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In vivo role of 20-hydroxyecdysone in the regulation of the vitellogenin mRNA and egg development in the American dog tick, *Dermacentor variabilis (Say)*

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Abstract

Injection of the hormone 20-hydroxyecdsyone (20-E) into partially-fed (virgin) female adults of the American dog tick, *Dermacentor variabilis,* while they are attached and feeding on the rabbit host, initiated the expression of the vitellogenin (Vg) gene, and vg protein secretion and uptake by the ovary. The induction of egg production by 20-E in this bioassay was dose dependent in the range of 1-50 times the concentration normally found in a replete, vitellogenic female. Ticks examined 4 d after the 50X treatment were still attached to the host, had numerous enlarged vitellin filled (brown) oocytes in their ovaries, but had not engorged to repletion. The ovaries reached weights similar to those found in untreated, replete (mated) females (pre-oviposition) while solvent injected controls demonstrated no increase in oocyte size or increase in ovary weight. An increase in the levels of a putative Vg protein was observed in hemolymph samples collected 1, 2 and 3 d post-20-E injection but was not observed in the corresponding solvent controls as determined by native PAGE. Analysis of the ecdysteroid-induced protein by tryptic digestion-mass fingerprinting and BLASTP found that the putative Vg had the strongest match to GP80 (U49934), the partial sequence for the vitellogenin protein from *Boophilus microplus.* A partial Vg cDNA was cloned and sequenced from replete, females of *D. variabilis* with a high similarity to GP80. Using this message as a probe, Northern blots conducted with RNA collected from partially fed, virgin females 1, 2 and 3 d post-20-E injection showed upregulation of the Vg mRNA on all 3 days. Controls injected with solvent only showed no Vg mRNA. Injections with juvenile hormone III did not stimulate Vg expression, oocyte growth or full engorgement. These studies indicate that ecdysteroids and not JH can initiate expression of the Vg gene, Vg protein synthesis and release into hemolymph, and Vg uptake into developing oocytes under bioassay conditions mimicking normal feeding on the host.

Keywords: 20-hydroxyecdysone, juvenile hormone, vitellogenin, vitellin, tick

1. Introduction

Hormonal regulation of reproduction in insects and other invertebrates has been the subject of intense investigation for many years. In insects, synthesis of vitellogenin (Vg), the precursor protein of vitellin (Vn) in eggs, has most commonly been attributed to juvenile hormone (JH). In view of their apparent similarity with insects, ticks have long been assumed to also use JH to initiate Vg synthesis, secretion into hemolymph and uptake by the developing ovary (Gilbert et al., 2000). Indeed, when argasid ticks were treated with precocene, which inhibits JH biosynthesis in insects, oviposition was disrupted; the effects were reversed and oviposition resumed when the ticks were treated topically with JH (Pound and Oliver, 1979). Other studies suggested that precocene was detrimental to tick development and reproduction (Gaber et al., 1983; Khalil et al., 1983), although this may have represented sublethal toxicity rather than true hormonal activity (Dees et al., 1982). No conclusive evidence for the existence of JH has been found in ticks. A search for this molecule by *Galleria* bioassay, radiobiosynthesis and/or selective ion EI GC-MS during egg, larval and adult development failed to demonstrate its occurrence in the ixodid tick, *Dermacentor variabilis*, or the argasid, *Ornithodoros parkeri* (Neese et al., 2000). Although JH has been shown to regulate vitellogenesis in a number of insect species, the evidence so far suggests that JH is not found in ticks and is not involved in egg development (Neese et al., 2000, Gilbert et al., 2000).

In mosquitoes, blood feeding initiates a cascade of regulatory events, including secretion of an ecdysteroidogenic hormone from the brain that stimulates the ovary to synthesize ecdysteroids. The latter stimulates the fat body to synthesize vitellogenin (Graf et al., 1997). In ticks, both a neuropeptide (Lomas et al., 1997) and 20-hydroxyecdysone (20-E) (Sankhorn et al., 1999; Friesen and Kaufman, 2002; Friesen and Kaufman, 2004) may have a similar role. 20-E increases in

concentration during feeding, reaching maximum levels following engorgement and the commencement of oviposition in *D. variabilis* (Dees et al., 1984). Sankhorn et al. (1999) found that ecdysteroids increased the levels of Vg in fat body organ culture of the same species, and Friensen and Kaufman (2002) found that ecdysteroids could increase Vg when injected into fed female adults of *Amblyomma hebraeum* forcible detached from the host prior to commitment to Vg synthesis. Studies by Oliver et al. (1992) suggested that the synganglion produces an egg development stimulation factor in the soft tick, *Ornithodoros parkeri*.

In the metastriate Ixodidae, vitellogenesis and egg maturation occurs in partially fed female adults following mating and only after the female has fed to repletion. Although the evidence is mounting that 20-hydroxyecdysone and not juvenile hormone is important in the regulation of vitellogenesis, no studies have yet been conducted to demonstrate 20-E regulation at the level of expression of the Vg gene. In addition, previous studies on the role of ecdysteroids in the regulation of Vg protein were conducted either in organ culture (Sankhorn et al., 1999) or in ticks forcibly detached from the host and injected with hormone (Friensen and Kaufman, 2002). In the current study, we have partially sequenced the Vg mRNA from the American dog tick and examined the role of ecdysteroids on the regulation of the Vg gene, on Vg protein synthesis and uptake by the ovary, and on egg development in partially fed, virgin females attached to the host. Our novel "in vivo" bioassay system where ecdysteroid or juvenile hormone is injected into the tick while still attached to the host is the closest approximation possible to the normal feeding habits of *D. variabilis* and simultaneously evaluates both the role of the hormone on feeding to repletion and on the regulation of vitellogenesis.

2. Materials and methods

2.1. Ticks

The American dog tick, *D. variabilis*, was reared as described previously (Sonenshine, 1993). Adult ticks were confined within plastic capsules attached to New Zealand White Rabbits (*Oryctolagus cuniculus*)[1] and allowed to feed as required for the experiments described below. Rearing conditions were 26±1° C, 92±6% relative humidity and 14:10 (L:D).

2.2. Chemicals

20-hydroxyecdysone (20-E), juvenile hormone (JH) III, dimethyl sulfoxide (DMSO) and TRI-Reagent were obtained from Sigma Chemical Co. (St. Louis, MO). For inoculation into ticks, 20-E and JH III were first dissolved in a mixture of ethanol and DMSO, and than an aliquot of these solutions dissolved into 0.15 M phosphate-buffered saline (0.13 M NaCl)(PBS), pH 7.0, to produce a final concentration of 1 or 0.2 µg/µl, respectively, that contained 0.05% ethanol/0.05% DMSO. Additional dilutions in PBS were conducted as needed to achieve the doses per tick as described later.

