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Hemolymph Ecdysteroids Do Not Affect Vitellogenesis in the Lubber Grasshopper

John D. Hatle,* Steven A. Juliano, and David W. Borst

The role of hemolymph ecdysteroids in the reproduction of non-dipteran insects is unclear. 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. Ovariectomized females had vitellogenin but no ecdysteroids, so ecdysteroids are not necessary for vitellogenin production. In addition, treatment of females with ecdysteroids altered neither Vg titers nor ovarian growth. Ovarian ecdysteriods increased at the same age in development as hemolymph ecdysteroids. In contrast to hemolymph ecdysteroids, ovarian ecdysteroids persisted until oviposition. Despite this, [^{3}H]ecdysone injected into the hemolymph was detected later only at very low levels in the ovary, suggesting that 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 ecdysteroids for the ovary. Arch. Insect Biochem. Physiol. 52:45–57, 2003.

KEYWORDS: oocyte development; phenotypic plasticity; comparative physiology

INTRODUCTION

The role of hemolymph ecdysteroids in the reproduction of non-dipteran insects is unclear (see Perrière 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 may stimulate vitellogenesis in *Locusta* (Girardie and Girardie, 1996; Girardie et al., 1992; 1996; 1998), the cockroach *Blaberus* (Perrière et al., 1993), and the cricket *Gryllus* (Behrens and Hoffmann 1983). In contrast, ecdysteroids have been reported to inhibit vitellogenesis in the cockroaches *Leucophaea* (Engelmann, 1971) and *Diploptera* (Friedel et al., 1980). Thus, there is no clearly defined role for ecdysteroids during reproduction in the Orthoptera.

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

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(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 (compare to 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. Moehrlin 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 the 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.

In this study, 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 corresponding 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. Animals that did not oviposit by 98 days were omitted from the study.

We collected hemolymph samples from each grasshopper about twice each week until oviposition. We immediately placed each sample in 0.5 ml acetonitrile and extracted twice with hexane. The lower phase of each sample was prepared for the RIA for ecdysteroids (Borst and O'Connor, 1972, 1974) by diluting it to a total volume of 2 ml with water and then drying two 400-µl aliquots for the assay. 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 [^{3}H]ecdysone (ecdysone, α -[23,24,-^{3}H(N)]; NEN; Boston, MA; specific activity = 1.9 TBq/mmol) and a mouse monoclonal anti-ecdysteroid antibody (diluted 1:20,000). 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 2,000*g* at 4°C for 5 min, and the supernatant (containing [^{3}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 (= α-ecdysone) about twice as strongly as 20-hydroxyecdysone (β-ecdysone or ecdysterone). 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

