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Endocrine Regulation of Vitellogenesis in the Ixodid Tick, *Amblyomma hebraeum* Koch

by

Kevin James Friesen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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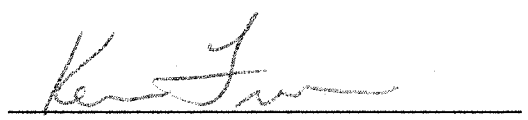

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Endocrine Regulation of Vitellogenesis in the Ixodid Tick, *Amblyomma hebraeum* Koch, submitted by Kevin James Friesen in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

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The broad-spectrum antiparasitic drug, MK-243, inhibited ovary growth in engorged ticks. However, haemolymph Vg-concentrations were not reduced in MK-243 treated ticks, suggesting that this drug specifically blocks Vg-uptake. The effects of the pyrethroid insecticide, cypermethrin (CyM), were also tested. CyM did not stimulate vitellogenesis in partially-fed ticks, as was hypothesized from its effects on argasid ticks (Chinzei et al., 1989, Invert. Reprod. Devel., 15, 19-26). Instead, CyM treatments caused a reduction of haemolymph Vg- and 20E-concentrations, as well as ovary growth, in engorged ticks.

In summary, the research presented here provides further support for the role of an ecdysteroid as the vitellogenic hormone in ticks. However, the other neurohormone(s) that stimulate ecdysteroid-synthesis have yet to be identified. Likewise, the lack of egg development following 20E-injections into partially-fed ticks indicates that a separate signal is required for yolk-uptake to occur.

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Abstract

The overall objective of this thesis was to determine the hormonal regulation of yolk synthesis (vitellogenesis) and yolk accumulation by developing oocytes in the ixodid tick, *Amblyomma hebraeum*. Using gel electrophoresis, the main yolk protein, vitellin (Vt) and its haemolymph-borne precursor, vitellogenin (Vg) were identified from engorged tick ovaries and haemolymph, respectively. Antibodies were raised against Vg, and used to develop an enzyme-linked immunosorbent assay (ELISA) to quantify Vg and Vt. The haemolymph profiles of Vg and the ecdysteroid, 20-hydroxyecdysone (20E), were then measured in normal engorged (vitellogenic) ticks as a standard against which to compare partially-fed (non-vitellogenic) ticks after various hormone treatments.

20E, juvenile hormone (JH)-III, triiodothyronine (T_3), and bovine insulin were all tested for vitellogenic stimulation in partially-fed ticks; 20E was the only hormone that stimulated Vg-synthesis by fat body and its release into the haemolymph. However, no yolk-uptake by ovaries occurred in 20E-treated partially-fed ticks; I tested whether this was because a Vg-receptor (VgR) is not up-regulated in partially-fed tick ovaries. Using immunoblotting techniques, an 86 kDa Vt-binding protein (a putative VgR) was identified from ovaries of engorged ticks. However, the 86 kDa protein was present in partially-fed tick ovaries, indicating that a situation other than the lack of VgR accounts for the inability of 20E to stimulate yolk-uptake.

Chapter 3

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*

Chapter 4

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List of Abbreviations

20E	20-Hydroxyecdysone
AP	Alkaline phosphatase
AVM	Avermectin
BSA	Bovine serum albumin
bw	Body weight
CP	Carrier protein
CyM	Cypermethrin
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FMev	Fluoromevalonate
FSF	Fat body stimulating factor
JH	Juvenile hormone
JHA	Juvenile hormone analogue
MK-243	Avermectin-analogue, MK-243
MOPS	3-(N-morpholino)propanesulfonic acid
PBS	Phosphate-buffered saline
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T_3	3, 5, 3'-Triiodothyronine

T_4	Thyroxine
TBS	Tris-buffered saline
TC 199	Tissue culture media 199
TTBS	TBS containing 0.04% (v/v) Tween 20
Vg	Vitellogenin
VgR	Vitellogenin receptor
VIF	Vitellogenesis inducing factor
Vt	Vitellin

Chapter 1: General Introduction

Ticks: General Background, Feeding, and Reproductive Strategies

Ticks are obligate ectoparasitic arthropods that infest all classes of terrestrial vertebrates (Sonenshine, 1991). Ticks are responsible for the transmission of numerous diseases, such as Lyme disease (a spirochete), Rocky Mountain spotted fever (a Rickettsia), tickborne relapsing fever (spirochete), and acute Q fever (Rickettsia). Heavy infestations of ticks may also result in malnourishment or death due to blood loss, or tick paralysis (Parola and Raoult, 2001).

Ticks are members of the Class Arachnida, in the Subclass Acari, Order Parasitiformes, Suborder Ixodida (Sonenshine, 1991). There are two main families of ticks (Fig. 1.1). Family Ixodidae ('hard ticks'; 694 species) are characterized by a hard cuticle in the unfed state. Family Argasidae ('soft ticks'; 177 species) have a more flexible, leathery cuticle in the unfed state. There are other differences of more phylogenetic importance.

Ticks have a three-stage life cycle, consisting of larvae, one (Ixodidae) or more (Argasidae) nymphal stages, and adult. All instars require a blood meal to complete development and moult to the subsequent stage or lay eggs (Sonenshine, 1991). Host selection and feeding is specific for each tick species. In general, argasid ticks, and most ixodid ticks, use a multihost feeding strategy, by which a new host is found for each developmental stage. However, in some species of ixodid ticks, two or more stages feed on the same host (2- and 1-host feeding strategies).

Argasid and ixodid ticks utilize different reproductive strategies to complete their life cycles (Diehl et al, 1982; Sonenshine, 1991). Adult female argasid ticks take relatively small blood meals, engorging to approximately 10 times their unfed weight. Blood meals are relatively rapid, ranging from a few minutes to a few hours. Mating

occurs either before or after the blood meal; provided a female has mated and fed, she will produce a batch of a few hundred eggs, and then seek another blood meal. In contrast, adult female ixodid ticks take a single, huge blood meal, which occupies 7 to 14 days. Mating occurs while both the male and female are attached to the host; mating provides a necessary stimulus for the female to enter a rapid phase of feeding after which she engorges to approximately 100 times her unfed body weight. An engorged female will lay a batch of several thousand eggs (up to 20,000 in *Amblyomma hebraeum*), and then die.

General Organization of the Female Tick Reproductive Tract

Unlike the paired ovaries in insects, the tick ovary consists of a single, hollow, horseshoe-shaped structure (Fig. 1.2A) which, at maturity, can reach several centimeters in length (Diehl et al., 1982). Each end of the ovary terminates in an oviduct, which connect to a common uterus and vagina. A single seminal receptical, which serves as the storage site for the spermatophore, is also attached to the uterus. The ovary is suspended in the surrounding haemolymph, and is anchored to the tick body only by tracheae and the oviducts.

Prior to the adult blood meal, the epithilial wall of the ovary consists entirely of oocytes and interstitial cells (Balashov, 1972; Diehl et al., 1982). Along the length of the ovary is a longitudinal groove, which contains oocytes at the earliest stages of development. It should be noted that the longitudinal groove is not analogous to the germarium in insect ovaries, as only primary oocytes, and not oogonia, are present (Brinton and Oliver, 1971). During oogenesis, oocytes begin to protrude into the surrounding haemolymph, while migrating peripherally away from the longitudinal groove (Fig.1. 2B). Eventually, mature oocytes are connected to the wall of the ovaries only by the surrounding basement membrane and a stalk of funicle cells.

Tick Oogenesis

Tick oogenesis has been divided into five distinct stages by Balashov (1972). Primary oocytes in Balashov's stage I, the period of small cytoplasmic growth (early previtellogenesis), first appear in engorged nymphs when they enter prophase of the first maturation division. The volume of oocytes at this stage increases only slightly, and ends when the meiotic prophase is arrested in diakinesis. Following the initiation of the adult blood meal, oocytes enter Balashov's stage II, or the great cytoplasmic growth phase (late previtellogenesis). This stage is marked by an enlargement of the nucleus and cytoplasm of the oocyte, resulting in the protrusion of the oocyte into the haemolymph. At the end of stage II, the endoplasmic reticulum and Golgi bodies are well developed, and the oocyte contains numerous mitochondria. The cell membrane of the oocyte at the end of stage II is lined with microvilli.

In the literature, the term 'vitellogenesis' is often used variably to describe either synthesis of the major yolk protein, vitellogenin (Vg), or Vg accumulation by developing oocytes, or both. In this thesis, the term 'vitellogenesis' on its own refers to the entire process: yolk synthesis and accumulation in the oocyte. The component events will be referred to as "Vg-synthesis", or "yolk-synthesis", and "Vg-uptake", or "yolk-uptake", respectively. As in most arthropods, Vg-synthesis in ticks occurs mostly extraovarially; Vg is synthesized in fat body in the argasid tick, *Ornithodoros moubata* (Chinzei and Yano, 1985), or both fat body and midgut cells in the ixodid tick, *Dermacentor variabilis* (Rosell and Coons, 1990). Vg is then released into the haemolymph and accumulated in large yolk granules by developing oocytes. Once incorporated into granules, yolk is generally referred to as 'vitellin' (Vt; Diehl et al., 1982), although Vg and Vt almost invariably remain immunologically similar (Hagedorn and Kunkel, 1979). The period of yolk-uptake comprises Balashov's stage III and IV of oogenesis, at the end of which (approximately 1-2 weeks post-engorgement) the oocyte is ready for ovulation (Diehl et

al., 1982). Tick Vts are multi-subunit haemoglycophospholipoproteins (Sonenshine, 1991) ranging from 370 kD (*D. variabilis*; Rosell and Coons, 1991) to an astounding 8700 kD (*D. andersoni*; Boktor and Kamel, 1976).

