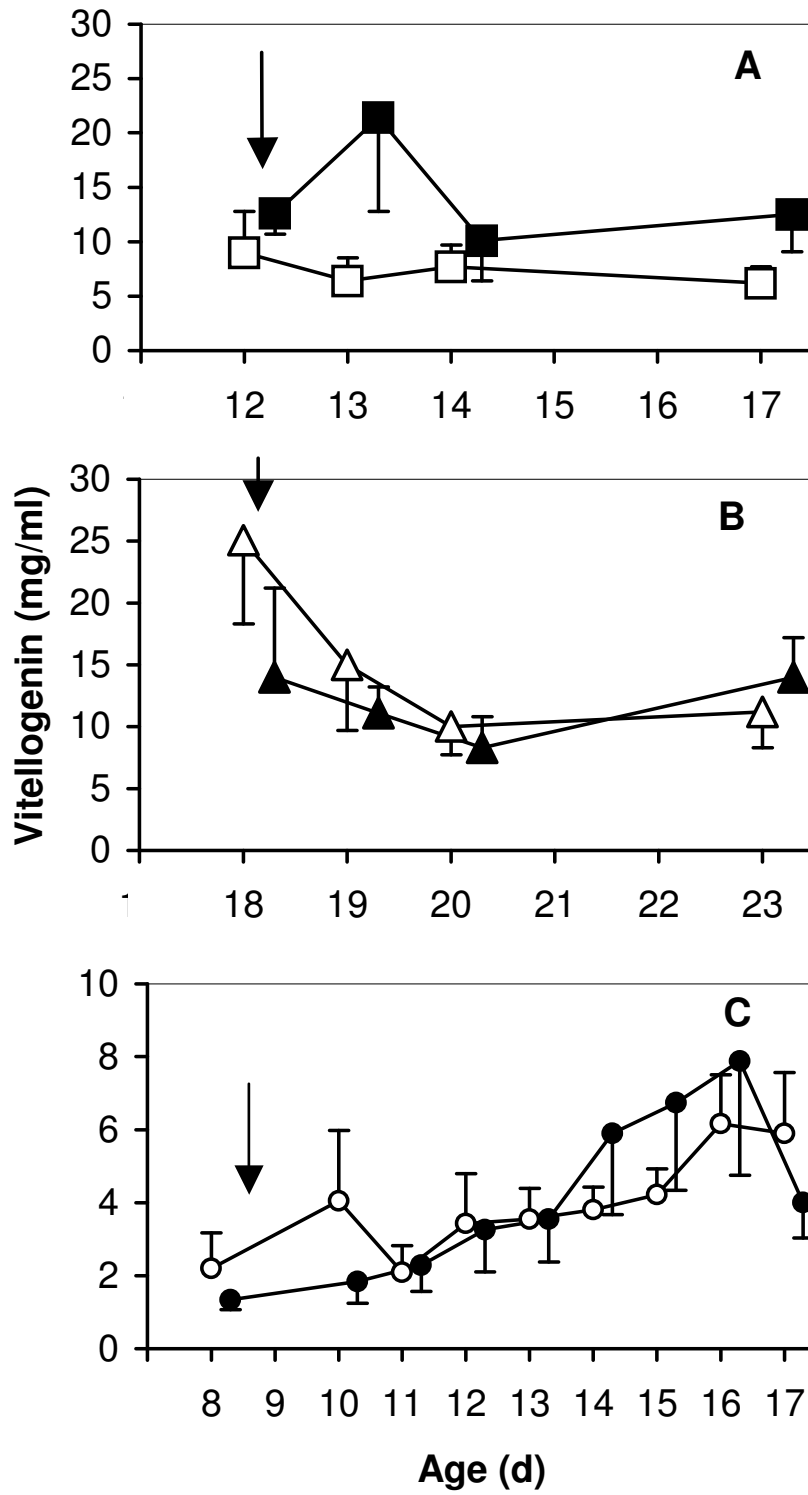


Figure 6

