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Quantification of juvenile hormone III, vitellogenin, and vitellogenin-mRNA during the oviposition cycle of the lubber grasshopper

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Abstract

The vitellogenic cycle of the lubber grasshopper (*Romalea microptera*) was studied by measuring levels of juvenile hormone (JH III), vitellogenin, and vitellogenin-mRNA through the first oviposition cycle. JH III and vitellogenin were measured by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), respectively. To measure vitellogenin-mRNA, a partial (753 bp) cDNA fragment of vitellogenin was isolated from the fat body of vitellogenic animals. The sequence of this cDNA was related to vitellogenin sequences in other insect species. Using these sequence data, an RT-PCR (reverse transcriptase polymerase chain reaction) assay was developed to quantify vitellogenin-mRNA levels during the oviposition cycle. Vitellogenin-mRNA levels in the fat body tissue from virgin females were measured on specific days after eclosion and compared to hemolymph levels of JH III and vitellogenin from the same individuals. The levels of all three compounds (JH III, vitellogenin, and vitellogenin-mRNA) showed similar changes throughout the oviposition cycle, being undetectable or nearly undetectable initially (day 3), rising to maximum levels on days 23 and 28, and then dropped to lower or undetectable levels on the day of oviposition. The ability to measure these characteristics will be useful for studying the effects of hormonal and nutritional manipulations on reproduction. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Romalea microptera*; Vitellogenin; Juvenile hormone; JH; Oviposition cycle; RT-PCR; Vitellogenin-mRNA

1. Introduction

Numerous studies have shown that hemolymph levels of vitellogenin are hormonally regulated, and there is a rich literature documenting how this regulation varies among insect species (Engelmann, 1983; Nijhout, 1994; Wyatt and Davey, 1996; Belles, 1998). In Orthoptera and related orders, the hemolymph concentration of juvenile hormone (JH) appears to be the critical factor that determines the onset and continuation of vitellogenesis (Chinzei et al., 1982; Wyatt and Davey, 1996). JH directly induces vitellogenin synthesis in several species (Engelmann, 1983; Wyatt and Davey, 1996). In *Locusta migratoria*, JH has been shown to have both a priming

effect (Wyatt et al., 1996) and a direct stimulatory effect on the transcription of vitellogenin genes in the fat body tissue (Zhang et al., 1993; Glinka and Wyatt, 1996). In addition, JH has been shown to regulate the uptake of vitellogenin by the ovary by interacting with membrane receptors of the follicle cells (Abu-Hakima and Davey, 1977; Davey et al., 1993).

One particularly attractive model for studying the regulation of vitellogenesis is the lubber grasshopper, *Romalea microptera* (= *guttata*). Unlike many other insect species, adult lubbers have an extended feeding and growth phase before the onset of vitellogenesis. Thus, the events of reproduction are separated from those of molting, a process which places its own energetic and regulatory demands on an organism. In addition, the large size (up to 12 g) of lubber grasshoppers makes it possible to obtain sequential hemolymph samples from the same individual. These samples

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can be analyzed for a variety of proteins and hormones, allowing the effects of environmental changes on individuals to be studied while controlling for individual variation. Indeed, a recent paper has shown that the level of nutrition and the timing of nutritional changes have marked effects on the reproductive output of this species (Moehrlin and Juliano, 1998). Studying the effects of nutritional and hormonal manipulations on reproductive responses should provide considerable insight into both the regulation of reproduction and the response of these organisms to variable environments.

As the groundwork for such studies, we have analyzed the levels of JH (by radioimmunoassay, RIA), vitellogenin (by enzyme-linked immunosorbent assay, ELISA), and vitellogenin-mRNA (by quantitative reverse transcriptase polymerase chain reaction, RT-PCR). The sensitivity of these methods allowed all three factors to be measured in each animal in this study. Our data indicate that these factors begin to rise toward the end of the second week of the cycle, reach a peak during the fourth week of the cycle, and drop to low levels at oviposition. These results are consistent with previous results for other Orthoptera and provide us with a baseline by which to evaluate the effects of other hormones as well as environmental factors on the vitellogenesis of this species.