During ovulation (Balashov's stage V), oocytes pass into the lumen of the ovary (Fig. 1.2B); peristaltic contractions of the ovary push oocytes through the lumen to the oviducts, and then into the vagina (Diehl et al., 1982). Oviposition results from a peristaltic contraction of the vagina. In ticks, eggs are passed from the genital pore to the mouthparts and then to Géné's organ, a lobed sac that can be extended from the camerostomal fold (soft articulation between the body and the mouth parts). Géné's organ secretes egg wax, which waterproofs oviposited eggs.

Hormonal Control of Vitellogenesis in Arthropods other than Ticks

Egg development has been extensively studied in insects. While there is no single model for the hormonal control of vitellogenesis applicable to all insects, Vg-synthesis in insects can be classified as being under the control of either juvenile hormones (JHs) or ecdysteroids (Nijhout, 1994). JHs are terpenoids derived from farnesoic acid; five structurally related forms have been identified from insects: JH-0, JH-I, JH-II, JH-III, and 4-methyl JH-I (Fig. 1.3). Ecdysteroids are sterol derivatives; 20-hydroxyecdysone (20E) and its prohormone, ecdysone (Fig. 1.4), are the most common forms in insects and other arthropods, although more than 60 ecdysteroids have been identified in arthropods (Nijhout, 1994).

Vg-synthesis occurs in the fat body of most insects, but may also occur to a lesser degree in the follicular cells surrounding the oocyte. In the Diptera, 20E stimulates Vg-synthesis and release into the haemolymph (Nijhout, 1994). In most other insects, JH, released from the corpora allata, stimulates Vg-synthesis. In addition, JH regulates yolk-uptake by causing the follicular cell layer surrounding developing oocytes to become

patent. Patency is characterized by the appearance of spaces between the follicle cells, via which Vg can gain access to the surface of the oocyte where it is taken up by endocytosis. JH induces patency by binding to a putative receptor on follicle cell membranes which then stimulates Na/K-ATPase and subsequent fluid loss by the cells (Davey and Gordon, 1996; Kim et al., 1999). Likewise, exposure of the ovary to JH in a newly moulted adult female mosquito is required for normal egg development. JH causes oocytes to become 'competent' to begin yolk-uptake by stimulating: (1) patency of the follicular epithelium, (2) primary follicles to advance to a quiescent developmental stage in preparation for vitellogenesis, and (3) differentiation of cellular machinery related to protein synthesis and endocytosis (Klowden, 1997).

Much less is known about the hormones which control vitellogenesis in other arthropods (Kaufman, 1997). There is some evidence suggesting a role during vitellogenesis for JH in mites (Oliver et al., 1985) and 20E in spiders (Trabalon et al., 1992). In crustaceans, methylfarnesoate (one of the JH-related family of hormones) has a well characterized role in regulating vitellogenesis (Laufer et al. 1987).

Hormonal Control of Vitellogenesis in Ticks

Several early studies established the importance of mating for ticks to undergo normal egg development (Galun and Warburg, 1967; Aeschlimann and Grandjean, 1973a, 1973b; Germond and Aeschlimann, 1977). In ixodid ticks, the spermatophore contains a chemical signal that induces engorgement in *D. variabilis* (Pappas and Oliver, 1972). Likewise, salivary gland degeneration, which normally occurs in engorged female ixodid ticks, is induced by a 'male factor' in *A. hebraeum* (Harris and Kaufman, 1984). Current studies are underway to determine whether 'engorgement factor' and 'male factor' are the same hormone.

The tick synganglion contains several regions of neurosecretory cells (Binnington and Obenchain, 1982). Using paraldehyde-fuchsin staining techniques, the activity of the neurosecretory regions was observed in argasid ticks (*Argas persicus*, Eisen et al., 1973; *Argas hermanii*, Shanbaky et al., 1990a); activity was measured by the amount of stain (and thus, neurosecretory material) these regions held. The neurosecretory cells were most active shortly after the blood meal, reaching a peak 1-3 days post-engorgement, followed by a decline in activity over the period of vitellogenesis. These authors proposed that a neurosecretory factor was released (decreased staining of neurosecretory cells) which stimulated egg development. Subsequently, vitellogenesis was stimulated in mated argasid ticks by injections of synganglia (*Argas hermanii*, Shanbaky et al., 1990b; *O. moubata*, Chinzei et al., 1992). Chinzei and Taylor (1990) proposed a model for the hormonal control of vitellogenesis in *O. moubata*, stating that a polypeptide ('vitellogenesis inducing factor'; VIF) from the synganglion stimulates some unknown tissue in the tick to release the vitellogenic hormone ('fat body stimulating factor', FSF). Because this model is reminiscent of vitellogenic control in both mosquitoes (Klowden, 1997) and oviparous vertebrates (Hadley, 1992), FSF was hypothesized to be a steroid hormone (Chinzei and Taylor, 1990). Other studies suggest that JH may play a role in egg production, although determining its precise role in vitellogenesis has not yet been realized (see below).

Effects of JH and JH blockers

Precocenes (Fig. 1.3) are cytotoxic to the corpus allatum of some insects; thus, sensitive insects treated with precocene suffer markedly reduced JH secretion and ovarian development (Retnakaran et al., 1985). Although ticks do not have a distinct corpus allatum, homologous (or at least analogous) tissue may be present as a diffuse population of cells associated with the synganglion (Binnington and Obenchain, 1982). The effects

of precocene described below have sometimes been interpreted as the results of cytotoxic activity on these aforementioned cells.

Argasid ticks can take several blood meals during their adult life; after each meal a mated female lays a batch of 50-200 eggs (Diehl et al. 1982). Precocene applied to fed mated female *O. parkeri* inhibited oviposition and the development of oocytes, an effect that was partially reversed upon application of JH-III (Pound and Oliver, 1979). Using precocene, Leahy and Booth (1980) inhibited oviposition as well as hatching in two argasid (*Argas persicus* and *O. coriaceus*) and one ixodid (*Rhipicephalus sanguineus*) species. However, application of JH-III did not reverse these effects. Likewise, precocene inhibited oviposition, but not yolk-synthesis, in *O. moubata* (Taylor et al., 1992). Among a number of inhibitors of JH synthesis used to block JH dependent vitellogenesis in *O. moubata*, the only compound which reduced the number of eggs laid was fluoromevalonate (FMev), an inhibitor of isoprenoid synthesis (Connat and Nepa, 1990). This inhibition also was not reversed by applying JH analogues (JHA).

Virgin female argasid ticks normally begin vitellogenesis after they have fed. However, the Vt accumulated by the eggs is subsequently resorbed if mating does not occur (Pound and Oliver, 1979). Mating is not the only stimulus for oviposition. JH applied to virgin female *O. moubata*, even up to 100 days after feeding, can induce some ticks to oviposit (Connat et al. 1983). In more direct studies, treatment of *unfed* virgin *O. moubata* (Chinzei et al., 1991) and *O. parkeri* (Taylor et al., 1991) with JH did not result in an increase of haemolymph Vg- or egg yolk-uptake. However, vitellogenesis was stimulated in these ticks using the pyrethroid insecticide, cypermethrin (CyM; see below), indicating that even unfed virgins are capable of yolk-synthesis if given an effective stimulus. Therefore, while JH does not seem to trigger vitellogenesis in *Ornithodoros* species, it may stimulate a subsequent stage of oocyte development—perhaps ovulation or oviposition.

There are fewer studies on the effect of precocene or JH in ixodid ticks. A female ixodid tick will feed only once during her adult life, after which she will lay one batch of several thousand eggs, provided she has mated and fed beyond a 'critical weight' (Kaufman and Lomas, 1996; Weiss and Kaufman, 2001). Eggs laid from precocene-treated *Boophilus microplus* were shriveled and lacked the wax layer present in untreated ticks (Booth et al., 1986). Thus, precocene seemed to be cytotoxic to the egg waxing organ (Géné's organ), while it did not otherwise adversely affect oogenesis. An attempt to block oviposition in *B. microplus* using various allatocidal compounds and inhibitors of JH-synthesis revealed that only precocene and FMev reduce the number of eggs laid (Connat, 1988). However, application of JHA did not reverse these effects, suggesting that precocene and FMev may be blocking oviposition by means other than blocking JH-synthesis.

In summary, JH may exert some control over reproduction in ticks, but apparently not at the level of yolk-synthesis. Whether JH is even present in ticks has been seriously challenged recently. Hexane extracts of *B. microplus* (whole body) inhibit the pupal-adult molt of the beetle, *Tenebrio molitor*[1], as well as induce oviposition in *O. moubata*, suggesting the presence of a JH-like compound (Connat, 1987). However, Connat (1987) and Neese et al. (2000) could not authenticate JH or JH-related molecules by gas chromatography/mass-spectrometry. The latter study in particular was compellingly negative.

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[1] Presence of JH during insect molting gives rise to larval characteristics, while its absence allows the formation of adult characteristics (Nijhout, 1994). Thus, JH treatment of an insect during the pupal-adult molt will sometimes inhibit metamorphosis. Treating insects with extracts thought to contain JH or JHA is a common bioassay to determine the presence of such molecules.

Thyroxine as a JH mimic

Thyroxine (T_4) is known to have physiological effects in insects, causing changes to protein and amino acid content of organs related to vitellogenesis (Chaudhuri and Medda, 1987). T_4 also increases the activity of ovary ATPases (Reddy et al., 1994). Likewise, T_4 (and the related T_3) induces 'patency' in the follicular epithelium of *Locusta migratoria* and *Rhodnius prolixus* by competing for the putative JH-receptor described above (Davey and Gordon, 1996; Kim et al., 1999). If a JH-like molecule is involved in tick oogenesis, T_4/T_3 treatments might mimic this effect. However, as oocytes from chelicerates, including ticks, lack a follicle cell layer (Kaufman, 1997), stimulation of vitellogenesis via potential JH-receptors would have to occur by some other mechanism than inducing patency.

