Effects of 20-Hydroxyecdysone and other Hormones on Egg Development, and Identification of a Vitellin-Binding Protein in the Ovary of the Tick, *Amblyomma hebraeum*

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Abstract

Partially-fed adult female *Amblyomma hebraeum* ticks were injected with 20-hydroxyecdysone (20E; up to 43 μg/g body weight [bw]), juvenile hormone III (JH III; up to 100 μg/g bw), bovine insulin (up to 2000 mU/g bw), or triiodothyronine (up to 200 ng/g bw) in an attempt to stimulate vitellogenesis. Of these, only 20E stimulated synthesis and release of vitellogenin (Vg). Immunoblot analysis revealed that Vg-synthesis occurred in the fat body. However, consistent with earlier observations suggesting that a distinct signal may be required for Vg-uptake, there was no significant Vg-uptake by oocytes of partially-fed, 20E-treated ticks. Because Vg-uptake commonly occurs via receptor-mediated endocytosis (i.e., a specific Vg-receptor), we attempted to identify a vitellin (Vt)-binding protein in ovaries of engorged female ticks. A single 86 kDa Vt-binding protein was identified, even under reducing conditions (2-mercaptoethanol), by a ligand-blotting technique. Sodium salt of suramin (5 mM) inhibited binding of Vt to the 86 kDa protein. However, this protein was also detected in ovaries from small partially-fed ticks (50-100 mg), suggesting that the inability of 20E to stimulate Vg-uptake in partially-fed ticks may not have been due to the absence of a Vg-receptor.

Keywords: ixodid ticks, *Amblyomma hebraeum*, vitellogenesis, vitellogenin, vitellin, 20-hydroxyecdysone, juvenile hormone, vitellogenin-receptor, insulin, triiodothyronine (T_3)

1. Introduction

In insects, synthesis of vitellogenin (Vg) is regulated by the juvenile hormones (JHs), and/or the ecdysteroids (Nijhout, 1994). Much less is known about vitellogenesis in ticks and other chelicerate arthropods (Kaufman, 1997; Chang and Kaufman, in press). A series of experiments by Chinzei et al. (1992) on the argasid tick, *Ornithodoros moubata*, indicates that Vg-synthesis is initiated by a 'vitellogenesis inducing factor' (VIF); VIF is a neuropeptide that acts on some unknown tissue to cause the release of a 'fat body stimulating factor' (FSF). FSF is the vitellogenic hormone. The identity of

FSF has not been established, but an ecdysteroid hormone has been implicated (Taylor et al., 2000; Ogihara, 2003). The weight of current evidence is in favor of an ecdysteroid being the vitellogenic hormone in ixodid ticks:

- Following engorgement, the rise in hemolymph ecdysteroid–titer correlates with the period of egg development in *A. hebraeum* (Kaufman, 1991; Friesen and Kaufman, 2002) and in *Ixodes scapularis* (James et al., 1997).

- Injections of 20E into small partially-fed ticks (*A. hebraeum*) stimulates enlargement of fat body, possibly a reflection of accelerated Vg-synthesis (Lunke and Kaufman, 1993). However, slow infusion of 20E into partially-fed ticks did not stimulate Vg-uptake by oocytes in the latter study.

- 20E, but not the JH-analogue, methoprene, stimulates Vg-synthesis by *Dermacentor variabilis* fat body *in vitro* (Sankhon et al., 1999). 20E also stimulates Vg-synthesis when injected into *A. hebraeum* (Friesen and Kaufman, 2002).

Numerous neurosecretory centers have been identified in the synganglion (= tick CNS) (reviewed by Binnington and Obenchain, 1982). Neurosecretory activity in general seems to increase shortly after a blood meal, and then declines during egg production (Eisen et al., 1973; Shanbaky et al., 1990), suggesting that a neuropeptide may be part of the pathway controlling vitellogenesis (Chinzei et al., 1992). Likewise, Lomas et al. (1997) demonstrated that a peptide from the synganglion stimulates ecdysteroid-synthesis in *A. hebraeum.* While no specific neurosecretory products have been fully characterized in ticks, antibodies against bovine insulin bind to three neurosecretory regions in the syngangion of *Ornithodoros parkeri* (Zhu et al., 1991). Vertebrate insulin has up to 68% nucleotide sequence homology to the ecdysteroidogenic peptide, bombyxin (Iwami et al., 1989). Both bovine and porcine insulins, acting through insulin-like receptors, can stimulate ecdysteroid-synthesis in isolated ovaries of the mosquito, *Aedes aegypti* (Graf et al., 1997; Riehle and Brown, 1999).

Engorged argasid ticks treated with precocene, an inhibitor of JH synthesis (Retnakaran et al., 1985), produced and laid fewer eggs than untreated engorged ticks, an effect which was partially reversed by topical application of JH (Pound and Oliver, 1979). Moreover, JH and a variety of JH-like compounds stimulated oviposition in fed, virgin argasid ticks (Connat et al., 1983). However, Taylor et al. (1991a) demonstrated that JH did not stimulate Vg-synthesis in unfed, virgin argasid ticks, even though vitellogenesis did occur in such ticks when treated with pyrethroid insecticides. Finally, although extracts of *Boophilus microplus* have JH-like bioactivity (Connat, 1987), no known JH has been identified in ticks by gas chromatography/mass spectrometry (Connat, 1987; Neese et al., 2000). Taylor et al. (1991b) concluded that, whereas JH appears not to stimulate Vg-synthesis in argasid ticks, it may have stimulated ovulation and/or oviposition in those studies in which ticks were treated with exogenous JH (eg: Pound and Oliver, 1979; Connat et al., 1983; Connat, 1987).

In some insects, thyroxine interacts with JH-receptors and mimics JH effects. For example, the protein and RNA content of the fat body in *Bombyx mori* increases following injection of thyroxine (Chaudhuri and Medda, 1987). Likewise, both thyroxine, and triiodothyronine (T_3) bind to putative JH-receptors on the follicular epithelium of *Rhodnius prolixus* and *Locusta migratoria*, and thus stimulate patency of the follicular cell layer in the same manner as JH (Davey and Gordon, 1996; Kim et al., 1999). Consequently, here we test whether T_3might also stimulate vitellogenesis in ticks.

In this study, the effects of 20E, insulin, JH-III, and T_3 on vitellogenesis were examined in *A. hebraeum*. As shown previously, 20E does not stimulate Vg-uptake in the oocytes of partially-fed ticks (Lunke and Kaufman, 1993), even though 20E stimulates Vg-synthesis *in vivo* (Friesen and Kaufman, 2002). The possibility arises that up-regulation of a Vg-receptor (VgR) in the ovary is controlled by a factor distinct from the vitellogenic hormone, a factor which is absent in small partially-fed ticks. Thus, in this study we also attempted to identify a putative VgR and determine when it appears in the ovary during the feeding cycle.

2. Materials and Methods

2.1 Ticks

Our colony of *A. hebraeum* was kept in darkness at 27°C and >95% relative humidity. Tick feeding occurred on rabbits as described by Kaufman and Phillips (1973). Ticks were allowed to engorge and detach spontaneously (weight range 900-3500 mg), or were forcibly removed from the host below the approximate 'critical weight' necessary to trigger vitellogenesis (< 250 mg; Kaufman and Lomas, 1996; Weiss and Kaufman, 2001). Just prior to conducting experiments, ticks were rinsed in tap water and weighed.