2. Materials and methods

2.1. Experimental design

The strategy used in this study was to measure the changes in JH, vitellogenin, and vitellogenin-mRNA levels in adult virgin females of the lubber grasshopper during reproduction. All three compounds were measured in each individual. Three to five grasshoppers were sacrificed at 3, 8, 13, 18, 23, 28, and 33 days after adult eclosion. Six additional individuals were sacrificed on the day of oviposition, which occurred around day 37.

2.2. Animals

A colony of *R. microptera* was established at Illinois State University using animals obtained in Copeland, FL, USA, in 1996 and 1997. On the day of ecdysis, virgin adult females were marked and placed in a communal cage without males. The cage was placed in an environmental chamber (photoperiod=14L:10D; 32:24°C) and the animals were fed Romaine lettuce and oatmeal *ad libitum* (Whitman, 1986). After 30 days, a sand container was provided for oviposition.

2.3. Hemolymph samples

Hemolymph was collected at the time of dissection. Samples for JH analysis were placed in 0.5 ml acetonitr-

ile and 1 ml 0.9% NaCl and extracted (2×1 ml) with hexane. The hexane phases were combined and held at -20°C until analysis. Hemolymph collected for vitellogenin analysis was diluted (1:50) in hemolymph buffer (100 mM NaCl; 50 mM Tris; pH 7.5; 1 mM EDTA; 0.1 mM DTT; and 0.1% Tween-20) and stored at -20°C until analysis.

2.4. Quantification of JH III

The hexane extract of each hemolymph sample was dried, resuspended in 30 µl methanol, and analyzed with a chiral-selective radioimmunoassay (RIA) for 10R-JH III as described previously (Hunnicuttt et al., 1989; Huang et al., 1994). The amount of JH III present in each sample was calculated from the standard curve and expressed as ng 10R-JH III/ml hemolymph.

2.5. Quantification of vitellogenin

Lubber vitellin and vitellogenin were characterized and this work will be reported elsewhere. As part of this characterization, vitellin was isolated from freshly laid eggs or mature oocytes, purified by anion exchange chromatography, and injected (i.m.) into New Zealand white rabbits (Myrtle's Rabbitry, TN, USA) with Freund's complete adjuvant. Booster injections (every 2 weeks) used Freund's incomplete adjuvant. As expected, the resulting anti-lubber vitellin antiserum cross-reacted strongly to its precursor, vitellogenin, allowing it to be used in an enzyme-linked immunosorbent assay (ELISA) for this protein. To this end, the IgG fraction of the antiserum was purified using protein A and a portion was derivatized with biotin using the procedure of Chang et al. (1998). Before use, both the underivatized and biotinylated IgG fractions were treated with an equal concentration of male lubber hemolymph and centrifuged to remove precipitated protein.

For the ELISA, the preabsorbed underivatized IgG fraction of the anti-vitellin antiserum was diluted (1:16000) in carbonate buffer (0.1 M, pH 9.2) and used to coat the wells of a microtiter plate (overnight at 4°C or 2 h at 37°C). After this and all subsequent steps, the wells were washed with PBS-Tween (0.01 M phosphate (pH 7.2), 0.136 M NaCl, 0.0027 M KCl plus 0.05% Tween 20). The wells were then blocked (5% dry milk/PBS-Tween; 30 min at 37°C), washed, treated with 50 µl of diluted hemolymph sample or hemolymph standard (37°C for 2 h), and washed again. Each well was treated with the biotinylated antiserum (1:16000 in PBS) for 2 h at 37°C, washed, treated with streptavidin conjugated to horseradish peroxidase (strep-HRP; 0.05 U/ml; Boehringer Mannheim, Indianapolis, IN, USA) at room temperature for 1 h, and washed. The amount of strep-HRP bound to each well (which was proportional to the amount of antigen present in the sample or standard) was

determined by adding a chromogen solution (0.1 M citrate buffer; pH 4; containing 0.04% ABTS [Sigma Chemical Co.; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] and 0.006% hydrogen peroxide). The plate was covered with foil and incubated at room temperature until the color developed (approximately 15 min). The absorbance of the solution in each well of the microtiter plate was measured at 410 nm. The antiserum detected no immunoactivity in serum samples from adult males and juvenile males and females.