2.3. In vivo tick bioassay

Ecdysteroid and JH induction of egg development was evaluated in this paper by a novel, in vivo bioassay developed by our laboratories using virgin adult females of the American dog tick attached to the rabbit host for 4 d. The rabbit was tranquilized with Azepromazine (Fort Dodge, IA) prior to the commencement of the inoculations. Inoculations of each hormone were done using a 50 µl Hamilton syringe and 30-gauge needle. The needle was inserted near the coxa of the 4th leg and 5 µl injected into each specimen. The needle was held in the body of the tick for 1 – 1.5 min, and if the inoculum leaked from the injection site, the test was rejected. Controls were injected with a similar

[1] All use of animals for this research was conducted at Old Dominion University and in accordance with protocols approved by the Old Dominion University Institutional Animal Care and Use Committee. The approved protocols are on file in the Old Dominion University Animal Care Facility Office.

volume of solvent only. Each tick was color coded with enamel paint to mark the specific treatment administered. Following completion of the inoculations, the ticks were allowed to continue feeding for up to 4 d before they were detached for further study.

20-hydroxyecdysone (20-E) or juvenile hormone III was injected into partially fed (virgin) female ticks on d 4 after attachment. The 20-E doses were 20 ng/tick (616 ng/g body weight), 200 ng/tick (6,160 ng/g body weight), and 1000 ng/tick (3,0750 ng/g body weight). The JH III dose was 1,000 ng/tick. The volume of the injection solution was adjusted with PBS to 5 µl for all treatments. The same volume was used for the solvent controls. The mean body weight of the ticks at the time of injection was 32.5 ± 13.1 (± 1S.D., n = 24) mg; this was determined from a separate cohort of ticks maintained under identical conditions on a different animal. Assuming complete mixing inside of the tick, the 20-E injections produced hormone concentrations that were 1X, 10X and 50X the maximum concentration per tick found in ovipositing females of the American dog tick (Dees et al., 1984). The controls were injected with solvent only and/or left untreated.

For studies on ovary development, ticks were examined 4 d after hormone injection to determine if they completed engorgement to repletion and then were forcibly detached and weighed using a Cahn Model 31 microbalance (Cahn, Inc., Cerritos, CA). After weighing, the ticks were dissected and the ovaries examined for evidence of vitellogenin uptake and oocyte development. Ovaries were scored according to the categories described by Balashov (1972), i.e., Stage 2, oocytes uniformly small and white; Stage 3, few oocytes slightly enlarged and light brown in color; Stage 4, many enlarged oocytes brown in color; and Stage 5, almost all oocytes enlarged and uniformly brown. To determine the effect of the hormonal treatment on ovary weight, the ovaries from treated versus solvent-injected controls were excised, washed in PBS and weighed.

2.4. Electrophoresis

Hemolymph was collected from partially fed (virgin) females that had been injected on the host (as previously described) with 20-E and bled 1, 2 and 3 d post-injection. Hemolymph was collected as described by Johns et al. (1998). Briefly, partially fed ticks were immobilized on slides, ventral side up on double-sided tape and one or two forelegs amputated with microdissecting scissors. While applying gentle pressure, clear hemolymph exuded from the opening to the body cavity was collected with a glass Drummond micropipette, transferred to an Eppendorf tube, and diluted 1:1 in tick saline PBS (defined earlier). Hemolymph was stored at -80°C until needed for further analysis. Newly oviposited eggs (n = 25) were collected from mated females and stored at -80°C. Later the frozen eggs were rinsed once with Dulbecco's phosphate buffer (Pierce, Rockford, IL) with Tween (PBST; pH 7.4, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M NaCl, 0.01 M potassium chloride, Tween 20 (Sigma) 0.05% v/v)) and homogenized in 500 µl of PBST with a mortar and pestle. The homogenate was clarified by centrifugation at 960 x g at 4°C. Total protein in the diluted hemolymph and the supernatant from egg homogenate was determined by the Bradford protein assay (BioRad, Richmond, CA) using bovine serum albumin (BSA, fraction V; Fisher Scientific, Fair Lawn, NJ) as a standard. The samples were then further diluted with native sample buffer (Invitrogen, Carlsbad, CA) to obtain 4 µg of total protein per lane for each sample to be tested. Native polyacrylamide gel electrophoresis was conducted with an Invitrogen XCell SureLock™ electrophoresis apparatus, 8 – 16% polyacrylamide Tris-Glycine gels, and the appropriate buffers from Invitrogen. Electrophoresis was conducted at 130 V for 4 h. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (45ml methanol, 10ml glacial acetic acid, 45ml water, 0.25g Coomassie Brilliant Blue R-250) overnight. The gels were destained with a mixture of 30% methanol and 10% acetic acid overnight, and then scanned using a Hewlett Packard ScanJet

5100C. To determine the relative molecular weight (rMW) of the putative Vn or Vg bands, molecular weight (MW) standards (BSA and apoferritin, Sigma) were run in a separate lane.

2.5. Digestion-mass fingerprinting

Gel slices from the native gel (described earlier) containing the purified (putative) Vg protein from hemolymph from partially fed (virgin) female ticks 2 d after injection with 20-E (a 50X dose) was submitted to the W.M. Keck Biomolecular Research Facility at the University of Virginia (Charlottesville, VA; www.healthsystem.virginia.edu/internet/biomolec/) for tryptic digestion-mass fingerprinting as described previously (Cohen and Chai, 1997; Rao et al., 2003). After digestion with trypsin, peptides were introduced into a Thermo-Finnigan LCQ DecaXP mass spectrometer, and the resulting spectra analyzed using the NCBI (BLASTP) non-redundant database.

2.6. Cloning of a putative Vg mRNA

Total RNA was isolated from the fat body of vitellogenic, replete female American dog ticks using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA was synthesized from this total RNA according to the GeneRacer protocol (Invitrogen). cDNA synthesis was primed using the GeneRacer Oligo dT primer (GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$, Invitrogen). The resulting single stranded cDNA was then PCR amplified using the GeneRacer 5' and GeneRacer 3' primers and High Fidelity Taq polymerase (Invitrogen) according to the manufacturer's recommendations using a combined annealing/extension of 7 min at 68°C for 25 cycles. Reaction products were subsequently incubated with Taq polymerase (Promega, Madison, WI) at

72°C to introduce an A-overhang. The mixture of reaction products was then introduced into the pCR4-Topo vector and used to transform Top10 competent cells, selected on LB plates with 50 μg kanamycin/ml media.