2.2 Hormone treatments

For injection of hormones, stock solutions were made as follows: 20E (Sigma, St. Louis, MO, USA) was dissolved in 70% ethanol to make a 5 mg/ml solution; bovine insulin (Sigma) was dissolved in dilute HCl (pH 2.5) to a final concentration of 5 mg/ml; T_3 (Sigma) was dissolved in 1 N NaOH to a final concentration of 4 mg/ml. On the day of injection, the stock solutions were diluted to working concentration in sterile-filtered medium TC199 (Gibco, Burlington, ON, Canada) containing 50 µg/ml gentamicin (Sigma). Experimental ticks were surface sterilized in 70% ethanol for 1 min, and injected through the camerostomal fold with 5 µl (for insulin and T_3 treatments) or 10 µl (for 20E treatment) per 100 mg body weight (bw) via a 30 gauge needle fitted to an AGLA micrometer syringe unit (Wellcome Reagents Ltd., Beckenham, Kent, England). Final doses of these hormones were within the range 0.0043-43 µg 20E/g bw, 20-2000 mU insulin/g bw, and 100-200 ng T_3/g bw. Vehicle-injected and/or non-injected ticks served as controls. Second and third bolus injections were given to each tick on days 2 and 5. Ticks were kept under colony conditions until their hemolymph and tissues were collected on day 10 (see below).

For topical application, JH III (Sigma) was dissolved in acetone to a concentration of 10 mg/ml; final concentrations for treatments were 2, 5, and 10 mg/ml. On days 0, 2, and 5 post-removal, partially-

fed ticks received a volume of 1 µl/100 mg bw JH III solution applied to the dorsal cuticle. Hence, the final doses were 20, 50, and 100 µg JH III/g bw. Control ticks were treated with 1 µl/100 mg bw acetone. Ticks were stored under colony conditions until day 6, when hemolymph and tissue samples were collected (see below).

2.3 Collection of tissue and hemolymph samples

Tick survival was determined at the time of dissection. Apart from those ticks injected with the highest dose of 20E (43,000 ng/g bw), 40% of which died by day 10, the survival of all other groups was greater than 96 %

For collection of hemolymph and ovaries, ticks were stuck to disposable petri dishes with cyanoacrylate glue and cooled in a refrigerator for 20 min. Cooling inhibits gut contraction, thereby reducing the risk of piercing the delicate gut wall and contaminating the hemolymph (Kaufman, 1991). A small incision was made in the cuticle, and hemolymph was collected in a calibrated glass micropipette; exact sample volume was calculated from the length of the fluid column. Samples were diluted at least 1:10 in 100% methanol for radioimmunoassay (RIA), or 1:4 in phosphate-buffered saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0) for electrophoresis or ELISA. Samples for RIA were stored at -15°C and all other samples were stored at -70°C until analyzed.

After collection of hemolymph, ticks were flooded with a modified Hank's balanced saline (200 mM NaCl, 8.9 mM D-glucose, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 27 µM phenol red, pH 7.2) and the dorsal cuticle removed using a microscalpel. Gut, salivary glands, Malpighian tubules, trachea, reproductive tract, and fat body were removed and immediately frozen on dry ice for immunoblot analysis.

Unlike most insects, ticks possess a single, U-shaped, tubular ovary, terminating in a pair of oviducts. In metastriate ixodid ticks (including *A. hebraeum*), these oviducts converge to an inconspicuous common oviduct, which in turn leads to the vagina (Diehl et al., 1982). A lateral groove

runs the length of the ovary. Oocyte development is asynchronous: immature oocytes are always found within the lateral groove throughout the extneded period of oogenesis, whereas mature ones are suspended from the ovarian wall opposite the groove. In common with all chelicerate arthropods, no follicular epithelium surrounds the oocytes (Kaufman, 1997; Chang and Kaufman, in press).

Ovaries were dissected out, and the length of the long axis of the eight apparently largest ovoid oocytes was measured using an ocular micrometer fitted to a compound microscope. The mean value for the eight oocytes was recorded for each tick. The ovaries were then gently blotted and weighed. Prior to analysis, all tissues were homogenized in PBS containing protease inhibitors (Protease Inhibitor Cocktail Set 1, Calbiochem, La Jolla, CA), centrifuged at 13,000 X g for 5 min and the pellet discarded. Supernatants were stored at -70°C for further analysis.

2.4 Assay for salivary gland degeneration

Because 20E triggers salivary gland degeneration (Kaufman, 1991), we also measured salivary gland function in this study using the technique of Harris and Kaufman (1984). Briefly, paired salivary glands were excised from each tick 5 or 10 days post-engorgement, and the main duct ligated using a very fine strand of silk thread (Dermalon®, Syneture, Norwalk, CT); the silk strand weighed less than the sensitivity of the balance [10 µg,]). The glands were gently blotted, weighed to the nearest 10 µg and incubated in medium TC 199 (Gibco) containing 10 µM dopamine (Sigma) for 10 min, blotted again, and re-weighed. Because dopamine stimulates salivary fluid secretion and the fluid cannot escape through the ligated duct, any fluid transported from the bathing medium accumulates within the acinar lumen and results in an increase in salivary gland weight. The net weight increase is thus a direct measure of fluid secretory competence. Loss of fluid secretory competence compared to controls is thus a quantitative measure of salivary gland degeneration (Harris and Kaufman, 1984).

2.5 Preparation of partially-purified vitellin (Vt)

Vt was partially-purified from the ovaries of day 10 engorged ticks as described previously (Friesen and Kaufman, 2002). Briefly, an ovary homogenate was passed through a gel filtration column (Superose 6B, Pharmacia; now Pfizer, Kirkland, QUE, Canada) and then an ion exchange column (DEAE-Sephacel, Pharmacia). The flow rate (1-3 ml/min) of the columns was maintained by a peristaltic pump. Tick Vg contains a heme moiety (Sonenshine, 1991), so the fractions eluted from each column which contained large amounts of both heme and protein (determined by spectrophotometry; Kaufman et al, 1986) were pooled and concentrated using Centriprep™ centrifuge tubes (Amicon, Beverly, MA, USA). The protein concentration of the resulting extract was measured using the Bradford assay (Bradford, 1976; Bradford reagent kit; Sigma), using bovine serum albumin (BSA; Sigma) as a standard, and the extract frozen at -70°C in 20 µl aliquots.

2.6 Electrophoresis, immunoblotting, and ligand blotting

Proteins from tissue homogenates were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis; Laemmlli, 1970) under reducing or non-reducing conditions. Samples (all at 100 µg, except for Fig. 3 where it was 30 µg) were diluted in electrophoresis sample buffer (60 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.0025% (w/v) bromophenol blue, 2% (w/v) SDS, with or without 2-mercaptoethanol (5%) for reducing gels and non-reducing gels, respectively) prior to being applied to the gels. Electrophoresis was performed using mini-Protean equipment (Bio-Rad, Hercules, CA). All gels consisted of a 3% stacking gel, and a 7.5% resolving gel. Electrophoresis was performed at 110V until the dye front reached the bottom of the gels. Proteins were then transferred to polyvinylidene difluoride membrane (PVDF; BioRad) for immunoblotting. Transfers to PVDF membrane were for 1 h at 0.3-0.5 mA. Antibodies used for immunoblots and ELISA (see below) were prepared against *A. hebraeum* Vg, which consists of two hemolymph polypeptides, Vg211 and Vg148, identified according to criteria presented by Friesen and Kaufman (2002). Membranes containing

transferred proteins were first kept overnight in blocking buffer (5% skim milk, 2% BSA, in Tris-buffered saline [TTBS: 60 mM Tris-HCl, 0.3 M NaCl, 0.04% (v/v) Tween 20, pH 7.5]). For antibody reactions, strips were then placed in blocking buffer containing either anti-Vg211 (1:500) or anti-Vg148 (1:250), or both. After 1 h, strips were washed three times for 5 min with blocking buffer, then placed in secondary antibody (alkaline phosphatase (AP)-conjugated, goat anti-rabbit IgG; BioRad) diluted 1:2000 in blocking buffer. One hour later, strips were washed three times for 5 min in TTBS and then stained for approximately 20 min using an AP-conjugate substrate kit (BioRad).