2.6. Cloning a cDNA for vitellogenin

Total RNA was isolated from fat body and ovarian tissue of adult females with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and used to produce a cDNA library using the λ TriplEx vector (Clontech, Palo Alto, CA, USA). Bacteria were infected with the λ TriplEx library and the resulting plaques transferred to pure nitrocellulose filters (Osmonics, Inc., Westboro, MA, USA) previously soaked in IPTG (Sigma Chemical Co, St Louis, MO, USA). Filters were blocked with 5% dry milk and treated with diluted (1:400) preabsorbed rabbit anti-vitelin antiserum. The filters were then treated with goat anti-rabbit IgG antiserum conjugated to horseradish peroxidase (Sigma Chemical Co.), washed, and then treated with a chromogen solution (PBS containing 2.8 mM 3,3'-diaminobenzidine; 0.1% CoCl_2 ; 0.03% hydrogen peroxide). Plasmids were isolated from the positive clones and cycle sequenced using the ABI 310 genetic analyzer (Perkin Elmer Biosystems, Norwalk, CT, USA). The sequence was compared with a BLAST search to other vitellogenin sequences deposited in Genbank.

2.7. Measurement of vitellogenin-mRNA by RT-PCR

Total RNA was isolated from fat body tissue dissected from female and male animals using Tri-Reagent. The total RNA concentration was quantified at 260/280 nm and diluted to 0.5 mg/ml. The RNA (1 μg) was reverse transcribed (total volume of 20 μl) using 80 units MMLV reverse transcriptase (Promega, Madison, WI, USA), random hexamer primers (100 ng), dNTPs (25 μM), DTT (10 mM) and the buffer supplied by the manufacturer. After incubation at 37°C overnight, aliquots of the RT reaction were amplified using primers (Operon Technologies, Alameda, CA, USA) that amplified a 211 bp sequence of the cloned vitellogenin-cDNA fragment. The 5' primer (5'-TTCTGCGAATCTTGAAGACC) was unlabeled while the 3' primer (5'-TTGGAGGAATCATGGACGAA) was labeled with 6-FAM at its 5' end. An aliquot (2 μl) of each RT reaction was added to 1 unit of Taq DNA polymerase (Perkin Elmer Biosystems), 0.2 μM of each primer, and 20 μM dNTP. This mixture was amplified

by the polymerase chain reaction (PCR; 15 s at 94°C, 30 s at 50°C, 1 min at 72°C) for the indicated number of cycles. The fluorescently labeled products were diluted with dimethylformamide (DMF) and analyzed with an ABI 310 Genetic Analyzer using the GeneScan protocol. A calibration standard containing fluorescently labeled DNA fragments of known size (GS-500-TAMRA; Perkin Elmer Biosystems) was added to determine the size of product produced by the sample.

The efficiency and linearity of the procedure was tested in three ways. First, cDNA samples with different amounts of vitellogenin-cDNA were amplified three times under identical conditions and the amount of product measured. For other studies, the cDNA fragment was amplified with the above primers and the product separated on a 2% agarose gel. The amplified band was removed, cleaned (GeneClean, Bio101, Vista, CA, USA), and quantified with a spectrophotometer. To determine the efficiency of the PCR amplification, a known amount of this purified cDNA (100–200 pg) was amplified in a double volume (40 μl) of PCR reactants for 15 cycles. After this and each of the next seven cycles, a small amount (2 μl) of the PCR reaction was removed and analyzed on the ABI310. In addition, known amounts of the purified cDNA (0.08 to 2.1 fmol) were amplified for 19 cycles using standard PCR conditions (see above) and the amount of fluorescent product determined. The slope of the resulting curve was used to determine the amount of vitellogenin-cDNA present in samples after reverse transcription.

2.8. Statistics

The natural or log transformed results were analyzed by one-way analysis of variance (ANOVA) followed by the Ryan-Einot-Gabriel-Welsch multiple range test (SAS Institute Inc., 1989).