Roles of ecdysteroids

Ecdysteroids are present in ticks (Connat et al., 1985), and an ecdysteroid (presumably 20E) is responsible for the degeneration of the salivary glands which occurs within a few days after feeding (Kaufman, 1991). However, haemolymph ecdysteroid-concentration rises to greater than 800 ng/ml haemolymph by day 10 post-engorgement (Lunke and Kaufman, 1992; Mao and Kaufman, 1999), an amount much higher than the 100-140 ng/ml required for complete salivary gland degeneration (Harris and Kaufman, 1985; Kaufman, 1991). In addition, the high ecdysteroid-concentration occurs 6 days after the salivary glands have completely degenerated (Mao and Kaufman, 1999). Thus, ecdysteroids probably also play a role in some other process. Much of the ecdysteroid synthesized after feeding is taken up by oocytes during vitellogenesis (Connat et al., 1985; Kaufman, 1991), and is believed to play a role in embryogenesis. The post-engorgement increase of haemolymph ecdysteroid-concentration also corresponds to an increase of Vg-concentration in haemolymph of *D. variabilis* (Dees et al., 1984) and fat

body of *Ixodes scapularis* (James et al., 1997). In addition, 20E stimulates *in vitro* Vg production in cultured fat body, an effect which is not observed using the JHA, methoprene (Fig. 1.3), and which is inhibited by actinomycin D (Sankhon et al., 1999). However, treatment of partially-fed *A. hebraeum* with 20E, JH, or the two combined, did not induce egg development, and injection of precocene did not inhibit vitellogenesis in engorged females (Lunke and Kaufman, 1993). Vg-synthesis was not monitored directly during these experiments, but a marked proliferation of the fat body was observed after 20E injection into partially-fed ticks. Thus, 20E may have stimulated Vg-synthesis, but did not permit uptake of Vg by oocytes.

Ecdysiotropic effects of insulin

20E is the vitellogenic hormone in mosquitoes and other Diptera (reviewed by Nijhout, 1994). Recently, vertebrate insulin has been shown to stimulate 20E synthesis by mosquito ovaries *in vitro* (Graf et al., 1997), and insulin receptors have been cloned from both *A. aegypti* (Graf et al., 1997) and *Drosophila* (Ruan et al., 1995; Fernandez et al., 1995). In the tick, *A. hebraeum*, ecdysteroid-synthesis occurs in epidermal tissue in response to a neuropeptide (Lomas et al., 1997). Synganglia from adult female ticks contain neurosecretory cells that are immunoreactive to bovine insulin antibodies (Zhu and Oliver, 1991; Davis et al., 1994), so it is possible that the ecdysiotropic neuropeptide (VIF?) is an insulin-like molecule.

Avermectin reduces haemolymph ecdysteroid-titre

Avermectins (AVMs) are a group of broad-spectrum anti-parasitic compounds originally isolated from the bacterium *Streptomyces avermitilis* (reviewed in Strong and Brown, 1987). Early research suggested that AVMs act as potentiators of γ-aminobutyric acid (GABA) systems in nematodes and arthropods, possibly through an agonistic action

on GABA-mediated chloride channels (reviewed in Campbell et al., 1983). However, more recent reports indicate that the main mode of action is to modulate glutamate-gated ion channels (Cully et al., 1994; Arena et al., 1995; Martin, 1996), channels which may also bind GABA (Ludmerer et al., 2002).

Treatment of host animals with AVM does not cause prompt death or detachment of ticks, but it does interrupt physiological functions such as feeding to engorgement, egg production, and larval hatching (Lancaster et al., 1982), but the mechanisms of these actions are not known. Engorged *A. hebraeum* treated with the AVM analogue MK-243 showed a marked decrease in ovary weight, oocyte size, and ovary yolk content, as well as a marked reduction in haemolymph ecdysteroid-concentration (Lunke and Kaufman, 1992). This effect of AVM further supports the potential role of an ecdysteroid during vitellogenesis in ticks.

Cypermethrin (CyM) and tick vitellogenesis

CyM is a pyrethroid insecticide. Pyrethroids stimulate continuous action potential firing in neurons of both the central and peripheral nervous systems of insects (reviewed by Ruigt, 1985). Neurosecretory cells of both *R. prolixus* (Orchard, 1980) and the stick insect, *Carausius morosus* (Orchard and Osborne, 1979), show increased spontaneous activity after pyrethroid applications. Stimulation of neurosecretion by sublethal doses of CyM results in ovarian development in the ladybird beetle, *Henosepulachna vigintioctopunctata* (Kono and Ozeki, 1987). Similarly, CyM treated unfed, virgin female argasid ticks undergo vitellogenesis to the same degree as fed, mated female ticks (Chinzei et al., 1989). Therefore, CyM presumably can stimulate release of VIF. The latter studies suggest that even without a blood meal and mating, a female argasid tick can synthesize Vg and produce eggs if given an effective stimulus. Although no one has

yet tested the effects of CyM on ixodid ticks, it may provide a tool for determining the competency of partially-fed females to undergo vitellogenesis.

Thesis Objectives

The main goal of this thesis was to define the roles of ecdysteroids and juvenile hormones during egg development in the ixodid tick, *A. hebraeum*. This overall goal was accomplished in four stages. (1) Vg and Vt were identified, and an enzyme-linked immunosorbent assay (ELISA) was developed in order to quantify Vg/Vt. (2) In order to establish a standard which experimentally treated ticks could be compared against, the characteristics of normal vitellogenesis that occur in engorged ticks were established. (3) Experimental treatments were performed to establish the (potential) roles of various hormones during vitellogenesis. Partially-fed ticks were treated with potential stimulators of vitellogenesis (20E, JH-III, bovine insulin, T_3, and CyM), and engorged ticks were treated with potential inhibitors of vitellogenesis (MK-243, and CyM). (4) Finally, in order to determine more about yolk-uptake in normal and hormone treated ticks, I have attempted to identify the Vg-receptor from engorged tick ovaries.

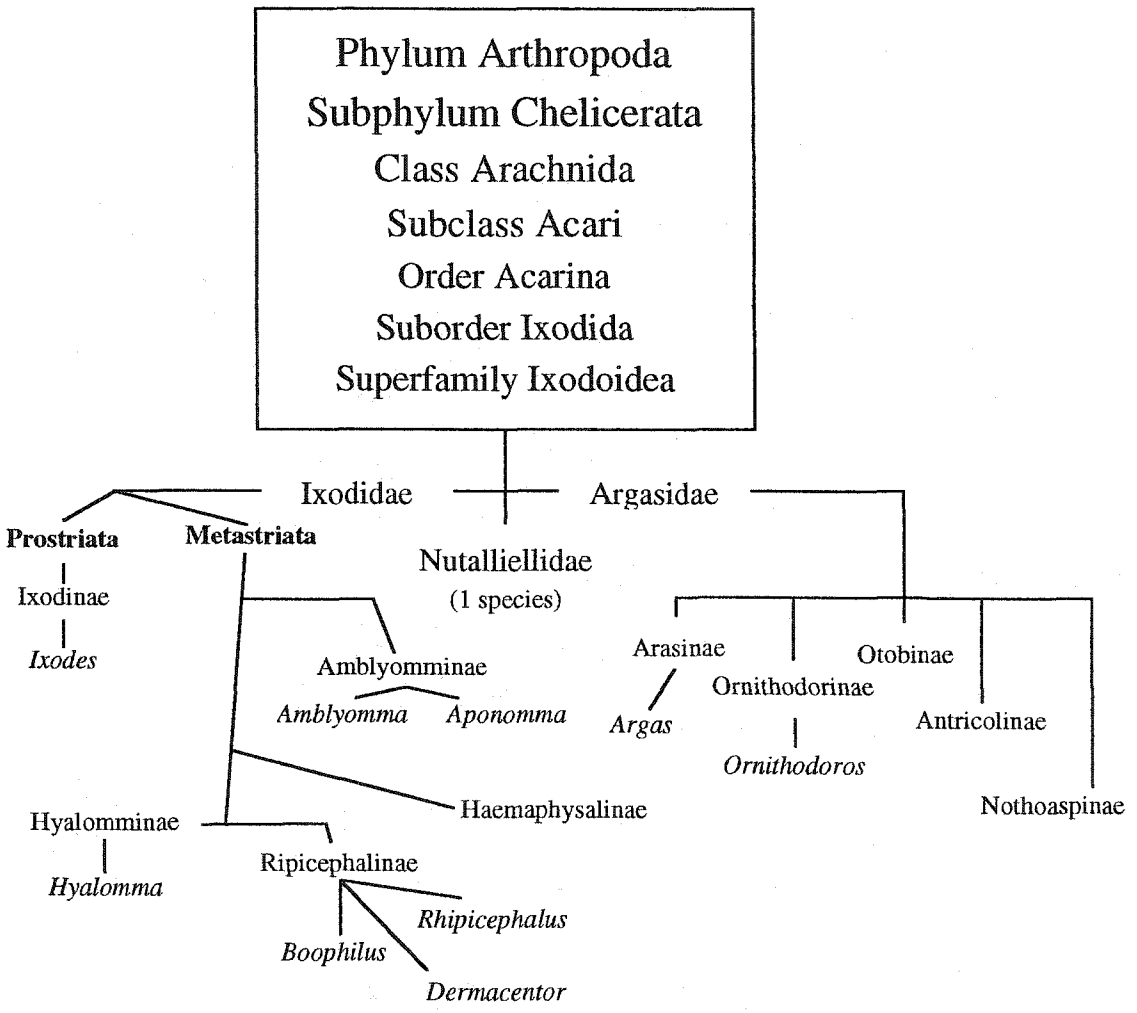

Figure 1.1. Schematic illustration of the phylogenetic relationships of ticks (Ixodoidea). Modified from Sonenshine (1991) and Hoogstraal (1983).