In order to identify the putative VgR, ligand blotting was performed in a manner similar to that described by previous authors (Dhadialla et al., 1992; Sappington et al., 1995; Romano and Limatola, 2000). Briefly, protein resolved by SDS-PAGE was transferred to PVDF membrane as described above for immunoblots, and then blocked for 30 min in blocking buffer. The membrane containing transferred proteins was then incubated overnight at 4°C with 1 mg/ml Vt (partially purified from day 10 engorged ovaries) in dilute blocking buffer (2.5% skim milk, 1% BSA, TTBS). Control strips were incubated with only dilute blocking buffer. In some cases, the sodium salt of suramin (5 mM; Sigma) was added to the Vt solution to test for inhibition of Vt binding (Dhadialla et al., 1992; Sappington et al., 1995). Following overnight incubation, the membrane was washed and treated with a combination of anti-Vg211 and anti-Vg148 antibodies, as described above for immunoblots. The latter method detects both endogenous Vg and/or Vt in ovary homogenates as well as any protein which binds exogenous Vt. Thus, for a polypeptide to be designated as a ‘Vt-binding protein', it must appear in blots exposed to exogenous Vt but not in control blots.

Immunoblots and ligand blots were converted to digital format using a Hewlett-Packard hpscanjet 3570c benchtop scanner and Adobe Photoshop® software. All images were scanned at a resolution of 600 pixels/inch. Mobilities of polypeptide bands were then measured using NIH Image software (National Institute of Health, U.S.A.), and the relative mobility of each band calculated in

relation to the molecular weight standards. In the case of Figure 7, some background discoloration was removed using Adobe Photoshop®.

2.7 RIA for ecdysteroid

Ecdysteroid-concentration in tick hemolymph was determined by the RIA procedure described by Kaufman (1990). Briefly, a methanol extract of each hemolymph sample was dried under vacuum and diluted in 0.2 M borate-BSA (5 mg/ml) buffer (pH 8.5). Samples were then incubated overnight at 4 °C with ~8000 cpm ^{3}H-ecdysone (New England Nuclear; now PerkinElmer Life and Analytical Sciences; Woodbridge, ON, Canada) per tube and an ecdysone antiserum (E-22-succinyl-thyroglobulin; supplied by Dr. L.I. Gilbert, University of North Carolina, and Dr. E. Chang, University of California, Davis). 20E standards (0-500 pg) were treated similarly. Antiserum-bound ligand was separated from free ligand by precipitation with protein A, prepared according to the procedures of Kessler (1981). The pellet was resuspended in 50 µl distilled water and 4 ml scintillation fluid (Scintiverse E; Fisher Scientific). Radioactivity of bound ligand was monitored by liquid scintillation spectroscopy. The limit of detection using this protocol was about 10 pg 20E. Further details are presented in Kaufman (1990). Since the ecdysone antiserum cross-reacts with several ecdysteroid analogues, all RIA data are expressed as '20E-equivalents'.

2.8 ELISA for Vg and Vt

Hemolymph Vg and ovary Vt were quantified using an indirect competitive ELISA as described by Friesen and Kaufman (2002). Partially purified Vt from day 10 ovaries was used as the standard for determining the concentration of unknown samples. Briefly, wells of a 96-well microtitre plate were coated with 1 µg partially-purified Vt. A mixture of anti-Vg antibodies, plus either known concentrations of Vt or unknown samples was added to each well. After incubation, the amount of antibody binding to the plate-bound Vt was quantified using an alkaline phosphatase (AP)-linked goat

anti-rabbit secondary antibody (BioRad) and an AP color substrate kit (p-nitrophenylphosphate and diethanolamine kit; BioRad). The color reaction was quantified by measuring the absorbance of each well at 405 nm using a microtitre plate reader (Bio-Tek Instruments, Winooski, VN). Absorbance values of known Vt-concentration were plotted on a logarithmic scale to create a standard curve from which concentration of Vg or Vt in unknown samples were calculated and reported as 'Vt-equivalents'. The sensitivity of this ELISA to *A. hebraeum* Vg was approximately 5 ng of Vt-equivalents

2.9 Statistical analysis

Results are reported as mean ± SEM (n). Statistical analysis was done using Statview 4.02 (Abacus Concepts, Berkely California). Differences among treatments were analyzed using a 1-way analysis of variance (ANOVA). Statistical significance is indicated as follows: (*) $0.01<p<0.05$; (**) $0.001<p<0.01$; (***) $p<0.001$.

3. Results

3.1 Effect of 20E

Injection of 20E had no significant effect on ovary weight (Fig. 1A) or oocyte length (Fig. 1B) in partially-fed ticks by 5 or 10 days post-treatment. However, 20E caused a marked increase in hemolymph Vg-concentration (Fig. 1C). Vg-concentration was highest on day 5 at 18.3 ± 3.8 mg/ml (approximately 30 times the day 5 EtOH control) and on day 10 at 35.0 ± 5.5 mg/ml (approximately 20 times the day 10 EtOH control), following 3 bolus injections of 4,300 ng 20E/g bw. On both days 5 and 10, ticks treated with 43,000 ng 20E/g bw had significantly lower hemolymph Vg-concentrations than those treated with 4,300 ng 20E/g bw ($p < 0.0001$). Moreover, when dissecting day 10 ticks that had been treated with the highest dose of 20E, we noticed that the hypodermis had fallen away from the surrounding cuticle, suggesting a very abnormal type of apolysis.

Salivary gland weight and salivary gland fluid secretory competence decreased in 20E-treated partially-fed ticks (Fig. 2). Compared to untreated controls, EtOH caused a significant increase in salivary gland fluid secretory competence by day 10 ($p < 0.009$; Fig. 2B). However, at both day 5 and day 10, salivary gland fluid secretory competence was significantly lower at 430 ng 20E/g bw and above than both untreated and EtOH controls (Fig. 2B).

Anti-Vg211 antibody (Friesen and Kaufman, 2002) bound to a single 219 kDa polypeptide that was present in the fat body of both day 10 engorged and 20E-treated partially-fed ticks (Fig. 3). Anti-Vg148 antibody bound to three polypeptides of 211, 169, and 78 kDa in fat body from engorged ticks. The 211 and 169 kDa polypeptides did not appear in fat body from 20E-treated (4,300 ng 20E/g bw) partially-fed ticks. However, the 78 kDa polypeptide appeared more intense in 20E-treated ticks than in engorged ticks. No polypeptides from day 0 engorged and EtOH-injected partially-fed tick fat bodies were specific to anti-Vg antibodies (Fig 3).