Individual transformed colonies were picked into 384-well master plates containing LB + kanamycin (50 μg/ml) media glycerol. These plates were allowed to grow overnight at 37°C. The 384-well plates were used to inoculate 96-well growth blocks containing LB Kanamycin media with 15% glycerol. The growth blocks were allowed to grow 16 to 20 h at 32°C with shaking at 260 rpm. The blocks were then centrifuged for 20 min at 4000 x g, and the media was removed by decanting. Plasmid DNA was isolated using the R.E.A.L. Prep 96 BioRobot Kit (Qiagen) with the Qiagen BioRobot 9600 following Qiagen's specifications. DNA sequencing reactions were then performed in 96-well plates using the BigDye Terminator Cycle Kit (Applied Biosystems, Foster City, CA). Each 10 μl reaction was composed of 4 μl of BigDye Terminator Matrix, 250-500 ng of purified plasmid DNA and 2 pmol of the T3 primer (provided by Ms. Winnell Newman, Nucleic Acids Facility, North Carolina State University, Raleigh, NC). Reactions were run on a GeneAmp 9700 thermocycler (Applied Biosystems) using the cycling parameters provided by the BigDye Terminator Cycle Kit. Samples were run on an ABI PRISM 370 DNA Analyzer (Applied Biosystems) by the Genome Research Lab at North Carolina State University. Chromatograms were viewed in Chromas (Technelysium Pty Ltd., Southport, Queensland, Australia). BLASTX and TBLASTN searches (Altschul et al., 1997) were conducted with these sequences using the NCBI nr database (www.ncbi.nlm.nih.gov) to determine putative protein function. Further sequence analysis was done using Vector NTI Suite 9.0.0 (Informax; Bethesda, MD).

2.7. Northern blots

Partially fed (virgin) females of the American dog tick that had been injected on the host (as previously described) with 20-E (or solvent only) were removed from the host at 1, 2 and 3 d post-injection and homogenized for 5 min on a Polytron homogenizer (Analytical Instruments, LLC, Golden Valley, MN) in TRI-Reagent (50 mg whole tick/ml reagent). RNA was isolated from the homogenate using TRI reagent according to the manufacturer's recommendations except that RNA pellets were dissolved with water containing 10 mM aurin trichloroacetic acid (ATA) to prevent degradation (Hallick et al., 1997). Samples were assayed for RNA content using a Molecular Devices Corporation Spectromax 384 Plus plate reader (Sunnyvale, CA) and stored at -80°C until needed for analysis.

Five micrograms of each RNA sample was denatured by glyoxal treatment and separated by electrophoresis in a 1.25% agarose gel according to the phosphate protocol of Sambrook and Russell (2001). RNA was transferred by capillary action to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) followed by UV crosslinking according to the manufacturer's recommendations. The Vg and ribosomal probes were digoxigenin-labeled by PCR amplification using the T3L and T7L primers. PCR conditions and reagents were according to the manufacturer's recommendations (Roche Diagnostics GmbH). Blots were pre-hybridized, hybridized and washed according to the manufacturer's recommendation, except that all elevated temperature manipulations were performed at 50°C instead of 68°C. Chromogenic detection was done using NBT/BCIP according to the manufacturer's recommendations.

3. Results

3.1. Effect of 20-E on oocyte development, ovary weight and tick weight

Inoculation of 20-hydroxyecdysone at the 1X dose into partially fed (virgin) female ticks while still attached to the rabbit host did not lead to the production of vitellogenic eggs. Examination of the ovaries showed that all of the eggs were white and uniform in size, representing category 2 of the Balashov scale and were similar to the appearance of the solvent-inoculated ticks and the untreated controls (Table 1). The same results were obtained each time the experiment was replicated. Representative images of unstimulated ovaries are shown in Fig. 1, A and B. At the 10X dose, many specimens showed evidence of Vg uptake into the oocytes. Numerous oocytes had turned brown by d 4 after treatment (Table 1). Microscopic examination of the ovaries showed that these eggs had enlarged and contained large brown clusters, presumably Vn granules (Fig. 1, C and D). Some eggs were only slightly enlarged while others were larger, representing categories 3 and 4, respectively, of the Balashov scale (Table 1). However, some eggs remained small, white and presumably unstimulated. Measuring representative regions of the ovaries showed that approximately 15% of the eggs had reached category 3 and 12% had reached category 4. A noteworthy finding was the large amount of presumed guanine that had accumulated in the rectal sac and Malpighian tubules over that of the controls at the 10X dose, suggesting increased metabolic activity in response to the injected hormone. When ticks were inoculated with the 50X dose of 20-E, 14 out of 19 specimens examined showed evidence of Vg uptake into the oocytes. Microscopic examination of the vitellogenic ovaries indicated that almost all of the eggs could be classified as very large and were uniformly brown, representing category 5 on the Balashov scale (Fig. 1E). Other eggs were large, category 3 or 4 and filled with yolk granules.

Positively correlated with the appearance of brown eggs was a significant increase in ovary weight for 20-E injected ticks. Mean ovary weight increased in a significant curvilinear relationship with age before and after drop off ($R^2 = 0.9668$)(Fig. 2). The mean weight of the ovaries from the

ticks injected with a 50X dose of 20-E was 10.24 ± 3.18 mg (4 d after treatment), similar to that reached by untreated, mated females on d 4 (Fig. 2). For the 20-E injected ticks, this represented a significant increase over that of the solvent injected control (*t*-test, $P \leq 0.05$)(Fig. 2). Again, the 20-E-injected ticks had a large build up of presumed guanine as determined by observation, so much so that the slightest incision and pressure on the body wall released a white cloud of waste material into the hemocoel.

Injection of 20-E had a statistically significant negative effect on the weight of partially fed (virgin) ticks still attached to the host as compared to controls. Komogorov-Smirnof statistics (PROC UNIVARIATE, SAS Institute 1999) revealed that tick weights were not normally distributed in our experiments. Consequently, tick weights were transformed (square root *x*) before statistical analysis. An analysis of variance (ANOVA) was conducted using a general linear model (PROC GLM, SAS Institute 1999) to determine whether ecdysone significantly affected the mean weight of partially fed *D. variabilis* females. In the analysis, tick weight was the dependent variable and ecdysone dose (1X, 10X and 50X) and treatment (ecdysone injected, solvent injected and uninjected) were independent variables. The main effect of treatment and the treatment (dose) nested effect were evaluated for statistical significance at $P \leq 0.05$. Pre-planned comparisons within each ecdysone dose were carried out between treatment means using pair wise *t*-tests. Least square mean weights were used in this analysis rather than arithmetic mean weights because different numbers of ticks were used in each treatment. The nested effect for treatment (dose) was highly significant ($P < 0.001$) and within each dose there were significant differences ($P < 0.05$) between the ecdysone injected ticks and those that were either injected with solvent only or not injected (Table 2). Following 20-E injection for all doses tested, tick weight increased 2.7-3.9 fold 4 d after treatment. In contrast, the solvent injected

controls and the ticks not injected demonstrated increases of 3.5-4.9 and 3.6-6.6 fold, respectively, so that the ticks injected with 20-hydroxyecdsone weighed less than the controls after 4 d (Table 2). The average weight of untreated and solvent injected ticks provided males and allowed to mate was 534.9±36.1 (± 1 SE, n=13) and 602.4±36.1 (n=14) mg, respectively. This was a significant increase (t-tests) over that of virgin females not provided males (Table 2) and is typical of mated female ticks feeding to repletion.