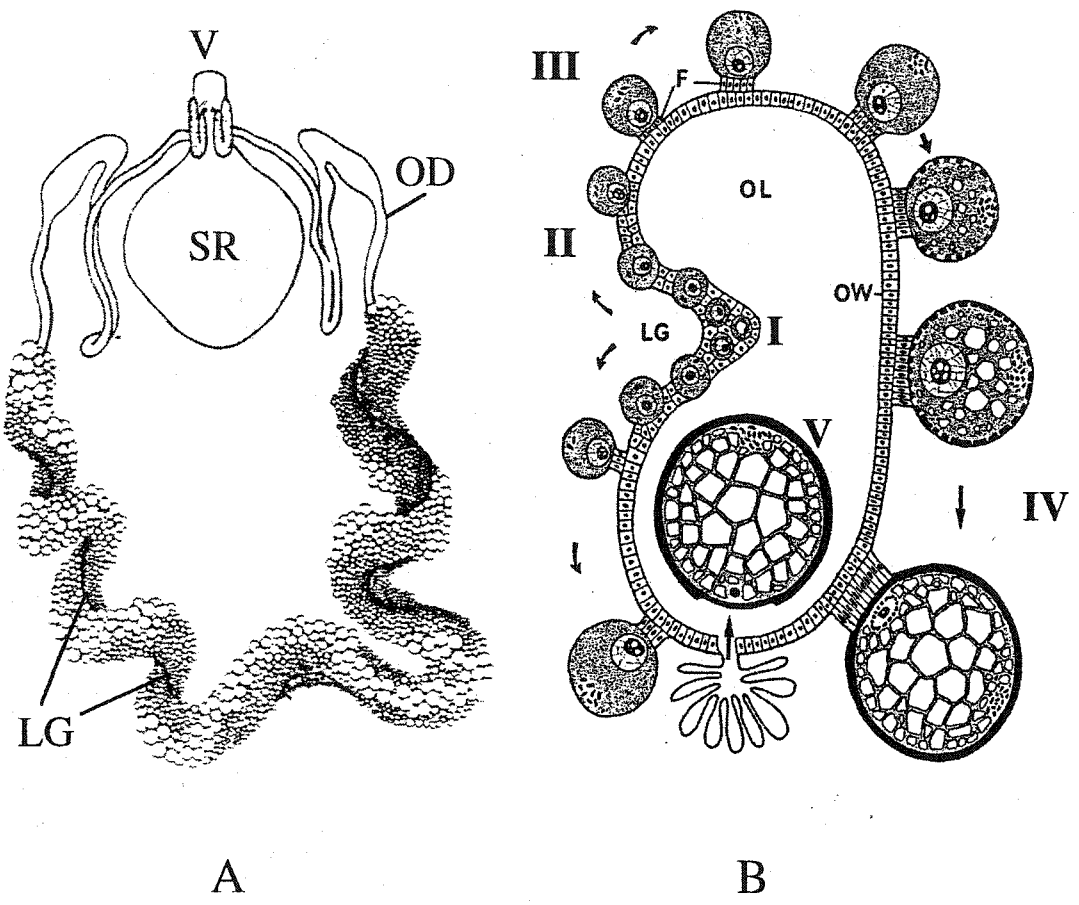

Figure 1.2. The tick ovary. (A) Female reproductive organs of a metastriate ixodid tick. (B) Schematic representation of the stages of oogenesis. (F) funicle cells, (LG) longitudinal groove, (OD) oviduct, (OW) ovary wall, (OL) ovary lumen, (SR) seminal recepticle, (V) vagina. Arrows indicate movement of oocytes around the ovary as development progresses through Balashov's five stages (I-V). Figures modified from Diehl et al. (1982).

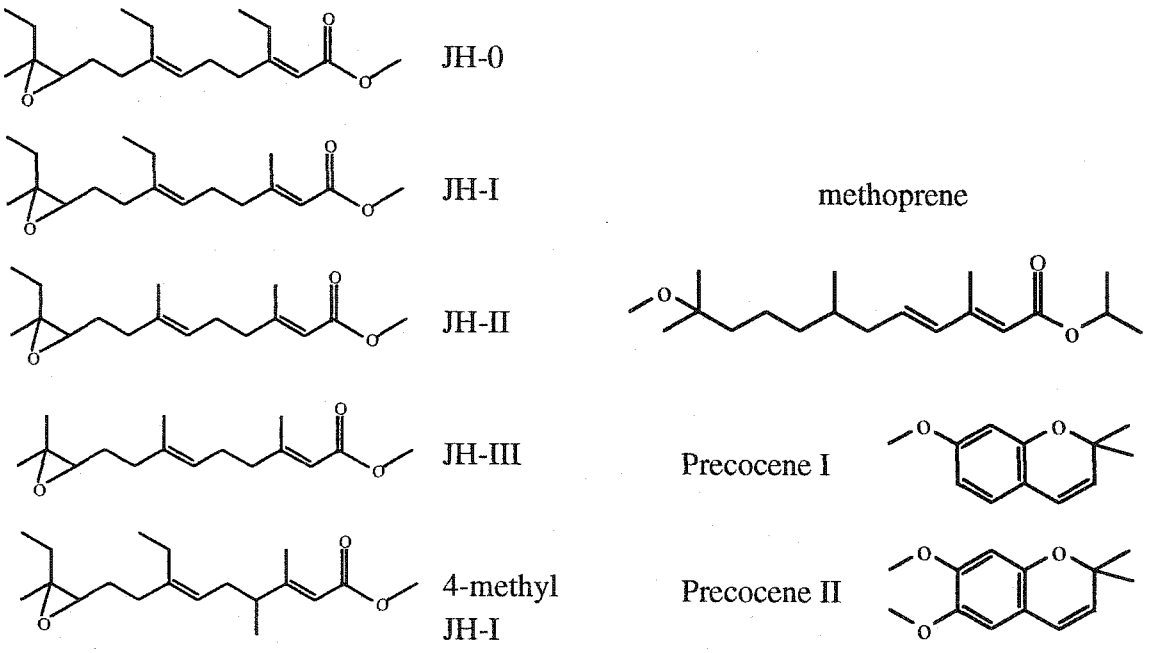

Figure 1.3. Structural diagrams of the juvenile hormones (JH) and other JH-related molecules.

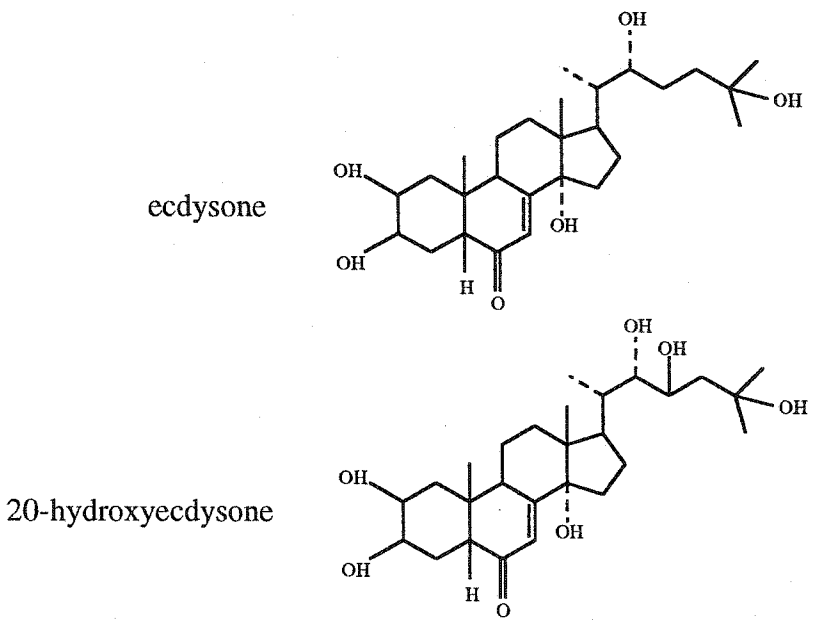

Figure 1.4. Structural diagrams of ecdysone and 20-hydroxyecdysone.

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Chapter 2

Quantification of Vitellogenesis and its Control by 20-Hydroxyecdysone in the Ixodid Tick, *Amblyomma hebraeum*[†]

Introduction

Synthesis of the major egg yolk protein, vitellogenin (Vg), and its uptake by oocytes are essential steps of egg maturation in all arthropods. While the roles of 20-hydroxyecdysone (20E) and juvenile hormones (JHs) during vitellogenesis have been well characterized in insects (reviewed by Nijhout, 1994), we know much less about the hormonal control of vitellogenesis in ticks. Vitellogenesis in the soft tick, *Ornithodoros moubata* (Family Argasidae), is triggered by a neuropeptide, 'vitellogenesis inducing factor' (VIF; Chinzei et al., 1992). This peptide triggers the release of 'fat body stimulating factor' (FSF) which, in turn, stimulates vitellogenesis in the fat body (Chinzei and Taylor, 1990). The identities of VIF and FSF are unknown, although evidence from several studies suggested that FSF may be a JH (Pound and Oliver, 1979; Connat et al., 1983), or an ecdysteroid (Sankhon et al., 1999; Taylor et al., 1997). However, attempts to confirm the presence of a JH in ticks by gas chromatography/mass spectrometry have failed (Connat, 1987; Neese et al. 2000).

There is some evidence that an ecdysteroid stimulates vitellogenesis in ticks. In the ixodid tick, *Amblyomma hebraeum*, haemolymph ecdysteroid-concentration rises after engorgement and, coincident with vitellogenesis, and peaks just before oviposition begins (Connat et al., 1985, Kaufman, 1991). Salivary gland degeneration, which occurs throughout the first week after engorgement, is regulated by an ecdysteroid in this tick (reviewed by Kaufman, 1989). However, the haemolymph ecdysteroid-concentration

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[†] A version of this chapter has been published. Friesen, K., and Kaufman, W.R., 2002, Journal of Insect Physiology, 48(8), 773-782.

rises far beyond that necessary for salivary gland degeneration, suggesting that an ecdysteroid may also be involved in other functions, such as vitellogenesis. Indeed, in *Ixodes scapularis* a rise in Vg-synthesis is associated with an increase in haemolymph ecdysteroid-concentration (James et al., 1997), and injections of 20E into partially-fed *A. hebraeum* females inhibits reattachment and feeding (Weiss and Kaufman, 2001).