3.2 Effect of insulin

Compared to vehicle-injected controls, multiple injections of bovine insulin into partially-fed ticks had no significant effect on ovary weight (n=6-15), salivary gland fluid secretory competence (n=12-24), hemolymph Vg-concentration (n=4-14), or hemolymph 20E-concentration (n=2-10) by days 5 or 10 (data not shown). The data for Vg-concentration was highly variable, however.

3.3 Effects of JH III and T_3

Topical application of partially-fed ticks with JH III had no significant effect on ovary weight (Fig. 4A), or hemolymph 20E- and Vg-concentrations (Fig. 4B) by day 6 post-treatment. Salivary glands of ticks treated with 10 μg JH/g bw weighed significantly less than acetone-treated controls by day 6 (1.88 ± 0.09 mg in JH-treated glands and 2.64 ± 0.19 mg in control glands; Fig. 4C). Salivary gland fluid secretory competence was greater in ticks treated with 2 μg JH/g bw than in acetone treated

controls (3.21 ± 0.19 mg/gland/10 min for JH-treated glands and 1.37 ± 0.13 mg/gland/10 min for controls). The latter effect was not apparent at 5 or 10 μg JH/g bw, however (Fig. 4D).

There were no apparent effects of T_3 treatment on ovary weight (n=3-4), hemolymph 20E-concentration (n=2-4), hemolymph Vg-concentration (n=2-4), or salivary gland fluid secretory competence (n=5-7) by day 5 or 10 (data not shown).

3.4 Identification of a Vt-binding protein

Immunoblots of days 2-4 engorged ovary homogenate were incubated first with partially purified Vt from day 10 engorged ovaries, and then by anti-Vg antibodies, to test for Vt-binding proteins. In addition to Vt already present in the eggs, days 2-4 ovary homogenates contained only a single detectable polypeptide (86 kDa) that bound to Vt (Fig. 5B). Subjecting ovary homogenates to non-reducing conditions did not appear to affect the size or Vt-binding function of this polypeptide (Fig. 5A); however, this polypeptide band appeared fainter under non-reducing conditions than under reducing conditions. Vt-binding to the 86 kDa polypeptide was attenuated by 5 mM suramin (Fig. 6). Moreover, the 86 kDa polypeptide was not detected in tissues other than ovaries, including the reproductive tract (seminal receptical, uterus, accessory glands), midgut, salivary glands, Malpighian tubules, and trachea (Fig. 7).

The 86 kDa Vt-binding protein was detected by ligand blotting in ovaries of engorged ticks up to day 4, but not on day 8 (Fig. 8). We do not interpret the lack of detection of this band on day 8 to a disappearance of the protein. All lanes of the gel were loaded with 100 μg protein (see methods). Because Vt is so abundant on day 8, the 86 kDa binding protein may have been present in too small an amount to show up on the gel. The 86 kDa band on ligand blots was visible in ovary homogenates from partially-fed ticks between 100-150 mg, and appeared more intense in ovary homogenates of partially-

fed ticks over 250 mg and day 0 engorged ticks. The 86 kDa protein was not detected in ticks weighing less than 50 mg, however.

4. Discussion

Fat body and midgut are the sites of Vg-synthesis in ticks (Chinzei and Yano, 1985; Rosell and Coons, 1990; Taylor et al., 1991b). Lunke and Kaufman (1993) demonstrated that injection of 20E into partially-fed ticks did not stimulate Vg-uptake by oocytes, although the fat body of these ticks enlarged noticeably, suggesting the synthesis of Vg. Here, we found that the fat body of engorged *A. hebraeum* 10 days post-engorgement contains at least 5 polypeptides that bind antibodies raised against two hemolymph Vgs (Vg211 and Vg148). Interestingly, both anti-Vg211 and anti-Vg148 bound to polypeptides in fat body that appeared larger than their corresponding hemolymph proteins (219 kDa and 211 kDa, respectively; Fig. 3). We do not know the events occurring between Vg-synthesis in the fat body and its release into the hemolymph that might account for these reductions in size.

Injections of 20E into partially-fed *A. hebraeum* stimulated Vg-synthesis by the fat body. However, the single 211 kDa polypeptide found in normal engorged tick fat body was not detected in fat body from 20E-treated partially-fed ticks (Fig. 3). 20E disappears rapidly from the hemolymph following injection into partially-fed *A. hebraeum* (between 85 and 99% removal within 7 h; Weiss and Kaufman, 2001), whereas in normal engorged ticks, hemolymph 20E titres remain elevated from days 4 to 10 (Kaufman, 1991; Friesen and Kaufman, 2002). We collected the fat body from 20E-treated ticks on day 10 (i.e., 5 days following the final injection of 20E) so hemolymph ecdysteroid titer should have been very low for much of this time. This abnormal hormonal profile may have resulted in an artificial snapshot of Vg-content of the fatbody.

Using immunoblots, Friesen and Kaufman (2002) recently demonstrated that 20E stimulates Vg-synthesis and release into the hemolymph of partially-fed *A. hebraeum*. Here we have quantified the increase in hemolymph Vg-concentration using an ELISA. In partially-fed ticks, 20E caused a dose-

dependent increase of Vg-concentration, followed by a decrease at the highest concentration tested (Fig. 1C). However, a corresponding increase in Vg-uptake by oocytes was not observed. During normal vitellogenesis, the ovary from an engorged tick begins to accumulate Vg by day 4-5 post-engorgement (Friesen and Kaufman, 2002). By day 10 post-engorgement, ovary weight is approximately 5% of the engorged body weight (Friesen and Kaufman, 2002), an increase that is largely due to yolk in the oocytes (Diehl et al., 1982). However, as seen in Fig. 1, and reported previously by Lunke and Kaufman (1993), no ovary or oocyte growth occurred in 20E-treated, partially-fed ticks. So we asked whether a putative VgR might be absent in small, partially-fed ticks.

In oviparous animals, Vg is taken up by oocytes via a VgR. VgRs are members of the low density lipoprotein (LDL)-receptor family, and share high sequence homology across invertebrate and vertebrate species (Sappington and Raikhel, 1998). Molecular weights of previously identified VgRs range from 95 kDa in chickens (Stifani et al., 1990) to 230 kDa in crabs (Warrier and Subramoniam, 2002). Here we have identified an 86 kDa Vt-binding protein from *A. hebraeum* ovaries (Fig. 5), which was not present in other tissues (Fig. 7), and which is a candidate VgR in this tick.

Suramin salt is a negatively charged hydrocarbon that inhibits Vg-binding to its receptor in birds (Stifani et al., 1988), crabs (Warrier and Subramoniam, 2002), and mosquitoes (Dhadialla et al., 1992). Suramin appears to inhibit ligand/receptor binding through a non-competitive mechanism, as demonstrated by its affect on the binding of α_2-macroglobulin to its receptor (a low-density-lipoprotein-related protein; Vassiliou, 1997). If tick VgRs are also members of the LDL-receptor family, suramin may have a similar effect on Vg binding in ticks. Because binding of partially-purified Vt to the 86 kDa protein in *A. hebraeum* was inhibited by 5 mM suramin (Fig. 6), this lends further support to the notion that the 86 kDa protein may be the VgR in this tick. However, previous studies have indicated that the effective binding of Vg to VgR is inhibited when VgR is subjected to disulfide reducing agents (Sappington et al, 1995; Stifani et al., 1990). Here, binding of Vt to the 86 kDa Vt-binding protein in *A. hebraeum* was not inhibited by treatment with 2-mercaptoethanol (Fig. 5A), suggesting that the 86 kDa

protein does not share all the properties of a VgR in the LDL-receptor family. Clearly, further investigation of this protein and its interaction with Vt is required.