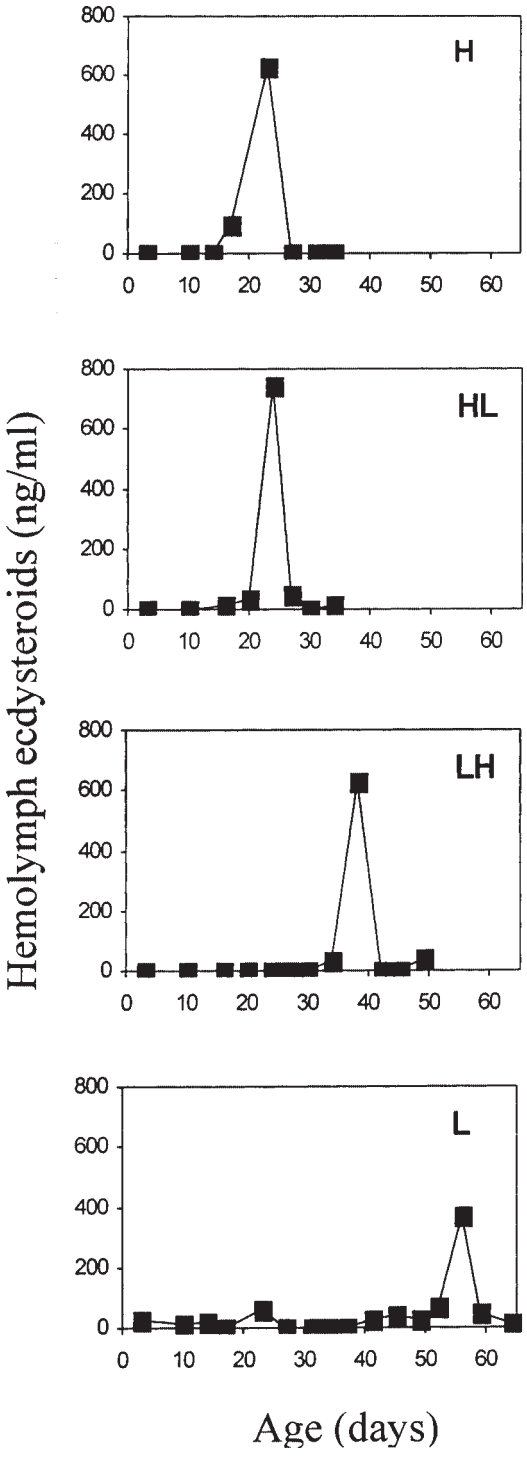

Fig. 1. Hemolymph ecdysteroid profiles of individual female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments. Ecdysteroids were measured by RIA and are reported as 20-hydroxyecdysone equivalents.

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 (Moehrlin 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 Vitellogenin Production?

We tested whether hemolymph ecdysteroids were necessary for vitellogenin production 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 µg of gentamycin was placed in the cavity, and the wound was sealed with Instant Krazy Glue® (Elmer's Products, Inc., Columbus, OH). 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 hemolymph samples, stored them at –20°C, and later measured Vg by enzyme-linked immunosorbent assay (ELISA; Borst et al., 2000) and ecdysteroids by RIA. The mean ± SE age at oviposition for the sham-operated females was 50 ± 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 by injection 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, MO). This dosage approximates 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.

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

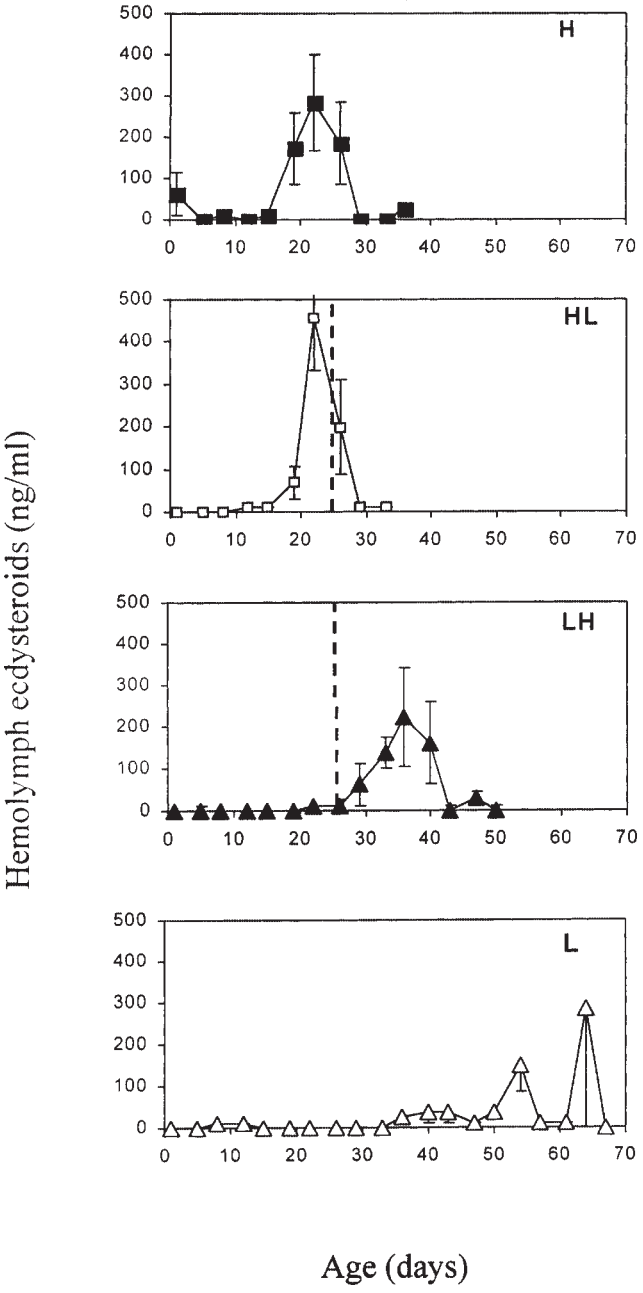

Fig. 2. Hemolymph ecdysteroid profiles of female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments; the vertical dashed lines indicate the age of the diet switch for HL and LH grasshoppers. The end of each profile is the mean age at oviposition for the grasshoppers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