In an attempt to establish the roles of JH and ecdysteroids during vitellogenesis, Lunke and Kaufman (1993) treated partially-fed (non-vitellogenic) female *A. hebraeum* with JH-III and 20E. Yolk uptake did not occur in response to JH-III, 20E or to combinations of the two. However, when they transplanted ovaries from partially-fed females to the haemocoels of engorged females, numerous oocytes accumulated a large amount of Vt, thus demonstrating that the ovaries of partially-fed ticks are fully competent to develop when placed in the hormonal milieu of a vitellogenic female. Finally, the anti-JH drug, precocene II, failed to inhibit egg development in engorged females. Unfortunately, haemolymph Vg was not quantified in the latter study. However, the authors noted a marked swelling of the fat body following 20E treatment. The fat body is a major site of yolk-synthesis in other ticks (Chinzei and Yano, 1985; Rosell and Coons, 1990), and the fat body of the ixodid tick, *D. variabilis*, synthesizes Vg when cultured *in vitro* with 20E, but not with the JH-mimic, methoprene (Sankhon et al., 1999). An *in vivo* study by Taylor et al. (1997) showed that 20E injections increased haemolymph Vg-concentration in unfed *O. moubata*, but the doses required were also toxic.

In brief, while there is tantalizing evidence to suggest that an ecdysteroid controls vitellogenesis in ticks, a clear effect has not yet been demonstrated *in vivo*, and the control of Vg-uptake remains unclear. In this study we describe our enzyme-linked immunosorbent assay (ELISA) for quantifying Vg in *A. hebraeum*, and we demonstrate that physiological doses of 20E can stimulate Vg-synthesis *in vivo*.

Materials and Methods

Ticks

Our colony of *A. hebraeum* was kept in darkness at 27°C and >95% humidity. Tick feeding occurred on rabbits as described by Kaufman and Phillips (1973). Ticks were allowed to engorge and detach spontaneously, or were forcibly removed from the host below the 'critical weight' necessary to begin vitellogenesis (Kaufman and Lomas, 1996; Weiss and Kaufman, 2001). In this study engorged weight ranged from 900 to 3500 mg, and partially-fed females (below critical weight) ranged from 100-250 mg. Ticks were cleaned with water, weighed, and stored individually in gauze-covered glass vials until needed for dissection and haemolymph collection.

Injections of 20E

20E (Sigma) was dissolved in 70% ethanol to make a 5 mg/ml stock solution, and was stored in a freezer until needed. On the day of injection, the stock solution was diluted to working concentration in sterile medium TC199 (Gibco) containing 50 µg/ml gentamicin (Sigma). Experimental ticks were surface sterilized in 70% ethanol for 1 min, and injected with 10 µl 20E/100 mg body weight (0.043-43 ng 20E/µl) via an AGLA micrometer syringe unit (Wellcome Reagents Ltd). Vehicle-injected and non-treated controls were run in parallel. Injections were repeated on day 2 and on day 5. Ticks were kept in the laboratory incubator until their haemolymph was collected on day 10.

Collection of egg, haemolymph, and ovary samples

Under our conditions for maintaining the tick colony, oviposition begins on day 10-12. At the onset of oviposition, and every other day, eggs were collected and weighed until oviposition ceased (approximately day 38). For collection of haemolymph 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 haemolymph (Kaufman, 1991). A small incision was made in the cuticle and haemolymph 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), 1:4 in 0.1 M MOPS (3-(N-morpholino)propanesulfonic acid, pH 7.5) for antiserum preabsorption, 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 chromatography, electrophoresis, or ELISA. Samples for RIA were stored in a laboratory freezer and all other samples were stored at -70°C until analyzed.

After collection of haemolymph, ticks were flooded with 1.2% NaCl (approximately isosmotic with tick haemolymph), the dorsal cuticle removed using a microscalpel, and the ovaries dissected out, gently blotted, and weighed. For chromatography (Fig. 2.3), tick ovaries were pooled and homogenized in PBS containing protease inhibitors (Protease Inhibitor Cocktail Set 1; Calbiochem). Homogenates were centrifuged at 13,000 X g for 5 min and the pellet discarded. Supernatants were used immediately for chromatography or stored at -70°C for further analysis.

Preparation of partially-purified Vt

A gel filtration column (Superose 6B, Pharmacia) was equilibrated with PBS at 4°C. Five ml of ovary homogenate from day 10 engorged ticks were applied to the column and subsequent eluant collected in 5-ml fractions. As tick Vg is known to contain a haem moiety (Sonenshine, 1991), the absorbance of each fraction was measured for both protein (280 nm) and haem content [taken as the difference between non-specific absorbance (500 nm) and haem-specific absorbance (400 nm)]. Adjoining fractions that were high in both protein and haem were pooled, concentrated using Centriprep tubes

(Amicon), and dialyzed against four changes of 4 l ion exchange running buffer (20 mM Tris HCl; pH 8.0) over 48 h in preparation for ion exchange chromatography.

Pooled fractions were applied to a DEAE-Sephacel ion exchange column (Pharmacia; equilibrated at 4°C with running buffer), and eluted by applying increasing concentrations of NaCl in running buffer in a stepwise manner. Similar to the gel filtration fractions, adjoining fractions which contained high concentrations of protein and haem were pooled and concentrated. The protein concentration of the resulting extract was measured using the Bradford assay (Bradford, 1976; Bradford reagent kit; Sigma), and the extract frozen at -70°C in 20 µl aliquots.

Electrophoresis and immunoblotting

Proteins from egg and haemolymph samples were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis; Laemmlli, 1970). Samples 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) to working concentration. Electrophoresis was performed using Protean or mini-Protean equipment (Bio-Rad) for large or mini-gels, respectively; mini-gels were used for immunoblotting, and large gels were used during Vg purification or for greater resolution of polypeptides. All gels consisted of a 3% stacking gel, and a 4-12% gradient or 7.5% continuous (mini-gels for immunoblots) resolving gel. Electrophoresis was performed at constant voltage until the dye front reached the bottom of the gels. Gels were then stained for protein with Coomassie brilliant blue, or the proteins were transferred to polyvinylidene difluoride membrane (PVDF; BioRad) for immunoblotting.

Following SDS-PAGE, egg and haemolymph proteins from gels were transferred to PVDF membrane for 1 h at 0.3-0.5 mA. After the transfer, each sample lane was sliced into 3 longitudinal strips so that the same sample could be stained with Coomassie

brilliant blue and the antibody for each Vg (see below). For antibody reactions, strips were first kept overnight at 4°C in blocking buffer (5% skim milk, 2% bovine serum albumin (BSA), in Tris-buffered saline (TTBS: 60 mM Tris-HCl, 0.3 M NaCl, 0.04% (v/v) Tween 20, pH 7.5). All subsequent steps occurred at room temperature. After blocking, strips were placed in blocking buffer containing either anti-Vg211 (1:500) or anti-Vg148 (1:250), or both. After 1 h, strips were washed 3 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 3 times for 5 min in TTBS and then stained for approximately 20 min using an AP-conjugate substrate kit (BioRad).

Gels were dried at 80°C for 1 hr using a slab drier (BioRad). Dried gels and immunoblots were converted to digital format using a benchtop scanner. Mobilities of polypeptide bands were then measured using NIH Image (National Institute of Health, U.S.A.) software, and the relative mobility of each band calculated in relation to the molecular weight standards.

Production of antibodies against Vg

Antibodies were produced against the two putative Vgs from engorged tick haemolymph (Vg211 and Vg148; see Results). Vg211 and Vg148 polypeptides were sliced from gels, homogenized in Freund's complete adjuvant, and injected subcutaneously into rabbits. Two booster injections of Vg were made using the same procedure but using Freund's incomplete adjuvant. Serum was collected after six weeks, by which time there was high antibody titre against Vg.

Engorged ticks were injected with anti-Vg antibodies in order to test the effect of these antibodies on blocking Vg-uptake. A mixture of equal amounts of undiluted anti-Vg211 and anti-Vg148 was injected into engorged ticks at volumes of 20 µl or 40 µl/g

bw. Control ticks were injected with a mixture of preimmune sera from the rabbits injected with Vg211 and Vg148. Ovaries were removed on day 10 post-engorgement, and analyzed for growth (see above).

IgG fractions were isolated from antisera using ammonium sulphate (50% saturated) precipitation and DEAE-Affigel Blue (BioRad) batch affinity chromatography. Precipitated antisera were centrifuged, and the pellet dissolved in DEAE-Affigel Blue running buffer (20 mM Tris-HCl, 25 mM NaCl, 0.02% sodium azide, pH 8.0). This solution was dialyzed overnight in 4 l running buffer and mixed with DEAE Affigel Blue beads in a sealed tube. The contents of the tube were mixed for 4 hours at 4°C, the beads were allowed to settle, and the enriched IgG fraction (supernatant) was retained.

Initially, IgG against both Vgs bound to haemolymph polypeptides other than Vg211 and Vg148 (data not shown). In order to further purify anti-Vg antibodies, IgG not specific to Vg was removed using Affigel 10 immunoaffinity beads (Bio-Rad) as follows. Affigel 10 beads were washed with cold 0.1 M MOPS (pH 7.5) in a sealed tube. Non-vitellogenic haemolymph (day 0 engorged or day 10 partially-fed) was added to the tube and mixed with the beads for 4 h at 4 °C. The beads were sedimented by gentle centrifugation, and the supernatant (containing excess haemolymph protein) discarded. So that IgG would not bind to the beads, any additional protein binding sites were blocked by adding 100 µl 1 M ethanolamine/ml beads and mixing for 1 h. The blocking solution was then removed by washing the beads 4 times with excess DEAE Affigel Blue running buffer. The enriched IgG fraction from the DEAE-Affigel Blue step above was then added to the reaction tube and allowed to incubate with the beads overnight at 4°C. The beads (containing IgG specific to non-vitellogenic females) were again settled by gentle centrifugation, and the supernatant (containing Vg-specific IgG) was removed and frozen at -70°C in 200 µl aliquots.