Reports of (a) insulin having ecdysteroidogenic activity in mosquitoes (Graf et al., 1997; Riehle and Brown, 1999), (b) the cloning of insulin receptors in both *Drosophila* (Fernandez et al., 1995; Ruan et al., 1995) and mosquitoes (Graf et al., 1997), as well as (c) identification of several insulin-like peptides from *Drosophila* neurons (Rulifson et al., 2002), suggest that the insulin family of peptides is highly conserved. Moreover, antibodies to bovine insulin bind to neurohemal organs in the synganglion of the tick, *Ornithodoros moubata* (Zhu and Oliver, 1991), suggesting that an insulin-like molecule may also be present in ticks. What physiological role such a molecule may have remains to be determined, but the results reported here do not support the suggestion that it functions as a putative VIF.

Whereas previous authors have proposed that JH stimulates egg development in argasid ticks (Pound and Oliver, 1979; Connat et al., 1983), this claim has been disputed (Taylor et al.,1991a, 1991b; Neese et al., 2000. Neither JH III nor T_3 stimulate 20E-synthesis or Vg-synthesis (Fig. 4 and Results, p. XXX). Likewise, salivary gland degeneration, a sensitive indicator of a rise in 20E-concentration, was not initiated in these ticks following JH or T_3 treatments. On the contrary, a significant increase in salivary gland fluid secretion occurred at a dose of 2 µg JH III/g bw (Fig. 4D). Consistent with this are observations by Shelby et al. (1989), who demonstrated that the JH analogs, methoprene and hydroprene, stimulated protein synthesis and Na/K-ATPase activity, and elicited other signs of differentiation, in the salivary glands of *A. americanum.* Lunke and Kaufman (1993) were not able to stimulate Vg-uptake by oocytes after combinations of 20E and JH treatments, suggesting that JH does not render ovaries competent to accumulate yolk, as occurs in some insects (reviewed by Nijhout, 1994). However, the 86 kDa protein is also present in ovaries of small partially-fed ticks (100 mg and above). This observation suggests that the failure of ovaries in 20E-treated partially-fed ticks (<250 mg) to accumulate yolk may not have been for lack of a VgR. As suggested earlier, (Lunke and Kaufman, 1993; Friesen and Kaufman, 2002), the possibility remains that Vg uptake in *A. hebraeum* requires a

signal in addition to the vitellogenic hormone. We do not know what the putative signal may be, nor the mechanism whereby it promotes Vg-uptake. However, we assume that it acts directly on the oocyte because, as mentioned earlier, the oocytes of chelicerate arthropods are not ensheathed by a follicular epithelium (Kaufman, 1997; Chang and Kaufman, in press). Identifying the factor which controls yolk-uptake, and determining its mechanism of action, will be a challenge for the future.

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Figure Legends

Figure 1. Effect of 20E injections on partially-fed ticks on days 5 and 10 post-treatment. (A) ovary weight, (B) oocyte length, (C) hemolymph Vg-concentration. EtOH (here and in subsequent figures): ethanol-injected control. Vg-concentration is reported as 'Vt-equivalents' (see Materials and Methods). All data (here and in subsequent figures) are reported as mean ± SEM. Significant differences from EtOH injected control (in this and in subsequent figures) are as follows: * $0.05>p>0.01$, ** $0.01>p>0.001$, *** $0.001>p$.

Figure 2. Effect of 20E injections on partially-fed ticks on days 5 and 10 post-treatment. (A) salivary gland (SG; in this and subsequent figures) weight, (B) SG fluid uptake.

Figure 3. Immunoblot of fat body homogenates of days 0 and 10 engorged, and of 20E-injected partially-fed ticks. 20E-treated ticks were given an injection of 4,300 ng 20E/g bw on days 2 and 5, and the fat body collected on day 10. EtOH: vehicle-injected partially-fed ticks. All lanes were loaded originally with 30 μg total protein. Each lane of the immunoblot was cut into 3 strips, which were treated as follows: (a) Coomassie blue, (b) Anti-Vg148 antibody, (c) Anti-Vg211 antibody. Vg polypeptides specific for anti-Vg148 or anti-Vg211 antibodies indicated by white or black arrowheads, respectively.

Figure 4. Effect of topical applications of JH III on partially-fed ticks 6 days post-treatment. (A) ovary weight, (B) hemolymph 20E-concentration (open circles) and Vg-concentration (grey bars), (C) SG weight, (D) SG fluid uptake. Vg-concentration and 20E-concentration are reported as 'equivalents' for reasons described in Materials and Methods.

Figure 5. Ligand blot demonstrating the binding of Vt to an 86 kDa polypeptide from day 2-4 engorged ovary homogenates. A) non-reducing, SDS-PAGE ; B) reducing, denaturing SDS-PAGE. All lanes were loaded originally with 100 µg total protein. Strips were treated as follows: (C.b.) Coomassie blue stain; (+Vt) with exogenous ligand; (-Vt) without exogenous ligand; (kDa) molecular weight markers. For this and subsequent figures, all strips, with the exception of those stained with Coomassie blue, were immunostained with a combination of anti-Vg antibodies. Bands representing endogenous Vt were identified from the third strip (-Vt) of panels A and B. The 86 kDa band (arrowheads) is the only one detected in the presence of exogenous Vt, and is thus considered to be a candidate VgR.

Figure 6. Ligand blot demonstrating the effect of suramin on Vt-binding to the 86 kDa ovary polypeptide. All lanes were loaded originally with 100 µg total protein. Strips were treated as follows: (C.b.) Coomassie blue stain; (-Vt) without exogenous ligand; (+Vt, +Sur) with exogenous ligand and 5 mM suramin; (+Vt) with exogenous ligand. Only the 86 kDa band (arrowhead) was significantly fainter in the presence of suramin.

Figure 7. Ligand blot demonstrating the absence of the 86 kDa Vt-binding polypeptide (arrowhead) in various tissue homogenates from ticks 2-4 days post-engorgement. All lanes were loaded originally with 100 µg total protein. (Ov) ovary, (RT) reproductive tract (seminal receptical, uterus, accessory glands), (Gut) midgut, (SG) salivary gland, (Mt) Malpighian tubule, (Trach) trachea. (-Vt) without exogenous ligand, (+Vt) with exogenous ligand.

Figure 8. Profile of the 86 kDa Vt-binding protein (arrowhead) at different stages of feeding, as demonstrated by ligand blot. All lanes were loaded originally with 100 µg total protein. Partially-fed ticks are categorized by weight range (mg), and engorged ticks by days post-engorgement.