3.2. Effect of JH III on oocyte development

Injection of partially fed (virgin) females of the American dog tick still on the host with 1,000 ng of JH III per tick which was equivalent in mass per tick to the highest dose tested for 20-E, produced no effect on oocyte development as determined by observation. Four days after injection, no brown eggs were found in the ovaries of the 18 specimens examined (Table 1). There was no apparent growth of the white eggs, and they all appeared as stage 2 on the Balashov scale. The same observations were noted for the solvent-injected controls. A non-injected control was not conducted in these experiments since no differences were obtained between the solvent and non-injected controls in the earlier experiments that were conducted (Table 1). Also, the mean weight of the females injected with JH III, 107.5 ± 68.1 mg, was not significantly different from the solvent controls (*t*-test, $P \leq 0.05$).

3.3. Effect of 20-E on vitellogenin levels in hemolymph

The most prominent band resolved by native PAGE of the proteins from the eggs of *D. variabilis* demonstrated a molecular weight of 367.5 ± 7.8 k (Fig. 3, lane 2) as determined by comparison with protein standards (Fig. 3, lane 1). This band is the putative vitellin (Vn) from the

American dog tick. A band at a slightly higher molecular weight (394.7 ± 1.2 k) was found in the hemolymph of the 20-E injected (partially fed, virgin) ticks (Fig. 3) 1, 2 and 3 d after treatment (lanes 5, 7 and 9, respectively). This higher molecular weight band, tentatively identified as vitellogenin (Vg), was present only in hemolymph from the 20-E injected ticks and not in the solvent injected controls (Fig. 3, lanes 4, 6 and 8, respectively) and the non-injected control (Fig 3, lane 3). The level of this putative Vg was highest on d 2 post-injection. The same results were observed for all three replicates. Tryptic digestion-mass fingerprinting of the putative Vg band (Fig. 3, lane 7) yielded peptide fragments with cross-correlation values ≥1.5 (Eng et al., 1994) and which were 100% identical to peptide sequence from GP 80, vitellogenin from the cattle tick, *Boophilus microplus* (Table 3). Fragment EPLVSTLPVHYLEEKK had an e-value of 4e-8 to GP80. Carrier protein (CP) (Gudderra et al. 2001 and 2002a,b) was found in all of the hemolymph samples analyzed (Fig. 3). However, it appeared that 20-E injection reduced its abundance as compared to the solvent injected control while the reverse occurred for Vg.

3.4. Cloning and partial sequence of the vitellogenin mRNA

Random sequencing of 192 ESTs from a *Dermacentor variabilis* fat body cDNA library yielded three ESTs with similarity to NCBI accession number U49934, GP80 from *Boophilus microplus* (Tellam et al, 2002). This clone is one of two tick Vg sequences currently in the NCBI nr database. The other is an EST from *Amblyomma americanum*, accession number BI273562; this short sequence originated from the salivary glands of male ticks and has low similarity to other Vg sequences on the database.

The three ESTs obtain in our studies are from three different cDNA clones. The three clones are short (approximately 500 base pairs) and have similar sequences. The longest of the

three inserts (pUA03) was sequenced in its entirety. The partial sequence of *D. variabilis* Vg cDNA is shown in Fig. 4 along with its conceptual translation.

TBLASTN analysis using the partial sequence of pUA03 returned the *Boophilus microplus* clone, GP80 (NCBI U49934) with an expect value of $2e^{-33}$. In an alignment (Fig. 5), the pUA03 insert is 69.6% identical at the nucleotide level and 58% similar and 53.3% identical at the amino acid level to GP80 from *B. microplus*. One of the tryptic digests sequenced (EYPTRHEYPTR, Table 3) overlaps the translation of pUA03 (Fig. 4, underlined). The sequence, EYPTRHEYPTR, is repeated multiple times with minor variations in the carboxy-terminal region of the translated GP80 protein. A similar sequence, EYPTHHKYPTH, repeats in the pUA03 insert (also underlined in Fig. 4). The sequence is repeated exactly twice, and an additional five times with a single amino acid substitution. Based on these similarities with a known tick vitellogenin sequence, we preliminarily assigned the identity of the protein represented by pUA03 as vitellogenin, the tick yolk protein of *D. variabilis*.

3.5. Effects of 20-hydroxyecdsone on expression of the vitellogenin mRNA

Northern blots of total RNA from partially fed (virgin) females of the American dog tick collected 1, 2 and 3 d after the injection of a 50X dose of 20-hydroxyecdysteroid and probed with the pUA03 insert showed up-regulation of the Vg gene (Fig. 6). There was no Vg RNA detected in ticks injected with solvent alone on d 1, 2 and 3 after injection (Fig. 6, lanes 4, 6 and 8, respectively). As expected, there also was no Vg mRNA in untreated partially fed (virgin) females 7 d after attachment (Fig. 6, lane 3). However, strong Vg expression was observed in RNA samples extracted from ovipositing females (Fig. 6, lane 2). In addition, Vg mRNA was found in 20-E injected ticks at 1, 2 and 3 d post-injection (lanes 5, 7 and 9, respectively). The Vg mRNA was slightly higher at 1 and 2

d (Fig. 6) with the Vg protein levels highest on d 2 (Fig. 4). Similar results were observed in all three replicates. Identical blots probed with tick ribosomal protein L10a (NCBI Accession No.: CS663255) as a loading control showed no differences in RNA levels in any lane, indicative of equivalent loading of the RNA samples (data not shown).

4. Discussion

4.1. Role of 20-hydroxyecdysone in the regulation of reproduction

There is mounting evidence that ecdysteroids may have a role in vitellogenesis. For example, several investigators have found a positive correlation in increased hemolymph ecdysteroid levels and vitellogenesis (Dees et al., 1984; Connat et al., 1985; Kaufman, 1991), which might suggest that these hormones are responsible for the initiation of Vg biosynthesis. Kaufman (1991) also found that ecdysteroids accumulate in the ovary, presumably in the oocytes during vitellogenesis, where it plays an important role in embryogenesis. Sankhon et al. (1999) reported that ecdysteroids induced vitellogenesis in fat body from unfed adult females of the American dog tick in organ culture. In these experiments, they found significantly higher levels of Vg (by ELISA) in the incubation medium as compared to controls. These results are somewhat corroborated by earlier studies of Taylor et al. (1997) who found that ecdysteroid injections increased hemolymph Vg concentration in unfed *O. moubata* but only at concentrations that were toxic. More recently, Friesen and Kaufman (2002) found that when ecdysteroids were injected into partially fed, female *Amblyomma hebraeum*, these ticks which would otherwise be non-vitellogenic, contained Vg in their hemolymph as determine by Western blots. In these studies, female ticks were forcibly detached from the host prior to commitment to egg production, were responsive to ecdysteroid treatments, and would release Vg into the hemolymph (Friesen and Kaufman, 2002; Friesen and Kaufman, 2004).