RIA for ecdysteroid

Ecdysteroid-concentration in tick haemolymph was determined by the RIA procedure described by Kaufman (1990). Briefly, a methanol extract of each haemolymph sample was dried under vacuum and diluted in a 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 (NEN) per tube and an ecdysone antiserum (E-22-succinyl-thyroglobulin; supplied by Dr. L.I. Gilbert, University of North Carolina, or 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'.

ELISA

Partially purified Vt (see above) was used as a standard for an indirect competitive antibody capture ELISA. This ELISA was developed by modifying the procedure described by Rosell and Coons (1991b); the final procedure is as follows. With the exception of blank control wells, each well of a 96 well microtiter plate was coated with 1 µg purified Vt in 100 µl PBS (pH 7.0, 0.02% sodium azide). Plates were placed in an airtight container with a small volume of water to hinder evaporation. At the same time, samples were quantified using the Bradford assay, diluted to constant concentration (0.1 and 0.01 mg/ml) in PBS, and mixed 1:1 with anti-Vg211/Vg148 IgG diluted 1:250 in 6% BSA in PBS. Both the samples and coated microtiter plates sat

overnight at 4°C. All subsequent treatment took place at room temperature in the sealed container. Wells were washed twice with PBS and then blocked with 150 µl of 3% BSA in PBS for 30 min. Wells were again washed twice with PBS, and 100 µl of each sample/antibody mixture were added to the appropriate well; 100 µl PBS were added to control wells. Two hours later, the wells were washed three times with PBS, and 100 µl secondary antibody (AP-conjugated goat anti-rabbit IgG; BioRad), diluted 1:500 in 1% BSA in PBS, were added to each well except for the blank control wells. Two hours later the wells were again washed three times with PBS, and 100 µl AP substrate (p-Nitrophenylphosphate and diethanolamine kit, BioRad) were added to each well. After 30 min, the colour reaction was stopped by adding 100 µl 0.4 M NaOH per well. Absorbance was read at 405 nm using a microtitre plate reader (Bio-Tek).

Absorbance values of known Vt-concentration were plotted on a logarithmic scale to create a standard curve (Fig. 2.1) by 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 is approximately 5 ng of Vt-equivalents.

Statistics

All graphs were generated using Microsoft Excel 98 software. Unless otherwise stated, data are presented as mean ± SEM (n).

Results

Post-engorgement growth of ovary and egg production

Ovaries of ticks maintained at 27 °C gained weight slowly for about 4 days, reaching 0.5±0.1% bw; they then grew more rapidly between days 6-12 (due to yolk uptake), reaching a maximum of 7.3±0.2% bw (n=10-26) at the onset of oviposition (Fig. 2.2). The ovaries then gradually lost weight as oviposition proceeded.

Forty-eight hour egg production increased steadily, reaching a plateau on day 16 post-engorgement. Subsequent egg production declined steadily from day 20 to day 36-38, by which time oviposition ceased. Cumulative egg production (calculated as the total egg weight taken as a percent of initial tick body weight) over the complete oviposition period averaged 42±3% of engorged body weight (n=26).

Isolation and characteristics of egg Vt and haemolymph Vg

Partial purification of Vt by gel filtration followed by ion exchange chromatography is shown in Fig. 2.3. The only haem containing peaks eluted in fractions 25-35 of the Superose 6B column (Fig. 2.3A). When this fraction was run on a DEAE Sephacel column, one major, and two minor haem containing fractions were detected (Fig. 2.3B). Fractions corresponding to the major peak (fractions 40-60) were kept as the standard Vt for gel electrophoresis and ELISA assays (see below). The two minor peaks were not analyzed further.

The Vt rich fractions were further resolved by SDS-PAGE into 7 major polypeptides with molecular sizes of 157, 104, 98, 83, 68, 66, and 62 kD (average 6 trials; lanes B and G, Fig. 2.4). Two polypetides (211 and 148 kD, average of 5 trials) present in the haemolymph of female ticks 10 days post-engorgement (lane E, Fig. 2.4), but not in day 0 engorged females (lane D, Fig. 2.4), nor day 10 partially-fed females (lane F, Fig. 2.4), nor males (data not shown), were also present in day 10 ovaries as minor polypeptides. In some gels, each of these two polypeptides from ovaries appear as a pair of bands (lane B, Fig. 2.4; see Discussion). However, for the purpose of identification, we designate both the haemolymph and associated ovary peptides as 211 and 148 kD. The haemolymph concentration of both the 211 and 148 kD polypeptides increased steadily from day 0 to day 10 post-engorgement (Fig. 2.5). It is on the basis of the above criteria (haem-containing protein, not detected in males and non-vitellogenic

females) that we identify these two polypeptides to be an unambiguous source of Vt. The most abundant haemolymph proteins at all stages of adult ticks were two polypeptides of approximately105 and 95 kD which also appeared to be present in eggs (lanes B-G, Fig. 2.4; all sample lanes, Fig. 2.5). Although they share similar molecular weights, these haemolymph polypeptides are probably not the same as the polypeptides found in eggs and day 10 ovaries (see Discussion).

Antibodies for immunoblots and ELISA were raised against Vg211 and Vg148. Following injections, these antibodies inhibited ovary and oocyte growth in engorged ticks (Fig. 2.6). Anti-Vg211 and anti-Vg148 IgG bound specifically to their antigens in day 10 engorged tick haemolymph, but not to any haemolymph polypeptides from non-vitellogenic females (Fig. 2.7). In addition, anti-Vg211 bound to the 211, 171, 157, 148, and 98 kD polypeptides from day 10 ovaries, and anti-Vg148 bound to the 148, 66, and 62 kD polypeptides from day 10 ovaries, indicating that a significant degree of Vg-processing had occurred following uptake. We also stained SDS-PAGE gels with tetramethylbenzidine (TMBZ) to determine the haem-content of polypeptides. Vg211 and its associated ovary polypeptides reacted with TMBZ, but no reaction occurred with Vg148 and its associated ovary polypeptides (results not shown).

Haemolymph 20E and Vg concentrations

On the day of engorgement, haemolymph titre of 20E was relatively low (20±2 ng/ml; n=8), but rose to 83±7 ng/ml (n=6) by day 4 (Fig. 2.8 inset). Following day 4, the rate of increase accelerated, and the 20E titre eventually peaked at 1737±232 ng/ml (n=6) on day 18 before falling to 1168±74 ng/ml (n=5) on day 20. Haemolymph Vg-concentration was also relatively low on day 0 (0.3±0.1 mg/ml; n=5), rising to 2.2±0.7 mg/ml (n=6) on day 2 and 13.5±3.6 mg/ml on day 4 (Fig. 2.8, inset), and then increasing a further 2.4-fold to an early peak on day 8 (33±9 mg/ml; n=6). The haemolymph Vg-

concentration appeared to drop transiently at the onset of oviposition to 19±4 mg/ml (n=7) on day 12, and then recovered (41±11 mg/ml; n=3) on day 16.

Effect of 20E on partially-fed ticks

Traces of Vg211 and Vg148 were detectable in haemolymph of ticks that received low doses of 20E, but similar traces also appeared in haemolymph of non-injected and ethanol injected controls (Fig. 2.9). However, doses of 430 to 4300 ng 20E/g body weight caused Vg211 and Vg148 concentrations to increase dramatically in haemolymph of partially-fed females (Fig. 2.9).

Discussion

As in insects, vitellogenesis in ticks occurs extraovarially; Vg is synthesized in fat body in *O. moubata* (Chinzei and Yano, 1985; Taylor et al., 1991), or both fat body and midgut cells in *D. variabilis* (Rosell and Coons, 1990). Vg is then released into the haemolymph and accumulated in large yolk granules by developing oocytes (reviewed by Diehl et al., 1982). Tick Vt is composed of 'haemoglycophospholipoproteins', with molecular weights ranging from 370 kD (*Dermacentor variabilis*; Rosell and Coons, 1991a) to 8700 kD (*D. andersoni*; Boktor and Kamel, 1976). Using SDS-PAGE, tick Vts have been resolved into 6 to 9 polypeptides, in *D. andersoni* (Boktor and Kamel, 1976), *O. moubata* (Chinzei et al., 1983), *Argas hermanni* (Shanbaky et al., 1990), *D. variabilis*; (Rosell and Coons, 1991a), *O. parkeri* (Taylor et al., 1991), and *I. scapularis* (James and Oliver, 1997).

In *A. hebraeum*, we have identified two Vg polypeptides (Vg211 and Vg148) in haemolymph from vitellogenic females, but not in haemolymph of males nor in non-vitellogenic females (Fig. 2.4). Vg211 and Vg148 are also present in day 10 ovaries,

although they each appear as a pair of bands on SDS-PAGE gels (Fig. 2.4). However, the antibody raised against haemolymph Vg211 binds to its corresponding pair in the ovary; likewise for the antibody raised against haemolymph Vg148 (Fig. 2.7). Yolk uptake by oocytes occurs continually throughout the post-engorgement period beyond day 4. Although Vg211 and Vg148 are detected as single polypeptides in the haemolymph, processing probably begins as soon as Vg is taken up by oocytes; the double bands appearing in the ovary may be a reflection of this early processing. These polypeptides were only minor bands in mature ovaries, and Vg211 itself was not detected in oviposited eggs (Fig. 2.4). But almost all of the major polypeptides of ovaries and eggs were recognized either by anti-Vg211 or anti-Vg148 antibody. Similar results were observed by Taylor et al. (1991), who compared haemolymph and egg polypeptides from *O. parkeri* and *O. moubata* by SDS-PAGE. In those species Vg contains two polypeptides that are not present in eggs, but are processed to form the Vt subunits. In ixodid ticks, haemolymph from vitellogenic *D. variabilis* (Rosell and Coons, 1991a) and *I. scapularis* (James and Oliver, 1997) also contain polypeptides with higher molecular weights than the polypeptides from eggs. In *I. scapularis*, these polypeptides also bind antibodies raised against Vt (demonstrated by immunoblot).