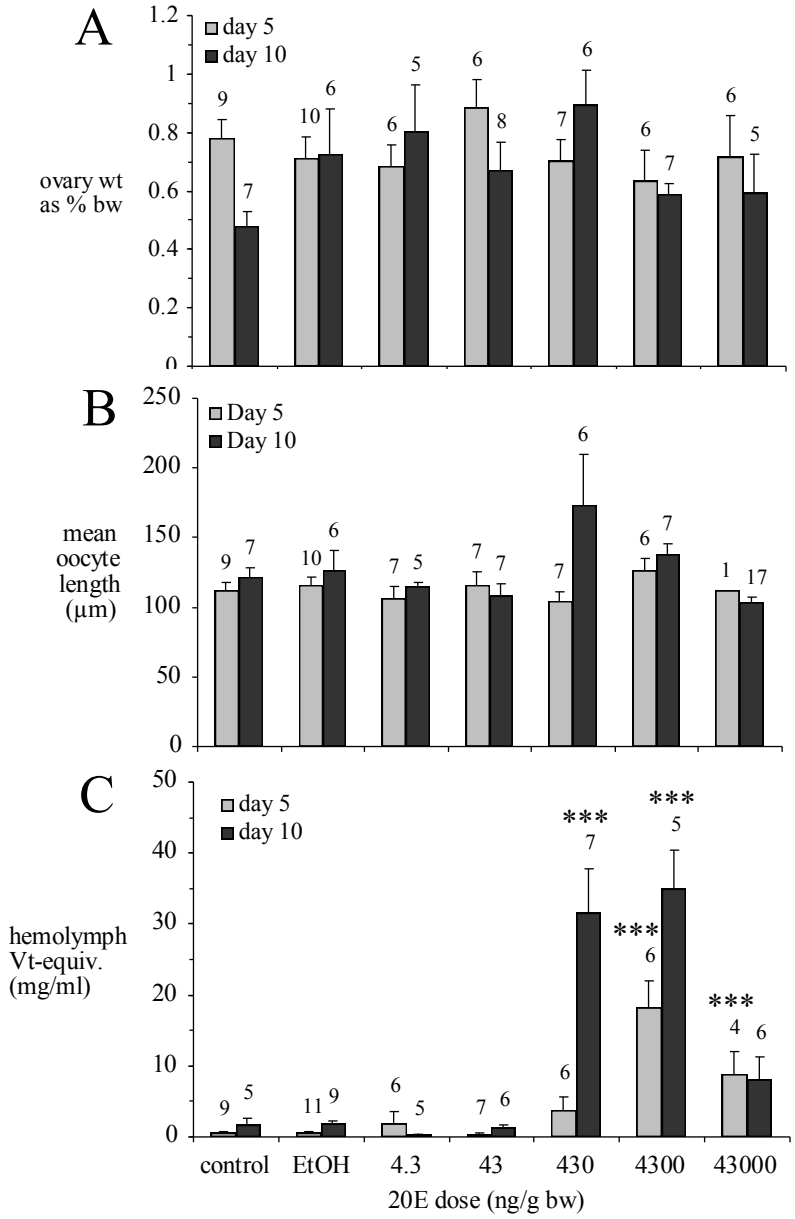
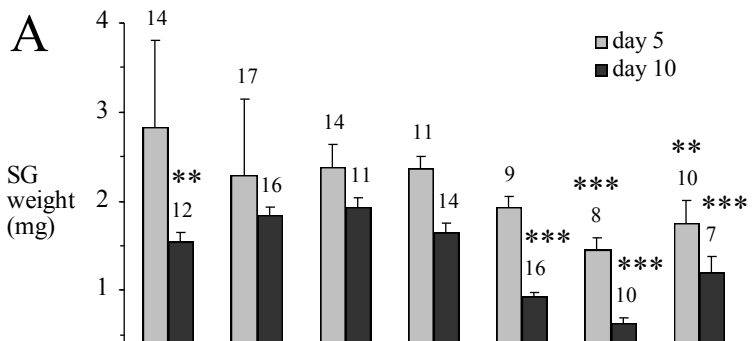

A
day 5
day 10
ovary wt as % bw
B
Day 5
Day 10
mean oocyte length (μm)
C
day 5
day 10
hemolymph Vt-equiv. (mg/ml)
control
EtOH
4.3
43
430
4300
43000
20E dose (ng/g bw)

Figure 1


A
day 5
day 10
SG weight (mg)

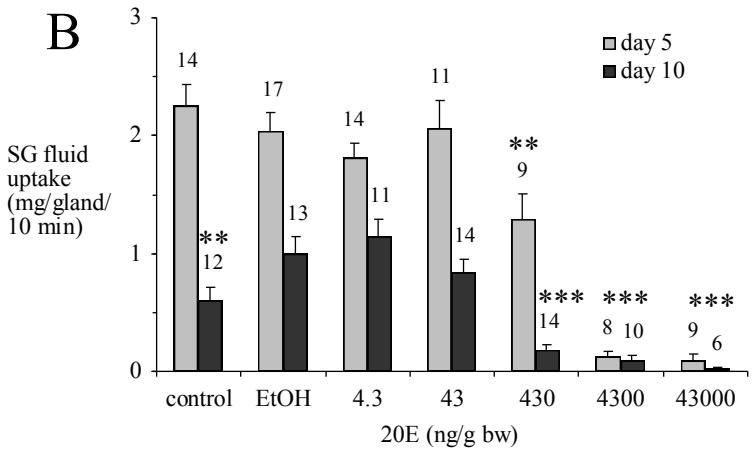

B
SG fluid uptake (mg/gland/ 10 min)
day 5
day 10
3
2
1
0
14
17
14
11
**
9
**
12
13
11
14
***
14
***
8
10
***
9
6
control
EtOH
4.3
43
430
4300
43000
20E (ng/g bw)