In partially fed (virgin) female adults of the American dog tick, mating is the trigger for the initiation of blood feeding to repletion and the onset of vitellogenesis and oocyte development. In the current study we were able to develop an assay where 20-hydroxyecdysone could be injected into partially fed (virgin) females while the ticks were still attached to the rabbit host. In addition, we partially sequenced the gene for Vg and using Northern blots could follow Vg gene expression. Vg protein in hemolymph could be observed simply by native PAGE where the protein identity was verified by tryptic digestion-mass fingerprinting and BLASTP and oocyte development determined by dissection and classification according to the Balashov scale (Balashov, 1972). It is clear from the results of our studies that the substitution of mating with the injection of 20-hydroxyecdysone while the ticks were still attached to the host was sufficient to initiate egg development. The response was dose dependent, i.e., no response at 1X, incomplete (33%) Vg uptake at 10X, and 74% Vg uptake at 50X the natural 20-E levels of *D. variabilis*, as determined by the percentage of ovaries with brown oocytes (Table 1). This dose response can be easily observed by comparing the photomicrographs between the solvent injected control which was identical to the 1X injection (Fig. 1, A and B) with the 10X (Fig. 1, C and D) and 50X doses (Fig. 1E), by the increasing number of brown eggs that were observed as the dose was increased (Fig. 1) and by the increase in ovary weight approaching that of replete (mated) females (Fig. 2). There was a significant reduction in overall tick weight for 20-E injected, partially fed (virgin) ticks as compared to controls (Table 2) 4 d after injection. This effect was the result of a decrease in weight gain for the hormone-treated ticks and may in part result from the increased metabolic activity associated with vitellogenesis and oocyte maturation. The latter is also supported by the obvious increase in the abundance of excreta observed for the 20-E treated ticks.

The physiological and molecular mechanisms for this 20-E induction of oocyte development although not fully understood, most certainly must include the initiation of synthesis of the Vg mRNA, synthesis of Vg protein, release of Vg protein from the site of synthesis and uptake of Vg into the developing oocytes. By Northern blots and using a partial sequence of the Vg gene from *D. variabilis* as a probe, it was clear that the Vg mRNA is not found in partially fed (virgin) females at any of the developmental times examined but is found in mated, replete (preovipositional) ticks as would be expected and in 20-E injected females (Fig. 6). Our putative, partial Vg clone (pUA03) was 69.6% identical at the nucleotide level and 58% similar and 53.3% identical at the amino acid level to Vg (GP80, U49934) from *B. microplus*. In addition EYPTHHKYPTH repeats were found in pUA03 similar to that of GP80, giving us a high level of confidence that our assignment of the translation product of pUA03 as the *D. variabilis* Vg was correct. In agreement with the appearance of the Vg mRNA after injection of 20-E, we also found a corresponding appearance of Vg protein in the hemolymph (Fig. 3) and it appeared that both the Vg mRNA and protein was abundant at d 2 after 20-E injection. *In toto*, it appears that 20-hydroxyecdysone is directly responsible for the initiation of vitellogenesis at the level of expression of the Vg gene, which ultimately results in the full development of oocytes in the tick ovary.

4.2. Role of juvenile hormone in tick reproduction

In view of the apparent similarity of ticks with insects, it has been assumed for some time that reproduction in the acari as in insects is regulated by juvenile hormone. This has been most recently reviewed by Roe et al. (2001) and Gilbert et al. (2000). Although there have been reports of JH treatment effects in ticks, Neese et al. (2000) was unable to detect any radiosynthesis of farnesol, methyl farnesoate, JH I, JH II, JH III, or JH III bisepoxide from common JH precursors in different

tissues and at different developmental stages in two tick species. In these studies, the lower level of detection for [*methyl*-^{3}H]-JH III and -methyl farnesoate was 1.27 fmol 10-synganglia^{-1} 3-h^{-1}. In addition, they were unable to find any of the common insect JHs in tick hemolymph by EI GC-MS and were unable to detect any compounds in whole body tick extracts that would produce a positive *Galleria* bioassay. It is apparent from the current study that the injection of 1,000 ng of JH III per tick had no effect on a single oocyte in the ovaries of 18 ticks tested in our studies (Table 1). 20-hydroxyecdysone at one-fifth this dose, produced brown eggs and at the same dose of JH III advanced most of eggs in the ovary to category 4 and 5 mature eggs (Table 1). JH III injections also had no effect on tick weight unlike that noted for 20-E (discussed earlier). Although it is difficult to prove a negative, so far there is no direct evidence that ticks have JH like that in insects, and it appears that an ecdysteroid (probably 20-E) and not JH regulates vitellogenesis.

4.3. Regulation of reproduction in ticks

When partially fed (virgin) adult females of the American dog tick are allowed to mate, mating initiates engorgement to repletion and then detachment from the host (Pappas and Oliver, 1972). Vitellogenin expression, oocyte growth and vitellogenin uptake follows detachment, usually within 1 or 2 d. The high dose of ecdysteroid injected into partially fed virgins in our current study not only was able to cause oocyte growth and vitellogenin uptake but egg development was accelerated over that found in nature. However, engorgement to repletion was not triggered by 20-E. Pappas and Oliver (1972) found an engorgement factor in the male gonad of the American dog tick. In another tick, Ambloyomma hebraeum, feeding is initiated by two small peptides found in the testes/vas deferens and which are introduced when the female is inseminated (Weiss and Kaufman, 2004).

There is also considerable evidence in soft ticks of hormones in the anterior of ticks that can initiate egg development. For example, Oliver et al. (1992) found that vitellogenesis was inhibited in *O. parkeri* when ticks were ligated between legs 2 and 3 within the first few days after blood feeding. Transplantation of synganglia into the posterior portion of the ligated tick was able to induce vitellogenesis and oocyte development to final maturation. Aeschlimann (1968) found that *O. moubata* virgin females could lay eggs after receiving a synganglion homogenate from fed mated females, and Shanbaky and Khalil (1975) found that injection of synganglion homogenate from vitellogenic females stimulated oocyte development in the ligated synganglion-less posterior of fed mated females of *Argas arboreus*. This research was reviewed by Chinzei and Taylor (1990), Sonenshine (1991), and Oliver et al. (1992). Lomas et al. (1997) also discovered a peptidic hormone from the synganglion that appeared to control ecdysteroid biosynthesis. Whether this hormone in turn regulates vitellogenesis is unknown. Although our understanding of tick reproduction has advanced greatly in the last few years and it appears that ecdysteroids and not JH regulate vitellogenesis, there are still many unanswered questions. For example, what is the tissue source of the ecdysteroids that regulate reproduction, what is the mechanism for the male induction of egg development and blood feeding in different tick species, what is the role of the female reproductive tract and the synganglion in the initiation of feeding and reproduction, and what factors regulate a possible Vg receptor. These and no doubt other questions will need to be addressed before we have a complete understanding of the regulation of tick reproduction.