Two polypeptides (approximately 105 and 95 kD) from tick haemolymph were the most abundant at all adult stages, including day 10 post-engorgement females (Figs. 2.4 and 2.5). Similar polypeptides were also found in mature ovary. Anti-Vg211 IgG bound to the 105 kD polypeptide in day 10 ovary, but not to the one in haemolymph. On the other hand, neither anti-Vg211 nor anti-Vg148 bound to the 95 kD polypeptides in ovary or haemolymph (Fig. 2.7). Similar haemolymph polypeptides (98 and 92 kD) have been identified from all stages of *D. variabilis*, and referred to as 'lipoglycoheme-carrier protein' (CP; Gudderra et al., 2001). In *D. variabilis*, CP is the most abundant protein in haemolymph, but is present only in small quantities in eggs. Because amino acid

sequence analysis showed CP to have a 61% homology to brine shrimp artemocyanin (a possible storage protein), the authors speculate that CP may be responsible for sequestering haem and lipid from the blood meal. In *A. hebraeum*, the concentrations of the 105 and 95 kD polypeptides increased soon after engorgement and the beginning of blood meal digestion (Fig. 2.5), suggesting that they may likewise be involved in the transport or storage of nutrients.

Ovary growth proceeded slowly during the first 4 days post-engorgement, a time corresponding to the period of late previtellogenesis ('great cytoplasmic growth') described by Balashov (1972), and then accelerated largely due to vitellogenesis (Fig. 2.2). Yolk uptake was preceded by a 6-fold increase in haemolymph Vg-concentration from day 2 to 4, and a further doubling of the concentration by day 8 (Fig. 2.8). The dip in Vg-concentration between days 10-14 suggests that the rate of Vg-uptake by oocytes may exceed the rate of Vg-synthesis at this time. Cumulative egg production by *A. hebraeum* was 42±3% of engorged weight, which is a very similar value to that previously reported for this species (Kaufman et al., 1986). *Ixodes ricinus* produces about 52% of their engorged weight as eggs, and *Boophilus microplus* produces about 62% of their engorged body weight as eggs (reviewed by Diehl et al., 1982).

Using an indirect ELISA similar to the one described here, Rosell and Coons (1991b) found that haemolymph Vg-concentration in *D. variabilis* peaks at 430 µg/ml by the third day of oviposition, and then declines rapidly to reach a very low concentration (approximately 25 µg/ml) by the eighth day of oviposition. In *A. hebraeum*, haemolymph Vg-concentration reaches its peak 2 days prior to oviposition and is maintained at a high level until at least the tenth day of oviposition (Fig. 2.8). Moreover, the absolute haemolymph Vg-concentrations observed in this study were approximately 10-fold those observed for *D. variabilis* by Rosell and Coons (1991b). Differential rates of Vg-synthesis and uptake probably account for the different profiles.

In insects, vitellogenesis is regulated primarily by juvenile hormones or ecdysteroids (reviewed by Nijhout, 1994). Vitellogenesis is under hormonal control in ticks (Chinzei and Taylor, 1990; Lunke and Kaufman, 1993), although the hormone that stimulates Vg-synthesis was unknown until recently. In *D. variabilis*, 20E stimulates Vg-synthesis by fat body *in vitro*, an effect that cannot be mimicked by the JH-analogue, methoprene (Sankhon et al., 1999). In *O. moubata,* haemolymph Vg-concentration increased 3-fold by 5 days after 20E injections (Taylor et al., 1997), although changes in ovary development were not observed because the ticks did not survive for 10 days. Lunke and Kaufman (1993) were not able to stimulate Vg-uptake by ovaries (as measured 7 days after a 24-hour infusion of 20E into partially-fed ticks), but they noted that the fat body increased in size after 20E treatment. Effect on haemolymph Vg-concentration in response to hormone treatments was not measured in their study. Here we demonstrate in *A. hebraeum* that haemolymph ecdysteroid rises 4-fold prior to a significant increase in Vg (Fig. 2.8, inset). Furthermore, treatment of partially-fed females (i.e. non-vitellogenic) with exogenous 20E stimulated a marked increase in haemolymph Vg-concentration (Fig. 2.9). The lower molecular weight peptides binding to anti-Vg antibodies in Fig. 2.9 are likely products derived from one or both Vgs.

The highest Vg-titres occurred after injecting 3 doses of 430 ng 20E/g body weight. As the haemolymph volume of partially-fed *A. hebraeum* is approximately 25% of body weight (Kaufman et al., 1980), the approximate haemolymph concentration at this dose should be 1230 ng 20E/ml haemolymph following each injection of 10 µl/100 mg body weight. This value is well within the physiological range, as 20E concentrations peaked at 1737±232 ng/ml in day 18 engorged females (Fig. 2.8). Rates of ecdysteroid clearance are very high in this tick (between 85 to 99% removed in 7 hours; Weiss and Kaufman, 2001). Thus, the multiple injections of 20E are probably necessary to maintain

an effective concentration in the haemolymph for just a few hours, but there has not yet been an exhaustive study to determine the most efficient protocol for hormone treatment.

The model for hormonal regulation of vitellogenesis by *O. moubata* developed by Chinzei and Taylor (1990) indicates that FSF is the ultimate hormone stimulating vitellogenesis. While the identity of FSF is uncertain, the authors speculated that it may be a steroid hormone because of the presence of ecdysteroid in ticks during vitellogenesis, and the similarity of their model to vitellogenesis in other animals. Vitellogenesis is regulated by a peptide in both mosquitoes (ovarian ecdysteroidgenic hormone; reviewed by Klowden, 1997) and oviparous vertebrates (follicle stimulating hormone; reviewed by Hadley, 1992), that stimulates release of a steroid (ecdysone in mosquitoes or estrogen in vertebrates) which triggers Vg-synthesis. The fact that ecdysteroid-synthesis in *A. hebraeum* is stimulated by a neuropeptide (Lomas et al., 1997) also fits this general model. However, because doses of 20E that are sufficient to stimulate vitellogenesis in partially-fed ticks do not stimulate yolk uptake (Lunke and Kaufman, 1993; Chapter 2), another factor must be necessary for complete oocyte maturation.

Figure 2.1. Standard curve for tick Vt using the ELISA described here. All haemolymph sample concentrations were adjusted to fall within the range of 20-60% bound. The sensitivity of this assay is ~5 ng Vt-equivalents (amount of Vt at 80% bound).

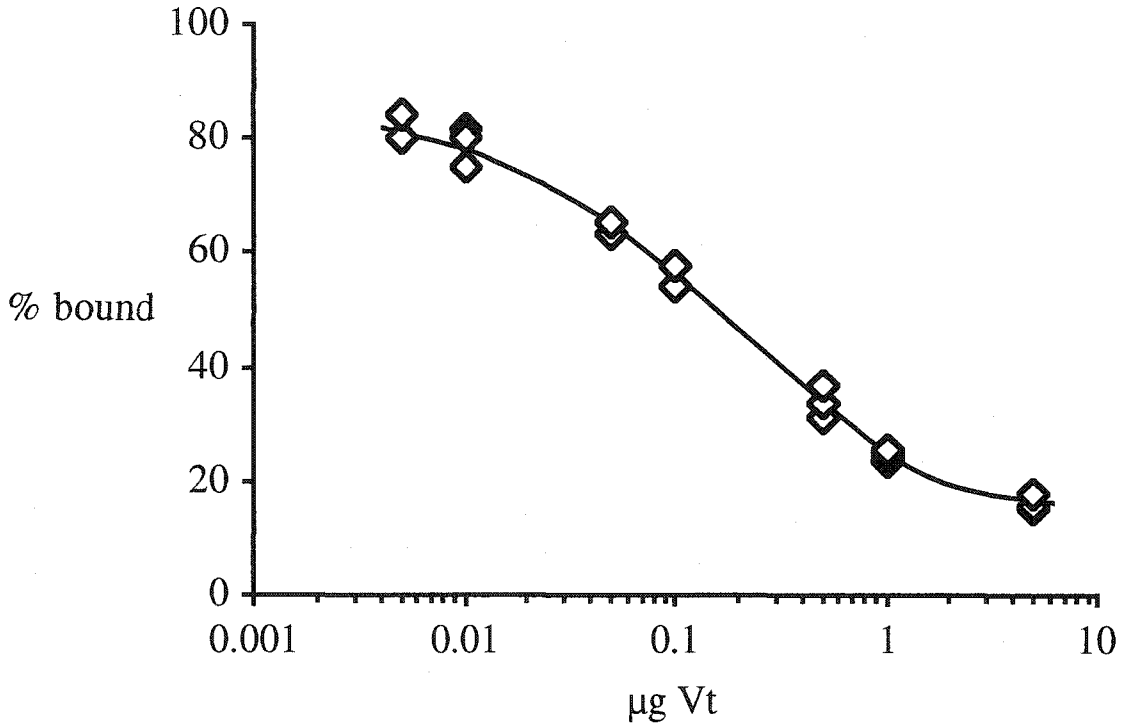


Figure 2.2. Profile of post-engorgement ovary growth (open circles) and egg production (open diamonds) in *A. hebraeum*. Eggs were collected from 26 females every 48 h after the onset of engorgement (day 10). Total egg production in this population was 42±3% engorged body weight. For ovaries n=10-26. SEMs are shown wherever they exceed the dimension of the symbols.