Figure 2

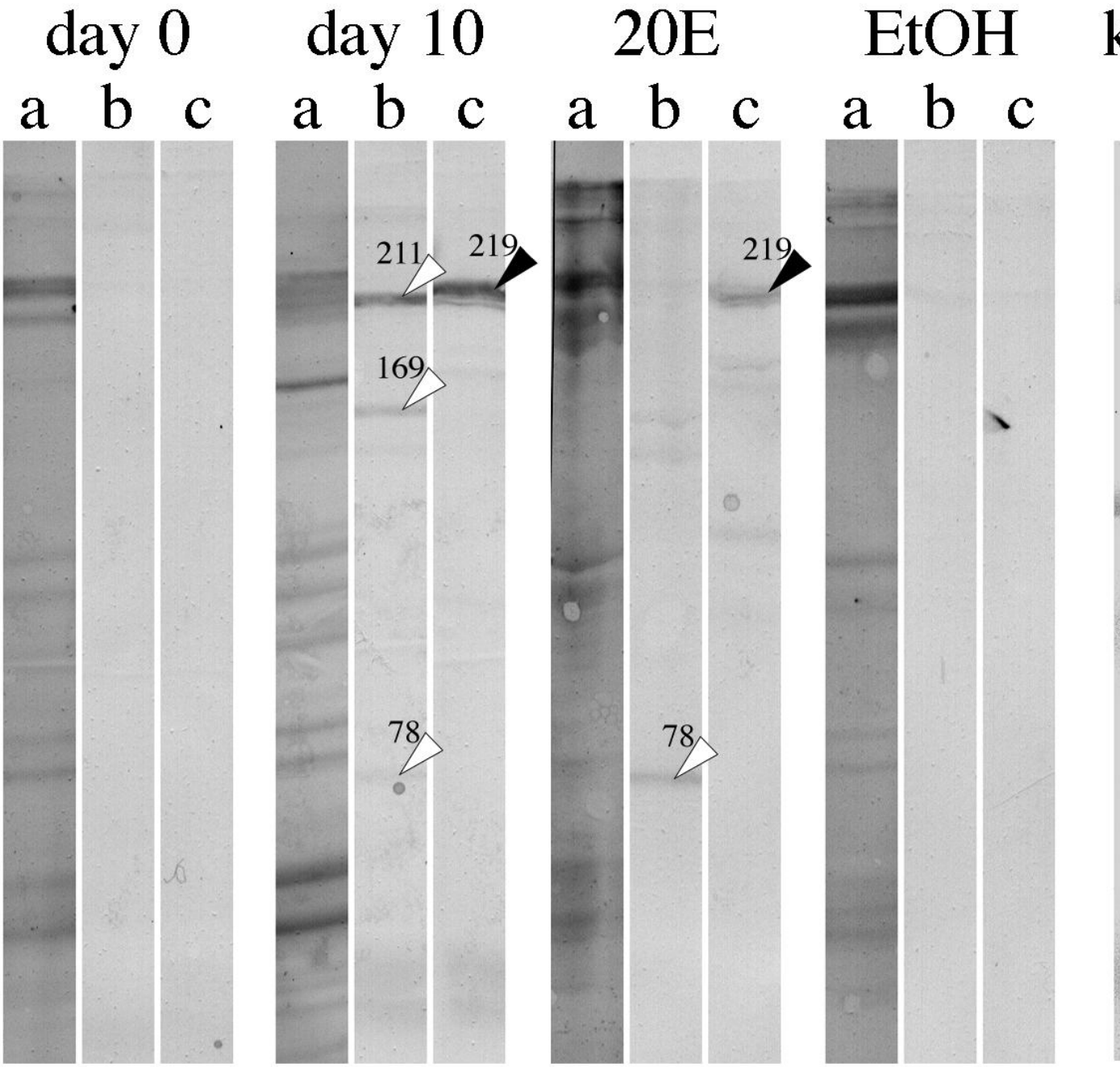


Figure 3

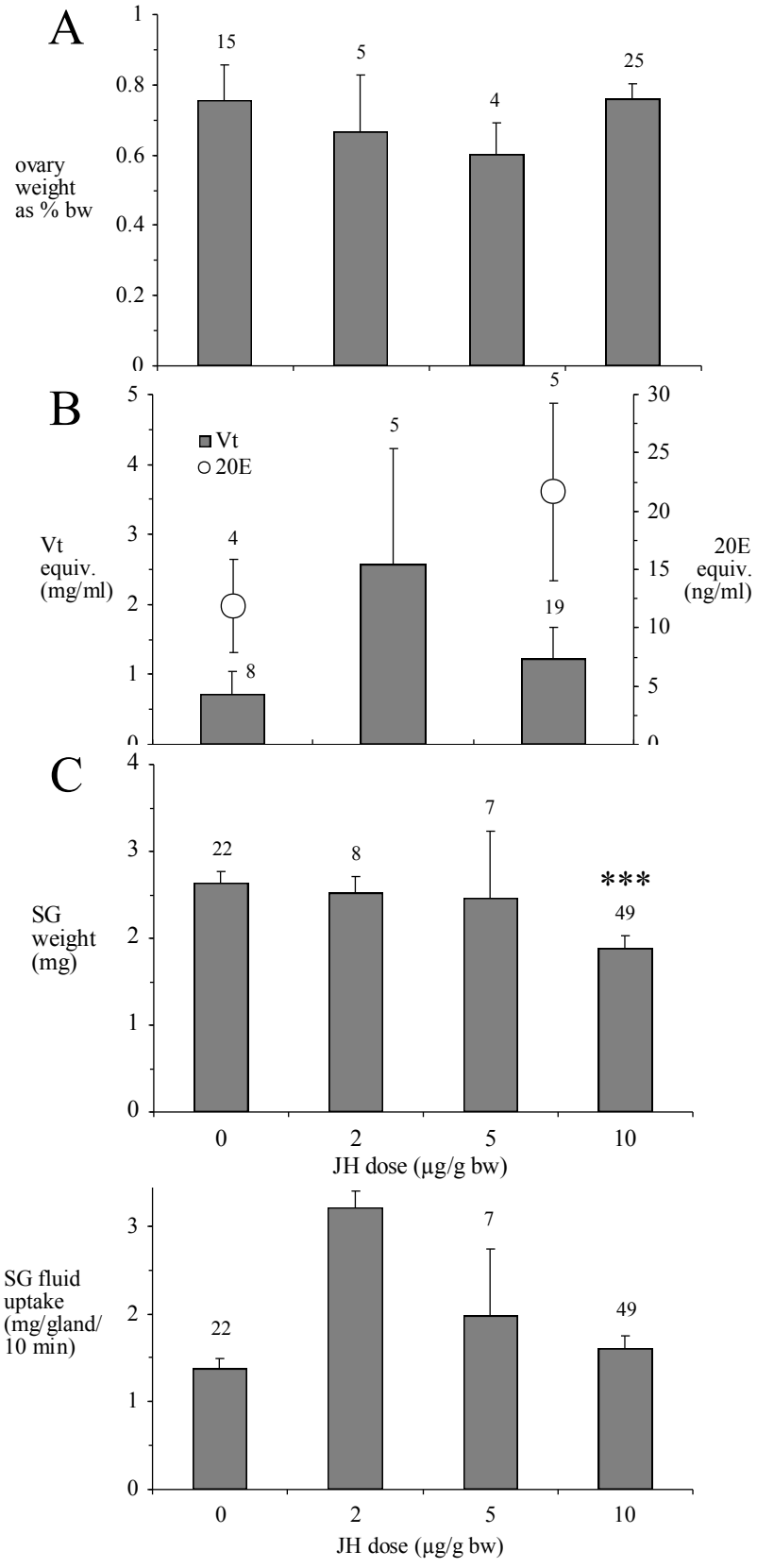

A
ovary weight as % bw
1
0.8
0.6
0.4
0.2
0
15
5
4
25
B
Vt
20E
Vt equiv. (mg/ml)
20E equiv. (ng/ml)
5
4
3
2
1
0
30
25
20
15
10
5
0
4
8
5
5
19
C
SG weight (mg)
22
8
7
***
49
0
2
5
10
JH dose (µg/g bw)
SG fluid uptake (mg/gland/ 10 min)
22
7
49
0
2
5
10
JH dose (µg/g bw)