3. Results

3.1. Cloning of a partial cDNA for vitellogenin

Twenty-five positive clones were isolated from the λ TriplEx cDNA library by screening with the anti-vitelin antiserum and several were characterized. One clone contained a 1.7 kb insert, from which a 753 bp fragment was subcloned and sequenced (Genbank accession #AF135420). In a BLAST search using the deduced amino acid sequence (251 a.a.) of this cDNA fragment, the proteins with the most significant alignments were vitellogenins from seven other insect species. The overall similarity of the deduced lubber amino acid sequence to these other insect vitellogenins was relatively low (from 24 to 31% of the amino acids were identical). However, a 60 amino acid region (amino acids 171 to

230) of the *R. microptera* sequence had a higher degree of similarity. For example, the lubber amino acid sequence was 49% and 47% identical to similar regions in the vitellogenins of *Bombyx mori* and *Athalia rosae* (Genbank accession # Q27309 and AB007850, respectively). Likewise, the lubber amino acid sequence was 46% identical to similar regions in the vitellogenins of *Aedes aegypti* and *Anthonomus grandis* (Genbank accession # Q16927 and M72980, respectively).

3.2. Measurement of vitellogenin-mRNA using RT-PCR

A 211 bp region of the lubber vitellogenin cDNA fragment was amplified by PCR using two 20-mer primers, one of which was fluorescently labeled. When these primers were used in the RT-PCR analysis of total RNA from fat body tissue, preparations from vitellogenic females yielded a fluorescent product with the anticipated size (Fig. 1). Total RNA from male fat body tissue (not shown) and newly eclosed (nonvitellogenic) females (see below) yielded no product.

For accurate quantification by RT-PCR it is necessary to measure product formation during the exponential phase of amplification, since amplification in the plateau phase is not quantitative (Freeman et al., 1999). Because of the relative abundance of vitellogenin-mRNA in vitellogenic animals, it was anticipated that most samples would reach the plateau phase after relatively few cycles. Therefore, the optimum cycle number was determined by amplifying the purified cDNA fragment in amounts similar to fat body levels of vitellogenin-mRNA observed in preliminary experiments. This test showed

that the PCR amplification was in the exponential phase from cycle 15 to 22 (Fig. 2; $R^2=0.9924$). The slope of this line indicated that the amplification factor for each cycle was 1.57. In addition, the linearity of the PCR reaction after 19 cycles was investigated with known amounts of the purified cDNA fragment. The level of fluorescence was linearly related to the amount of the cDNA being amplified (Fig. 3; $R^2=0.9701$). The quantification of mRNA levels by this method was highly repeatable. Several samples from vitellogenic females

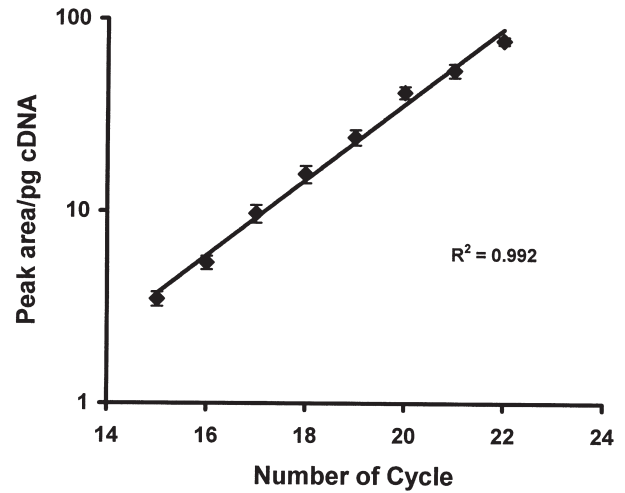


Fig. 2. The exponential phase of the PCR amplification. Amounts of cDNA similar to the levels of vitellogenin-mRNA measured in vitellogenic females were amplified as in Fig. 1 for 15 to 22 cycles. Since the PCR amplification must be in the exponential phase to be quantitative (Freeman et al., 1999), 19 cycles were used for subsequent analyses.