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Figure Captions

Fig. 1. Photomicrographs of ovaries from partially fed virgin females of the American dog tick, *D. variabilis,* injected with 20-hydroxyecdysone (20-E) or the solvent control 4 d after attachment and then examined 4 d post-injection. A and B, injected with solvent only (50X magnification, bars = 1.66 mm); C and D, injected with a 10 X dose of 20-E (60 X magnification, bars = 2 mm); E injected with a 50 X dose of 20-E (120 X magnification, bar = 2 mm).

Fig. 2. Changes in the ovary weight of normal mated females of *D. variabilis* before and after drop off from the rabbit host compared to that of partially fed virgin females injected with 20-hydroxyecdysone (a 50 X dose) or solvent only 4 d after attachment and then examined 4 d post-injection. Each value is the mean ± 1 standard error of the mean for a sample of 6 individuals. Mean ovary weight increased in a significant curvilinear relationship (Microsoft Excel 2000, Microsoft Corp., Seattle, WA) with age before and after drop off.

Fig. 3. Native gel stained for total protein showing the presence of vitellogenin (Vg) in hemolymph collected from partially fed virgin females of *D. variabilis* injection with 20-hydroxyecdysone (20-E, a 50 X dose) 4 d after attachment and examined 1, 2 and 3 d post-injection. Lane **1** = molecular weight markers of BSA and apoferritin (unlabeled protein, putative subunit of apoferritin); **2** = vitellin (Vn) from *D. variabilis* eggs; **3** = partially fed virgin females 7 d after attachment, not injected; **4** = d 1 after injection, solvent only; **5** = d 1, 20-E injected; **6** = d 2, solvent only; **7** = d 2, 20-E; **8** = d 3, solvent only; **9** = d 3, 20-E. CP = carrier protein; MW = molecular weight markers; Vn = vitellin.

Fig. 4. Sequence of a partial clone of *D. variabilis* vitellogenin. Numbering begins at the start of the pUA03 insert. Nucleotide sequence in the coding region is presented in upper case letters, sequence in the 3' non-coding region is presented in lower case letters. The predicted amino acid sequence is shown above the nucleotide sequence using single letter designations for amino acids. Two regions with similarity to one of the tryptic digest products are underlined. The sequence corresponding to the presumed poly-adenylation signal, AATAAA, is double-underlined.

Fig. 5. Alignment of pUA03 (Fig. 5) with the corresponding portion of GP80 (U49934). Numbering is according to the amino acid sequence of pUA03. Identities are shown with light shading. Similarities are shown with dark shading. A gap is indicated by dashes (-). The stop codon is indicated by an asterisk (*).

Fig. 6. Northern blot showing the time course of expression of the vitellogenin gene (Vg) in total RNA from partially fed virgin females of *D. variabilis* following injection with 20-hydroxyecdysone (20-E, a 50 X dose) 4 d after attachment to the host and examined 1, 2 and 3 d post-injection. Lane **1** = nucleic acid markers; **2** = replete, pre-ovipositing female; **3** = partially fed virgin female 7 d after attachment, untreated; **4** = d 1 after injected, solvent only; **5** = d 1, 20-E injected; **6** = d 2, solvent only; **7** = d 2, 20-E; **8** = d 3, solvent only; **9** = d 3, 20-E. Right arrow indicates Vg transcript. Nt = nucleotides.

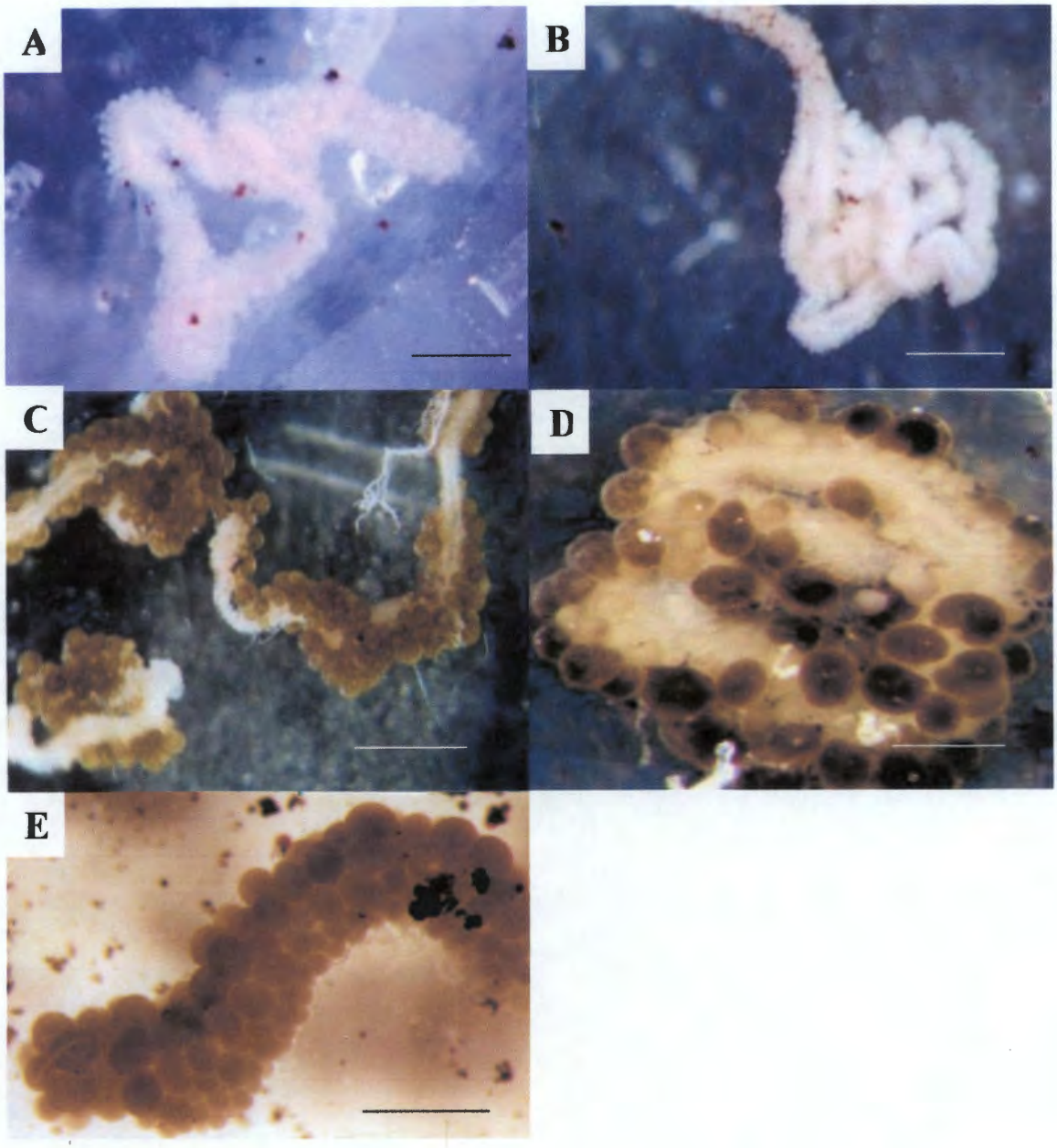
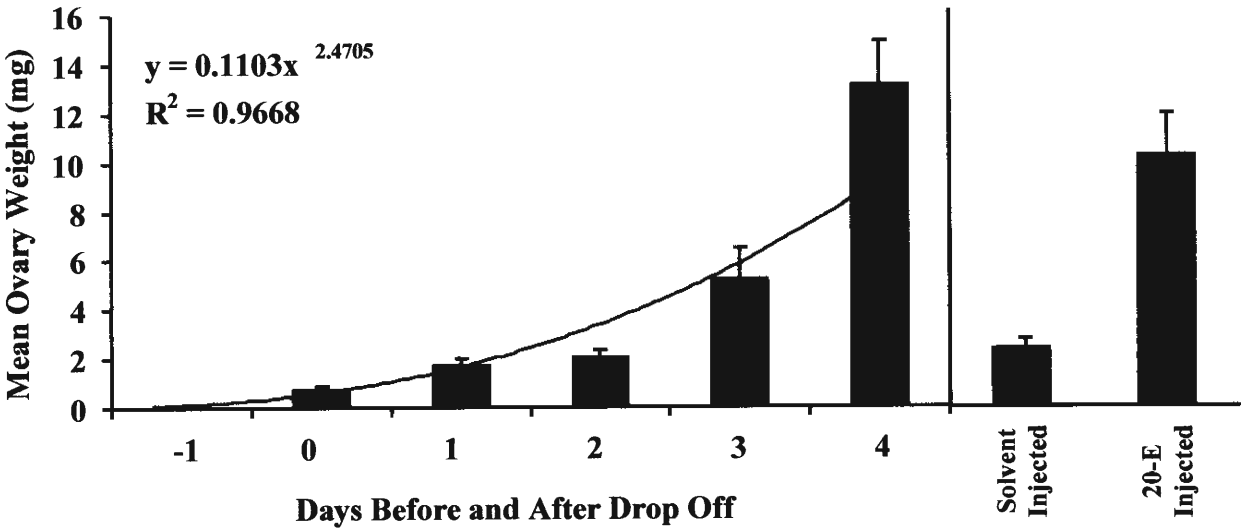