daily from age 8 days through age 17 days with 1.34 µg ecdysone plus 2.67 µg 20-hydroxyecdysone (n = 8 for both water- and ecdysteroid-treated grasshoppers). Thus, the daily ecdysteroid dose used in trial three was about 10-fold higher than the doses used 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. Grasshoppers were killed at the indicated ages (see Fig. 5; high-fed mean ± SE sample size per cohort = 5.1 ± 0.5, range 3–8; low-fed sample size per cohort = 5.5 ± 0.5, range 4–8). On the final day, we collected a hemolymph sample from each individual, and then weighed the ovaries and stored them in methanol at –20°C. Later, each ovary was homogenized in methanol using a Potter-Elvehjem tissue grinder with a teflon pestle. The homogenates were centrifuged (2,000*g* at 4°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 ~40% of [^{3}H]ecdysone added to representative samples. 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 80,000 DPM [^{3}H]ecdysone (n = 12). As a comparison, 11 grasshoppers were injected with 80,000 DPM of [^{3}H]water (NEN, Boston, MA; specific activity = 37 MBq/g). 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; $P = 0.412$), and this result was confirmed using a randomization ANOVA ($P = 0.410$). 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

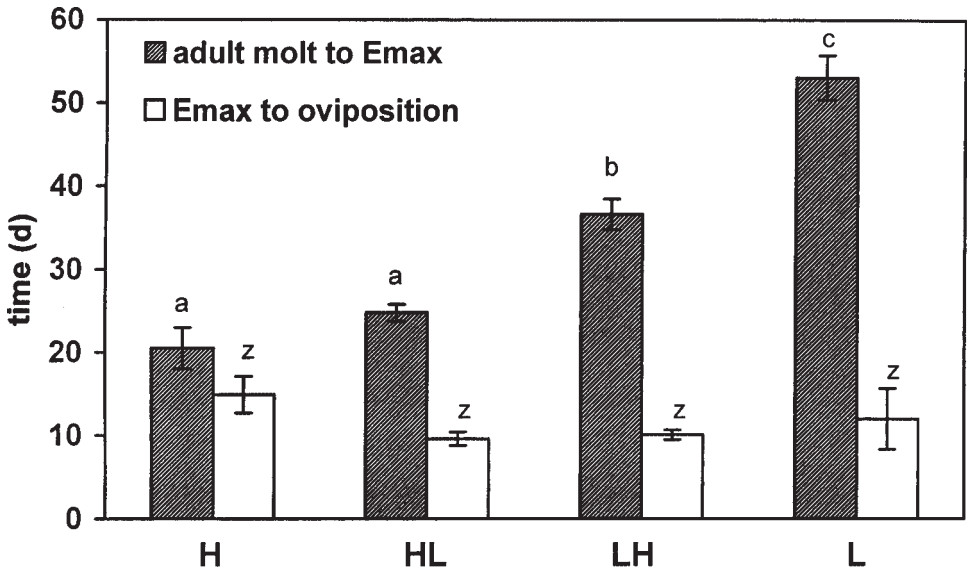

Fig. 3. Durations (mean ± SE) of two developmental periods for female lubber grasshoppers fed four different diet regimes. See text for details of diet treatments. Within a response variable, means associated with the same letter are not significantly different at experimentwise $\alpha = 0.05$, Ryan-Einot-Gabriel-Welsch multiple range test, SAS Institute Inc., 1989). The times from the adult molt to E_{max} mirror the times from the adult molt to oviposition (see Hatle et al., 2000).

egg production and specifically hypothesized that they stimulate vitellogenesis.

The E_{max} titers were not correlated with the numbers of eggs produced (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 ng/ml): H = 710 ± 150 (i.e., 1.4 ± 0.1 µM ecdysteroids); HL = 1,260 ± 300; LH = 1,000 ± 120; L = 500 ± 130.

Experiment 2: Are Hemolymph Ecdysteroids Necessary for Vitellogenin Production?