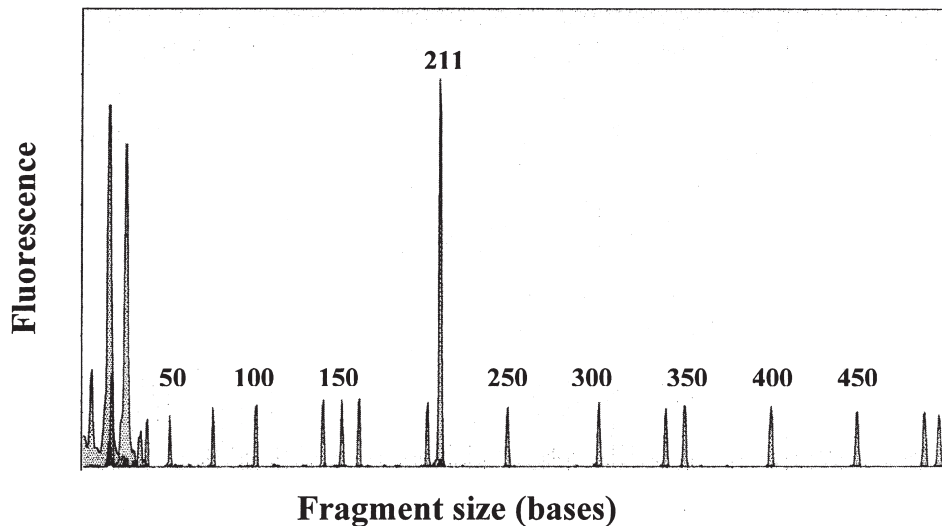


Fig. 1. RT-PCR analysis of vitellogenin-mRNA. Total RNA from the fat body of a vitellogenic female was reverse transcribed, amplified by PCR with a fluorescently labeled primer (6-FAM) and quantified with an ABI 310. The amplification yielded a single fluorescent product (211 bp). The small peaks of equal height were produced by the fluorescent (TAMRA) calibration standard, which has 16 DNA fragments from 35 to 500 bp.

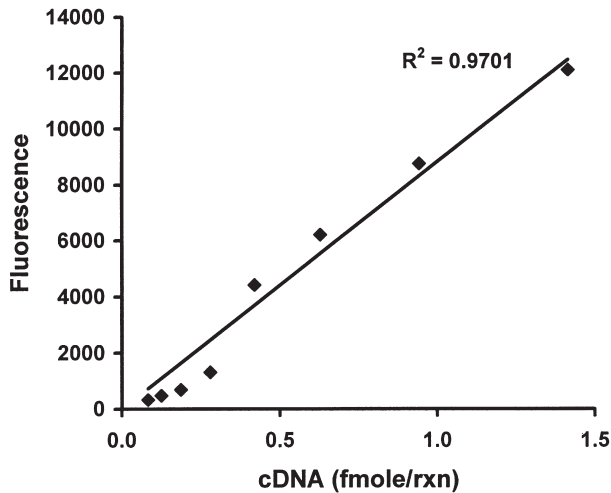


Fig. 3. The linearity of the PCR amplification. Known amounts of the vitellogenin-cDNA fragment were amplified for 19 cycles and analyzed as in Fig. 1. PCR amplification was linear over the range of vitellogenin-cDNA amounts used.

were amplified three times under similar conditions. The average coefficient of variation in the results from these samples was 10% (Table 1).

3.3. Changes in animal weight and ovarian index (OI) during the oviposition cycle:

Animal weight increased from 3.3 (± 0.5 , SE) g on day 3 to 6.5 (± 0.6) g on day 33, and then dropped to 5.1 (± 0.3) shortly after oviposition (37.2 ± 0.7 days). The OI (ovary wt/animal wt $\times 100$) was very low on day 3 (1.1 ± 0.1) and did not significantly change through day 13 (2.0 ± 0.1 ; $P > 0.05$). Thereafter, the OI rose rapidly by day 18 (10.4 ± 2), reached its maximum on day 33 (28.2 ± 2.1) before eclosion, and then fell to a low level (6.1 ± 0.6) shortly after oviposition. These results are similar to observations that we have made previously for this grasshopper (unpublished data) and for a related lubber grasshopper, *Taeniopoda eques* (Whitman, 1986).