Fig. 1

Fig. 2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14
12
10
8
6
4
2
0
y = 0.1103x 2.4705
R2 = 0.9668
-1
0
1
2
3
4
Solvent Injected
20-E Injected
Days Before and After Drop Off

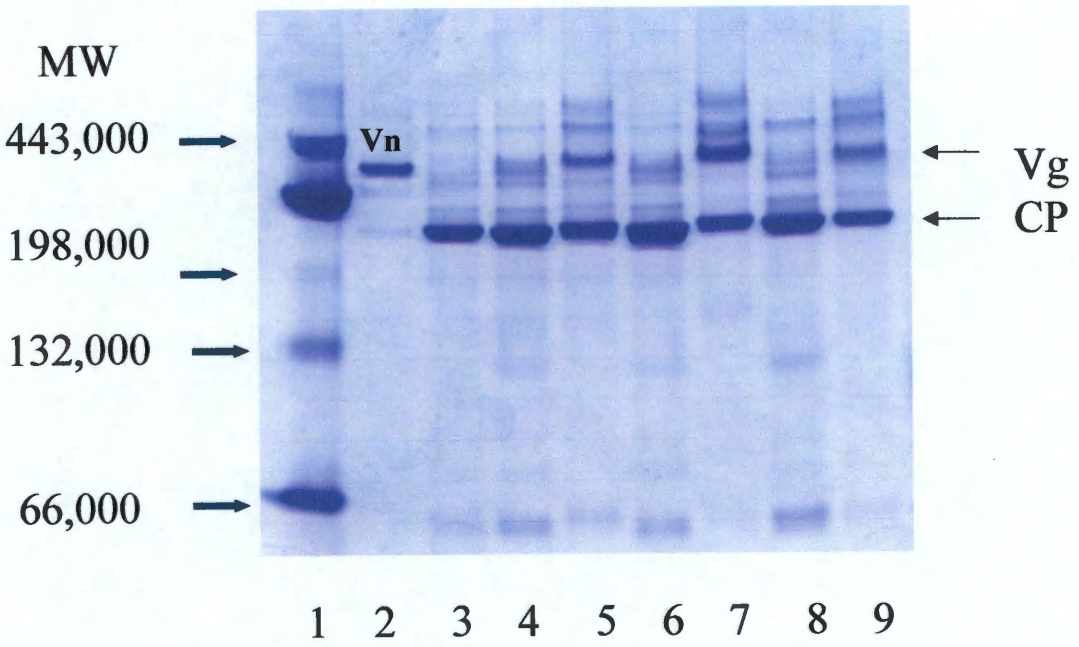

Fig. 3

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      P  S  H   S  K  Y   P  S  H  H   K  Y  P   S  H  K
1     ACCCCAGCCA CTCGAAGTAC CCGTCCCACC ACAAGTATCC GAGCCACAAG
      E  Y  P  T   H  H  K   Y  P  S   H  E  E  Y   P  T  H
51    GAGTACCCTA CCCACCACAA GTACCCCAGC CACGAAGAGT ACCCAACCCA
       H  K  Y   P  S  H  E   E  Y  P   T  H  H   K  Y  P  S
101   CCACAAGTAC CCCAGCCACG AAGAGTACCC AACCCATCAC AAGTACCCCA
        H  E  E   Y  P  T   H  H  K  Y   P  S  H   E  E  Y
151   GCCACGAAGA GTACCCAACC CACCACAAGT ACCCCAGCCA CGAAGAGTAC
      P  T  H  H   K  Y  P   S  H  E   E  Y  P  T   H  H  K
201   CCAACCCACC ACAAGTACCC CAGCCACGAA GAGTACCCAA CCCACCACAA
       Y  P  T   H  K  K  Y   P  T  H   K  H  P   T  H  H  E
251   GTACCCTACG CACAAGAAGT ACCCAACTCA CAAGCACCCG ACCCACCACG
        Y  P  T   H  H  K   Y  P  T  H   H  K  Y   P  S  H
301   AGTACCCGAC CCACCACAAG TACCCGACCC ACCACAAGTA CCCGTCCCAC
      H  E  Y  P   P  S  R   E  H  Y   P  Y  S  P   S  L  Q
351   CACGAGTACC CTCCCAGCCG CGAACACTAC CCGTACAGCC CGAGCCTCCA
       R  E  G   *
401   GCGCGAAGGG TAGcatattt gtttttaag acacgggaat atcacttcaa
451   aaaataggtt tcaggcgcaa cgtggcgtcg ctatgttcat ctacaagccc
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551   tcgggttgac atataaaaaa aaaaaaaaaa aaa
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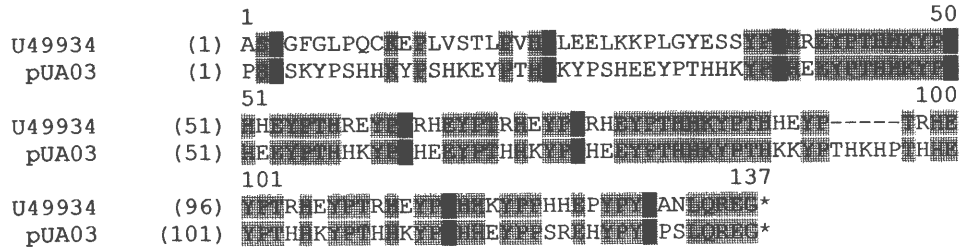
Fig. 4