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 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 MANOVA, between subjects effect; $F_{1,10} = 9.18$; $P = 0.013$). However, none of the individual days showed a significant difference in Vg level due to ecdysteroid treatment (all $P > 0.10$). Indeed, the elevated mean on day 13 in ecdysteroid-treated grasshoppers was largely due to two of the seven individuals. There was no significant effect of age (repeated measures MANOVA; Pillai's Trace; $F_{3,8} = 0.33$; $P = 0.804$). The interaction of age and treatment, which compares the trajectories of vitellogenin levels for the two treatments, was not significant ($F_{3,8} = 0.52$; $P = 0.678$). Ovarian masses at age 17 days also did not differ

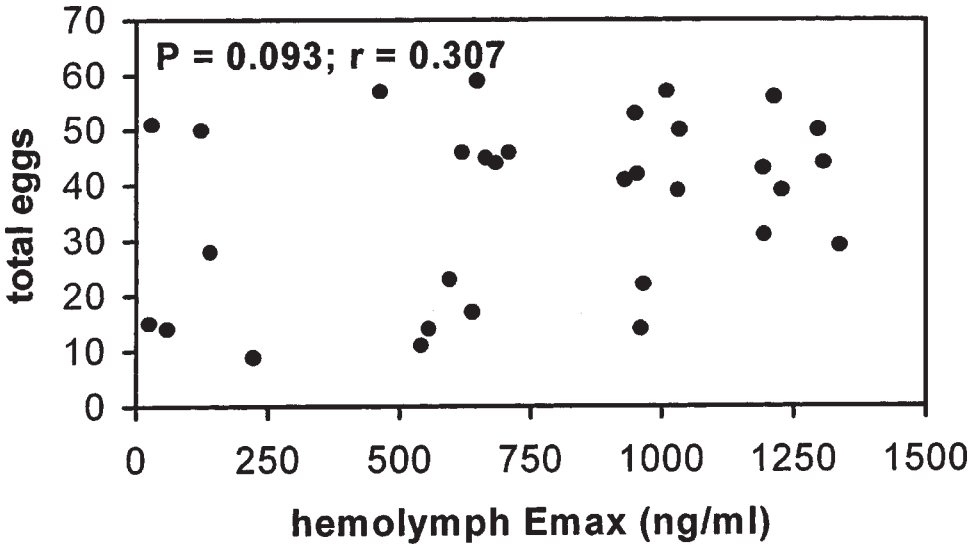

Fig. 4. Correlation of E_{max} titers with the total number of eggs produced by individual female lubber grasshoppers. Data are pooled from all four feeding treatments (see text for explanation). The E_{max} titer of an individual was not correlated with the number of eggs she laid. Ecdysteroids were measured by RIA and are reported as 20-hydroxyecdysone equivalents.