3.4. JH, vitellogenin, and vitellogenin-mRNA levels during the oviposition cycle

The levels of all three compounds (JH III, vitellogenin, and vitellogenin-mRNA) changed in a parallel

Table 1
Precision of vitellogenin-mRNA analysis

Sample	N	Mean ^a	SE
1	3	2224	108
2	3	9599	325
3	3	3224	298

^a Area of peak at 211 bp.

fashion throughout the oviposition cycle (Fig. 4). JH levels (Fig. 4a) were initially low, then rose modestly on days 8 and 13 to an intermediate plateau (approximately 60 ng/ml) that was about 5-fold higher than the initial level of JH. On day 18, JH levels began to rise again and reached maximum levels (approximately 450 ng/ml) on days 23 and 28. These values were 7-fold higher than the intermediate level observed on days 8 and 13 and over 30-fold higher than the initial level. Thereafter JH levels fell and were nearly undetectable at oviposition. Vitellogenin was first detectable (0.2 mg/ml) on day 8, and increased to a maximum (approximately 3 mg/ml) on days 18 and 23 (Fig. 4b). Thereafter, vitellogenin dropped to low levels (0.4 mg/ml) at oviposition. Finally, levels of vitellogenin-mRNA (Fig. 4c) were first detectable on day 8, and then rose to a maximum (about 4 fmol/ μ g total RNA) on day 23. Thereafter, vitellogenin-mRNA levels fell to an intermediate level (1 fmol/ μ g total RNA) at oviposition.

4. Discussion

In this study we report the partial cDNA sequence for a third orthopteran vitellogenin. In previous studies, partial sequences for vitellogenin in *L. migratoria* (Locke et al., 1987) and *Blattella germanica* (Martin et al., 1998) have been reported. In addition, partial or complete cDNA sequences are now available for vitellogenins in a number of other insect species. Analysis of their deduced amino acid sequences indicates that insect yolk proteins fall into two major families (Hagedorn et al., 1998). Members of the first family are similar in sequence to vertebrate serum binding proteins such as apolipoprotein B and are usually referred to as vitellogenins (Vgs). Members of the second family show some similarity to mammalian triacylglycerol lipase and are usually referred to as yolk proteins (Yps). Yolk proteins are typically synthesized in the ovary in contrast to Vgs that are usually synthesized and processed in the fat body and exported via the hemolymph to the ovary (Wyatt and Davey, 1996). Based on the sequence of the cDNA fragment and the expression of this mRNA in the fat body, the precursor protein for *R. microptera* vitellin appears to be a member of the insect vitellogenin family.

The analysis of JH, vitellogenin, and vitellogenin-mRNA in the lubber grasshopper showed that these compounds all had similar profiles through the first oviposition cycle. All three were low after eclosion, rose during the second week of the cycle, peaked during the fourth week of the cycle, and fell before oviposition. In view of the metabolic requirements for vitellogenesis, it is not surprising that these factors are tightly controlled during the cycle in order to maximize reproductive output.