1
50
U49934 (1)
pUA03 (1)
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100
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pUA03 (51)
101
137
U49934 (96)
pUA03 (101)

Fig. 5

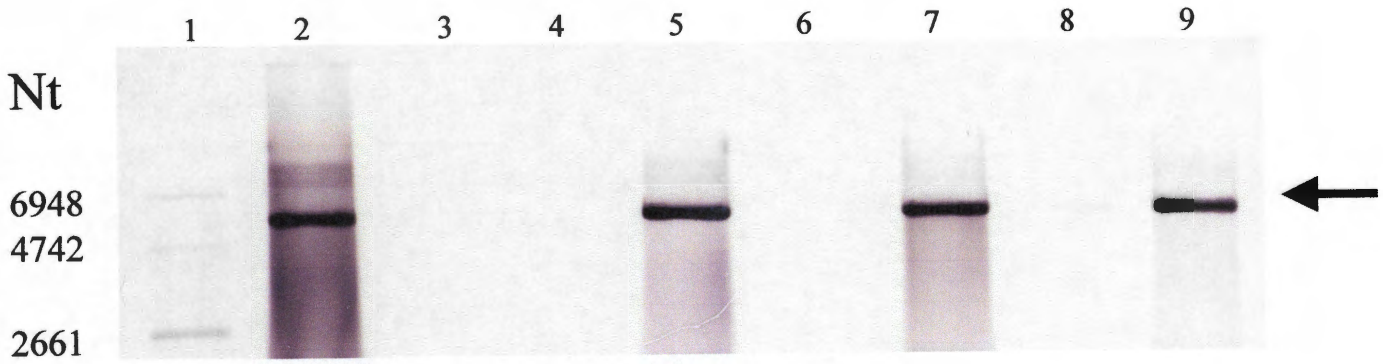

Fig. 6

Table 1. Stimulation of egg development after the *in vivo* injection of 20-hydroxyecdysone (20-E) or juvenile hormone III into partially fed virgin female adults of *Dermacentor variabilis* 4 d after attachment to the rabbit host.

Cpd/Dose		Hormone treated			Solvent control			Not injected		
20-E	Replicate	n	Ovary Color[1]	Category[2]	n	Ovary Color[1]	Category[2]	n	Ovary Color[1]	Category[2]
1X 20ng/tick	1	5	5 white	2	5	5 white	2	5	5 white	2
	2	5	5 white	2	5	5 white	2	5	5 white	2
	3	5	5 white	2	5	5 white	2	5	5 white	2
10X 200ng/tick	1	5	5 brown	3,4	5	5 white	2	5	5 white	2
	2	5	2 brown, 3 white	3	5	5 white	2	5	5 white	2
	3	5	3 brown, 2 white	3,4	5	5 white	2	5	5 white	2
50X 1000ng/tick	1	9	4 brown, 5 white	4,5	5	5 white	2	5	5 white	2
	2	5	5 brown	4	5	5 white	2	5	1 brown, 4 white	2,3
	3	5	5 brown	4,5	5	1 brown, 4 white	2,3	5	5 white	2
JH-III										
1000ng/tick	1	8	8 white	2	5	5 white	2		ENC[3]	
1000ng/tick	2	10	10 white	2	5	5 white	2		ENC	

[1]Ovaries from each tick contains either all white eggs (designated white) or at least 1 brown egg (designated brown).
[2]Categories based on Balashov (1972). For details, see text.
[3]ENC = Experiment not conducted.

Table 2. Effect of *in vivo* injections of 20-hydroxyecdysone (20-E) into partial fed virgin female *Dermacentor variabilis* (4 d after attachment) on tick weight (8 d after attachment).

	Least Squares Mean weight (mg) ±1 SE[1]					
Dose[2]	20-E injected	n	Solvent injected	n	Not injected	n
1X	127.3 (21.8)a	17	160.3 (21.8)b	17	214.3 (17.0)b	28
10X	86.3 (17.6)a	26	114.0 (15.0)b	36	117.7 (15.0)b	36
50X	98.0 (12.1)a	55	155.7 (16.7)b	29	186.0 (9.8)b	83

[1]Within each dose, means followed by the same letter are not significantly different ($P > 0.05$) by pairwise *t*-tests. Tick weights were square root transformed prior to statistical analyses.

[2]20-hydroxyecdysone doses used were 1X = 20 ng/tick, 10X = 200 ng/tick and 50X = 1000 ng/tick.

Table 3. Tryptic digestion/mass spectrometry analysis of the putative vitellogenin band (394,700 molecular weight) resolved from the hemolymph of partially fed virgin females of *Dermacentor variabilis* injected with a 50 X dose of 20-hydroxyecdysone 4 d after attachment and analyzed 2 d after injection (lane 7, Fig. 3).

Representative partial amino acid sequence	Mass (D) Peptide	X Corr.[1]	Identification[2] (%-identity with GP80)
EPLVSTLPVHYLEELKK	1995.1	1.892	Vitellogenin[1] (100%)
EYPTRHEYPTR	1448.7	1.510	Vitellogenin[1] (100%)
VLVGYLNALGK	1146.7	3.949	Vitellogenin[1] (100%)
YLVTSDCSAK	1143.5	3.247	Vitellogenin[1] (100%)
HPANHVLASR	1101.6	2.851	Vitellogenin[1] (100%)
HYYVDVK	923.5	2.267	Vitellogenin[1] (100%)
TLLEGK	660.4	1.944	Vitellogenin[1] (100%)
YNLGPYVK	953.5	1.706	Vitellogenin[1] (100%)
AVLFAK	648.4	1.500	Vitellogenin[1] (100%)

[1]Cross-correlation value (Eng et al., 1994).

[2]GP 80 vitellogenin protein from the cattle tick, *Boophilus microplus.*