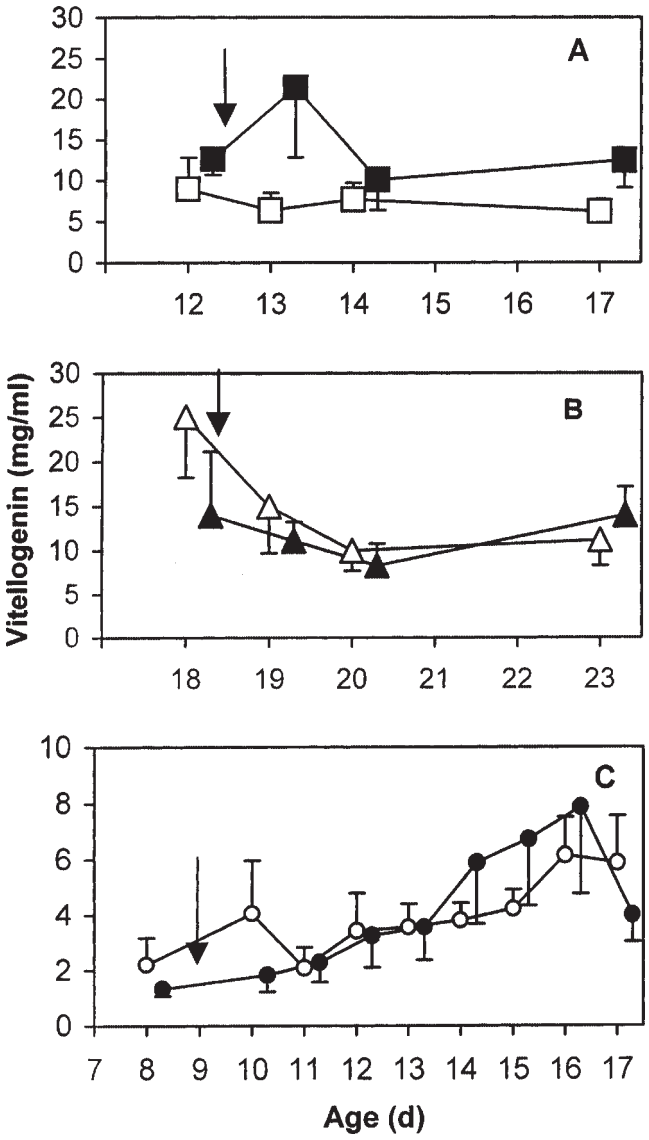

Fig. 5. Hemolymph vitellogenin titers for female lubber grasshoppers injected with either a cocktail of ecdysone and 20-hydroxyecdysone (filled symbols) or water (open symbols). Data for ecdysteroid treated grasshoppers are offset for clarity. **A:** In the first trial, grasshoppers were treated with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

between ecdysteroid-treated (363 ± 57 mg) and water-treated (431 ± 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; $F_{1,11} = 0.87$; $P = 0.370$). 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 age (MANOVA; Pillai's Trace; $F_{3,9} = 1.48$; $P = 0.283$). The interaction of age and treatment was not significant ($F_{3,9} = 0.38$; $P = 0.768$). Ovarian masses at age 23 days did not differ between these ecdysteroid-treated (1,180 ± 11 mg) and water-treated (1,130 ± 200 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; $F_{1,14} = 0.01$; $P = 0.923$). 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 age (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. The interaction of age and treatment was not significant ($F_{8,7} = 1.15$; $P = 0.434$). Ovarian masses at age 17 days also did not differ between ecdysteroid-treated (303 ± 60 mg) and water-treated (321 ± 120 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.

The hemolymph samples were also analyzed for ecdysteroids by RIA. We obtained these samples about 12 h after the last ecdysteroid treatment. We detected no ecdysteroids in these samples (detection minimum ~25 ng/ml). Because female lubber grasshoppers at this stage have a hemolymph

volume of about 2 ml (Knepp and Borst, unpublished data), and each individual was treated with 4 µg, we estimate that the maximum half life of ecdysteroids in these animals was about 100 min.

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 µg/ovary. The ovarian growth of low-fed grasshoppers was slower than that of high-fed grasshoppers (Fig. 6B). Low-fed grasshoppers had undetectable levels of ecdysteroids before 30 days, whereas after 30 days these grasshoppers typically had about 12 µg/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. 6C; compare 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 [^{3}H]ecdysone contained 1010 ± 470 DPM (1.3% of the injected counts) whereas extracts of [^{3}H]water-injected grasshoppers contained 122 ± 24 DPM (0.15% of the injected counts; two-tailed t-test with Welch's correction; $t_{10} = 1.89$; $P = 0.088$). Because < 2% of the ecdysone was detected in the ovary, hemolymph ecdysteroids are taken up by the ovary weakly, if at all.