Figure 4

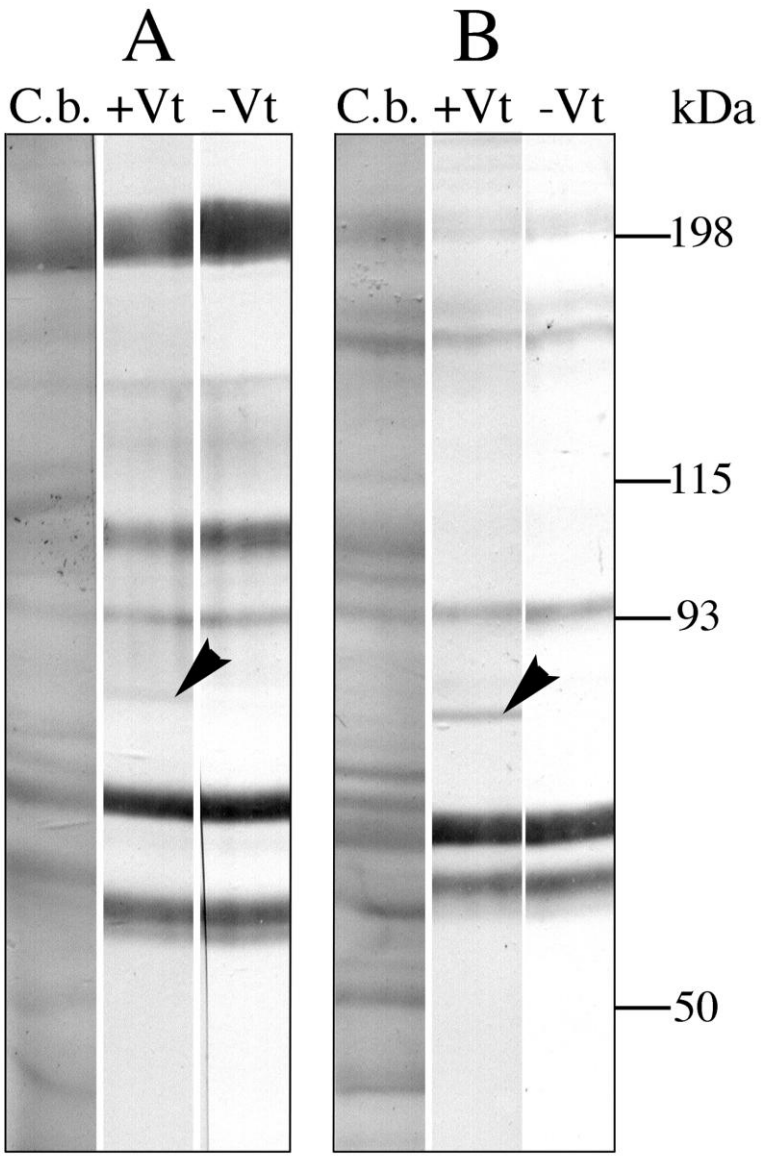


Figure 5

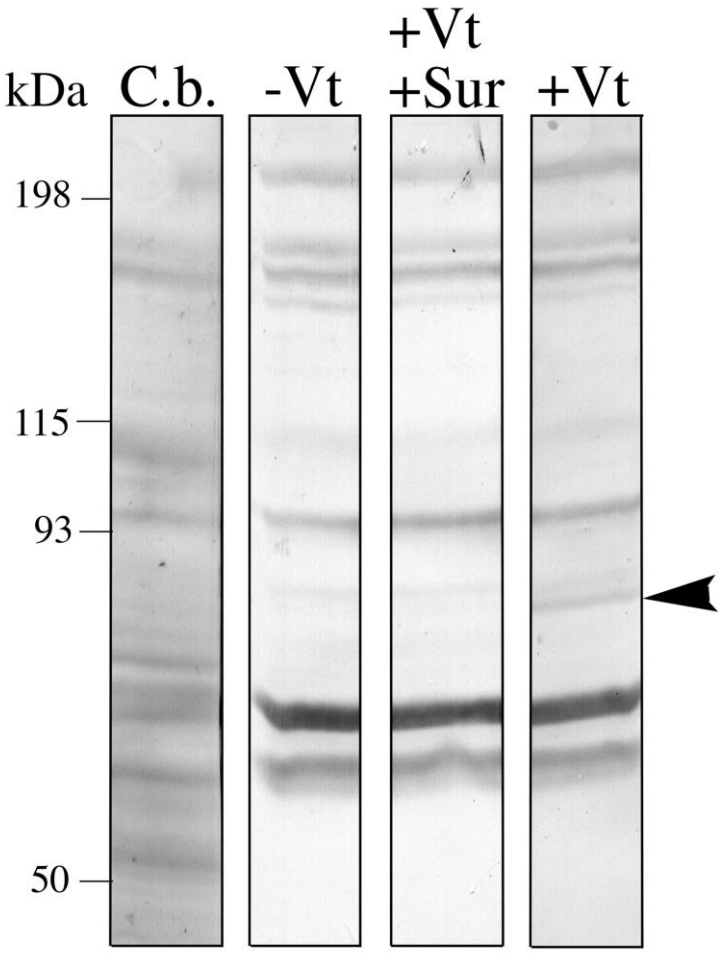


Figure 6

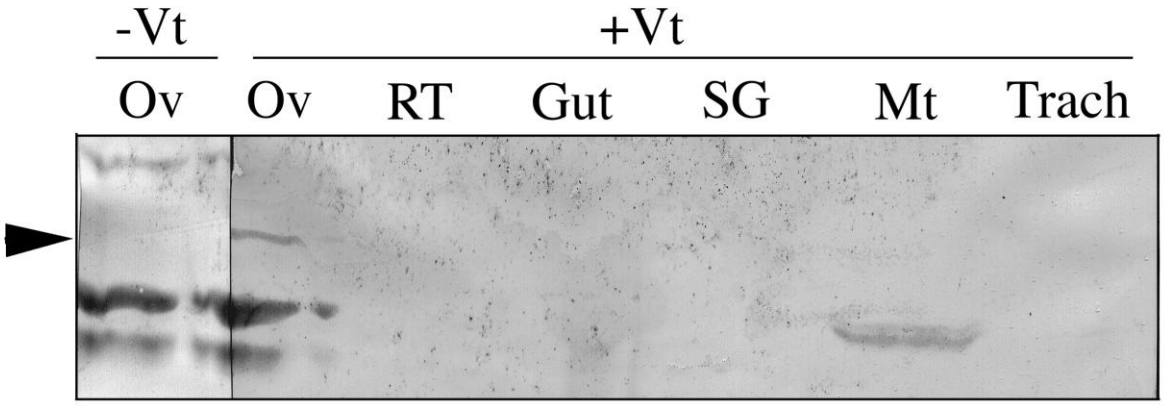


Figure 7

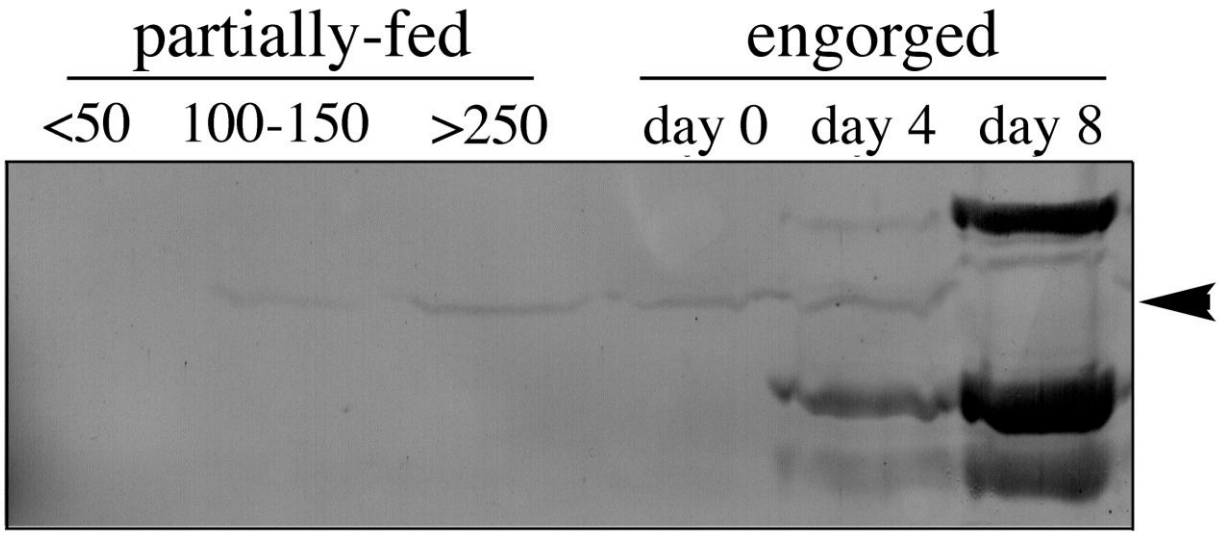


Figure 8