The coordination of JH, vitellogenin, and vitellogenin-

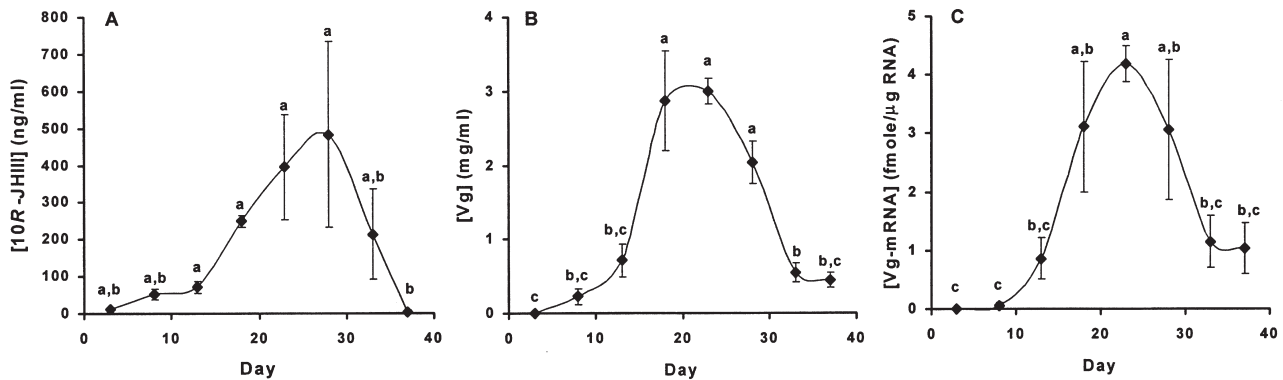


Fig. 4. JH III, vitellogenin, and vitellogenin-mRNA levels during the oviposition cycle. Virgin female lubber grasshoppers were analyzed on the indicated day after eclosion. Oviposition occurred on day 37. There were three to six individuals on each day. The results were analyzed by ANOVA; means with the same letter are not significantly different ($P > 0.05$). (A) Hemolymph levels of 10R-JH III were measured by RIA ($F_{7,22}=6.53$, $P=0.0003$). (B) Hemolymph levels of vitellogenin (Vg) were measured by ELISA ($F_{7,22}=16.37$, $P=0.0001$). (C) Vitellogenin-mRNA (Vg-mRNA) levels in fat body tissue were measured by RT-PCR ($F_{7,22}=6.14$, $P=0.0005$).

mRNA observed in lubber grasshoppers is similar to the regulation of these factors in *L. migratoria* (Chinzei et al., 1982; Chinzei and Wyatt, 1985; Glinka et al., 1995). Changes in the levels of vitellogenin-mRNA in the lubber grasshopper were generally consistent with changes reported for *L. migratoria* (Chinzei et al., 1982). A minor difference was observed at the end of the vitellogenic cycle. In the lubber grasshopper, vitellogenin-mRNA levels dropped to about 25% of the maximum at oviposition, while vitellogenin-mRNA levels in the locust decreased to about 70% of the maximum. The reason for this difference is not clear. Vitellogenin-mRNA may be less stable in the lubber grasshopper. Alternately, this difference may reflect a difference in the length of the oviposition cycle (37 and 17 days for the lubber and locust, respectively), which would lengthen the period at the end of the cycle during which the vitellogenin-mRNA could be broken down. In any case, the relatively low level of vitellogenin in the hemolymph at oviposition indicates that the remaining fraction of vitellogenin-mRNA is not being efficiently translated at this period. This suggests that the production of vitellogenin in lubber grasshoppers may be both transcriptionally and translationally controlled.

Our study does not address the relationship of JH levels to vitellogenin-mRNA and hemolymph levels of vitellogenin in the lubber grasshopper. Nevertheless, in other Orthoptera (e.g. *L. migratoria*), JH both primes the fat body and stimulates production of vitellogenin from the hemolymph (Wyatt and Davey, 1996). In view of these results and the close correlation observed between the levels of JH, vitellogenin, and vitellogenin-mRNA, it seems likely that the intermediate levels of JH observed at days 8 and 13 stimulate the synthesis of vitellogenin-mRNA in the lubber. This in turn would lead to an increase in its hemolymph levels of vitellogenin. Likewise, we suspect that the higher levels of JH

observed later in the cycle (day 18) might stimulate vitellogenin uptake by the oocyte. This would be in agreement with the rapid rise in the OI that occurred between days 13 and 18. A similar requirement for different JH levels to stimulate vitellogenin synthesis and vitellogenin uptake has been demonstrated in *Rhodnius prolixus* (Davey et al., 1993). We are currently testing these hypotheses by treating animals with defined amounts of JH III and the JH analog methoprene.

Knowledge about the interactions among JH, vitellogenin, vitellogenin-mRNA, and other factors that regulate reproduction is critical for understanding the molecular and physiological mechanisms that control this process. In addition, these interactions may also help explain how reproductive output is varied in response to variable and unpredictable environments. These issues are central to understanding the mechanisms of phenotypic flexibility (plasticity) and inflexibility (canalization) that occur during reproductive development (Scheiner, 1993; Schlichting and Pigliucci, 1998). An analysis of some of these mechanisms and their relationship to the onset of reproductive canalization in the lubber grasshopper is given in a companion paper presented at this conference (Hatle et al., 2000). Ultimately, these mechanisms must have a major role in regulating reproductive output that occur in response to environmental variation.

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