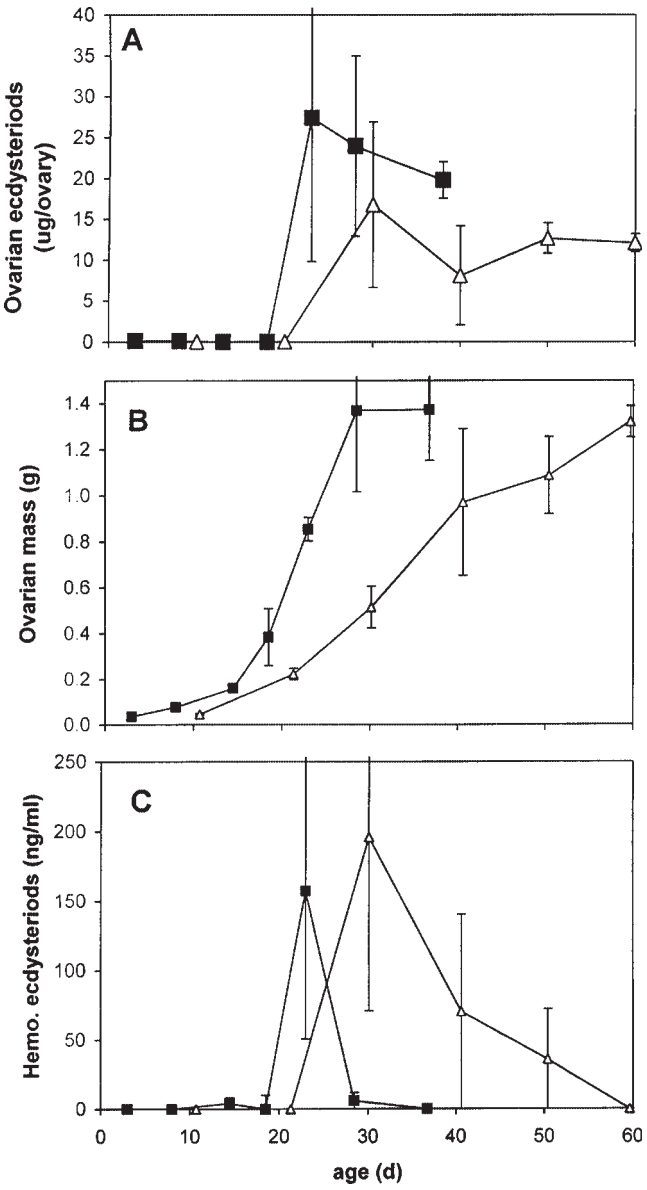

Fig. 6. Ovarian ecdysteroids levels (**A**), ovarian masses (**B**), and hemolymph ecdysteroids (**C**) of adult female lubber grasshoppers fed high- (filled squares) or low- (open triangles) diets. See text for details of diet treatment. Ecdysteroids were measured by RIA as 20-hydroxyecdysone equivalents.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

DISCUSSION

Our data indicate that the timing of the peak of hemolymph ecdysteroids in lubber grasshoppers is plastic (Figs. 2, 3, 6C) 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 necessary for vitellogenesis. Further, treatments with exogenous

ecdysteroids altered neither Vg titers (Fig. 5) nor ovarian growth. Therefore, hemolymph ecdysteroids do not affect vitellogenesis in the lubber grasshopper.

Hemolymph Ecdysteroids Peak During the Canalized Phase of Egg Production

After E_{max} titer was attained, 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). It seems likely *S. gregaria* has an early plastic phase followed by a later canalized phase, and 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 study) 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). The role of these peptides in coordinating JH, Vg, TP, and ecdysteroid profiles should be studied.

Ecdysteroids Do Not Affect Vitellogenesis

Hemolymph ecdysteroids are not necessary for vitellogenin production 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 (see Experiment 1) and Vg levels (see 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 vitellogenin production 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, and had Vg profiles similar to those observed in previous studies (Borst et al., 2000; Hatle et al., 2001). In addition, there was no correlation between E_{max} titers and the number of eggs produced by an individual grasshopper (Fig. 4).

This differs from the role of hemolymph ecdysteroids in several other orthopterans. Ecdysteroid treatment stimulated vitellogenesis in locusts (Girardie and Girardie, 1996) and decapitated cockroaches (Perrière 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).

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 appears to act partly by reducing JH synthesis by 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).

Hemolymph Ecdysteroids Are Produced by But Are 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, 6C) 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, the ovary has gained approximately 50% of its final mass (Fig. 6B; Sundberg et al., 2001). Taken together, these data indicate that ecdysteroids are produced at a specific time 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 ecdysteroids might enter the hemolymph and then be sequestered by the ovary. The sequestration of ecdysteroids likely would require active transport (perhaps bound to some hemolymph protein), 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 little if any of the hemolymph ecdysteroids are sequestered by the ovary. Less than 2% of the ecdysone injected into the hemolymph was recovered in the ovary.

Overall, our results suggest that hemolymph ecdysteroids are produced in the ovary and not an extra-ovarian site. Some of these ecdysteroids remain in the ovary and some are released into the hemolymph. 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's maximum titer is less important than the time at which the maximal level of that hormone occurs (Nijhout, 1999; Gilbert et al., 2000). If hemolymph 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 it is the timing of that maximal level that is important. 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 at least 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 strength of the relationship between egg number and E_{max} (Fig. 4).

This study demonstrates a second physiological parameter by which the lubber grasshopper differs from other Orthoptera. In previous studies, lubber grasshoppers were shown to be unresponsive to AKHs at developmental stages during which locusts are hyperlipemic to AKHs (Gaede and Spring, 1989; Hatle and Spring, 1998). In this study, ecdysteroids are shown to have no effect on vitellogenesis. It appears that the natural history of this flightless, sluggish-moving grasshopper has led to the development of physiological mechanisms distinct from those of other orthopterans.

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