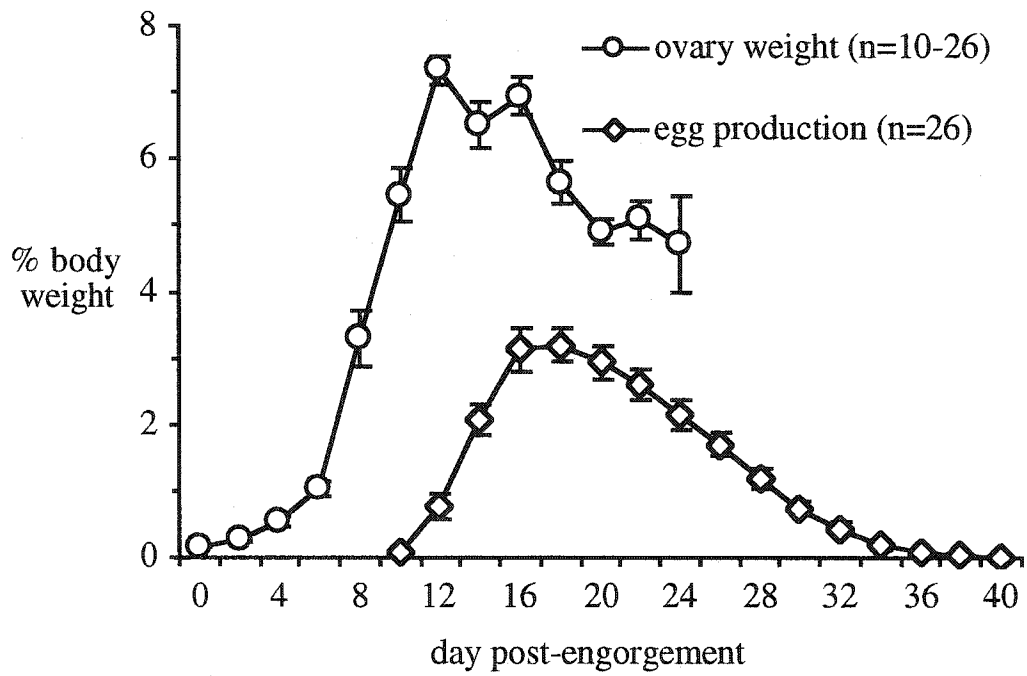


Figure 2.3. Separation of proteins from ovary homogenate by gel filtration (A) followed by ion exchange chromatography (B). Absorbance of eluant fractions was measured spectrophotometrically at 280 nm for protein (dashed line) and the difference between 400 nm and 500 nm for haem (solid line; see Kaufman et al. 1986). Gel filtration fractions 28 through 35 (containing large quantities of haem and protein) were pooled and separated further using a stepwise increase of NaCl on an ion exchange column. Ion exchange fractions 39 through 55 contained high quantities of haem and protein, and thus were assumed to be rich in Vt and pooled for further analysis. NaCl steps in running buffer (20 mM Tris-HCl, pH 8.0): a) 0.2M NaCl b) 0.22 M NaCl c) 0.25 M NaCl d) 0.5 M NaCl.

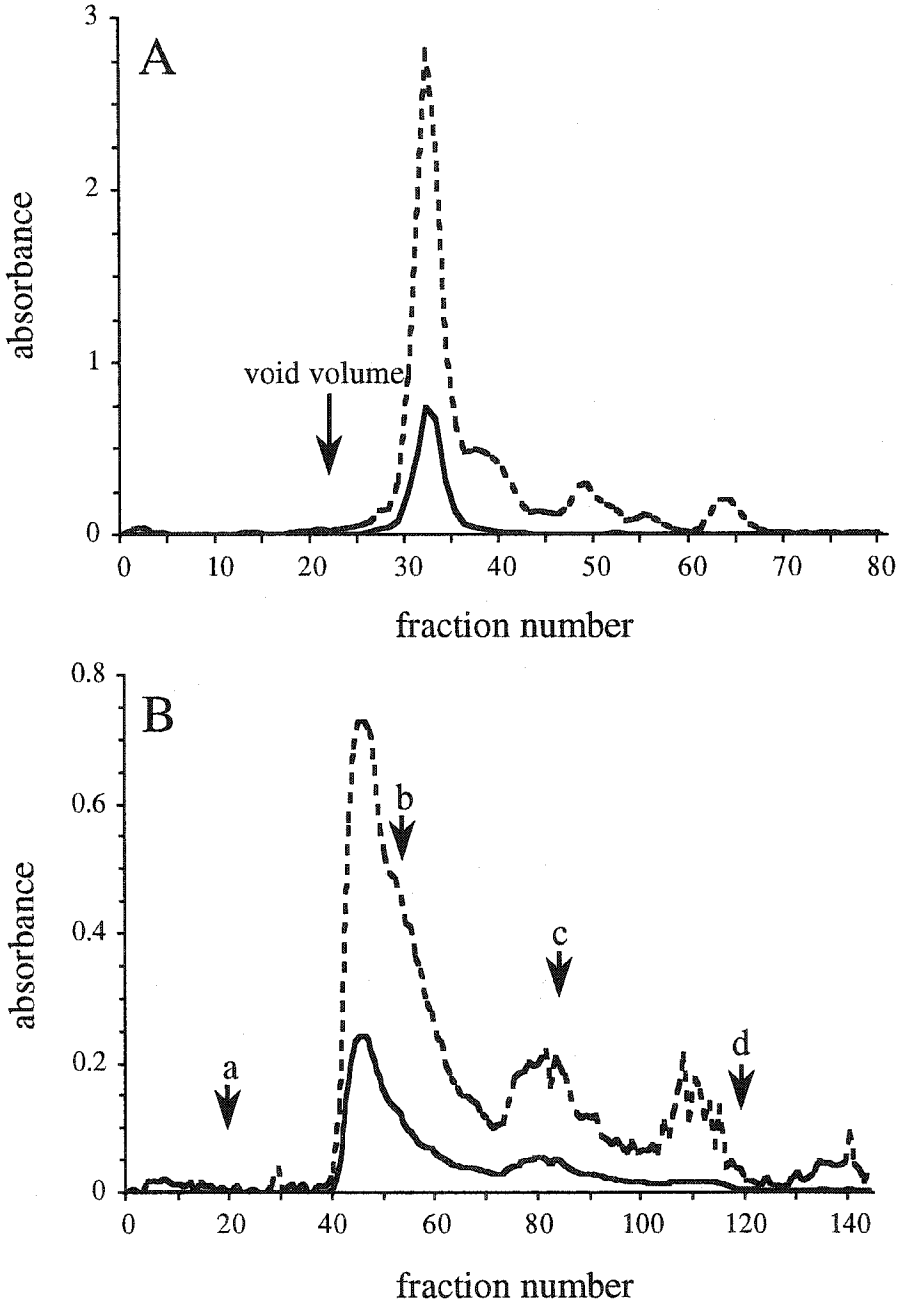


Figure 2.4. Separation of ovary, egg and haemolymph polypeptides by 4-12% SDS-PAGE. Lanes contain (A) 20 µg crude ovary homogenate from day 0 engorged ticks (B) 20 µg crude ovary homogenate from day 10 engorged ticks, (C) 20 µg crude egg homogenate 24 h after oviposition, (D) 0.5 µl day 0 engorged female haemolymph, (E) 0.5 µl day 10 engorged female haemolymph, (F) 0.5 µl day 10 partially fed female haemolymph and (MW) molecular weight markers. Lane G contains 30 µg crude ovary homogenate separated by 7.5% SDS-PAGE resolving gel to demonstrate clearer distribution of polypeptides. Black arrowheads indicate major egg polypeptides (see Results). Two minor polypeptides (asterisks; 211 and 148 kD) are putative Vgs as they are present both in day 10 ovaries and day 10 engorged tick haemolymph, but only in small amounts in non-vitellogenic female (day 0 engorged and day 10 partially-fed) haemolymph.

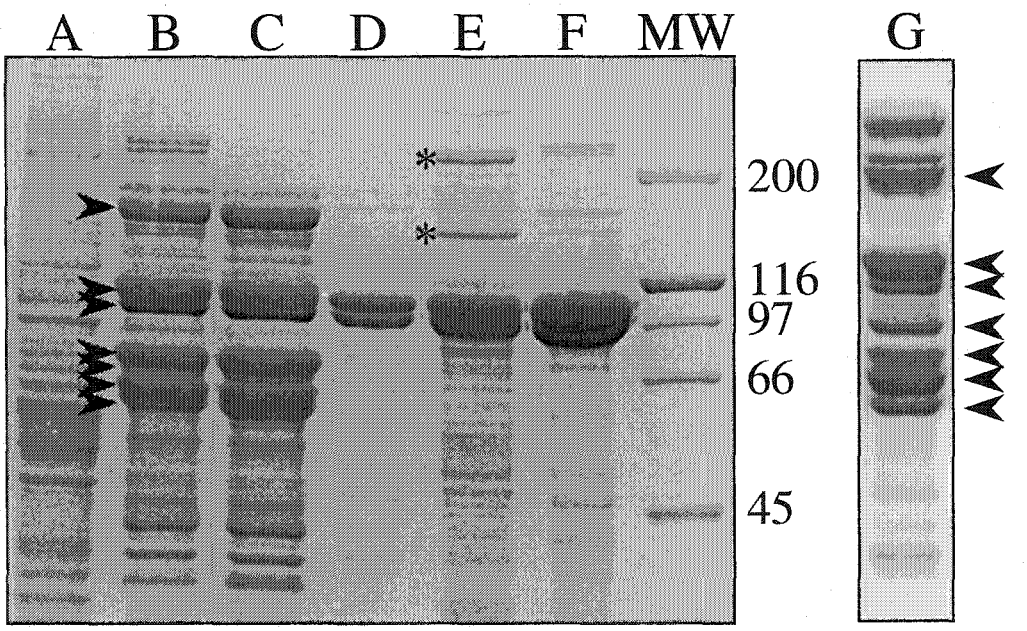


Figure 2.5. Changes in Vg211 and Vg148 (black arrowheads) in haemolymph during the first 10 days post-engorgement. Pooled samples were diluted to 0.5 µl haemolymph/lane and applied to a 4-12% resolving SDS-PAGE gel. 'Ov.' lane contains 40 µg day 10 ovary homogenate. Vg polypeptides increased approximately 15 fold from day 2 to day 10 post-engorgement, as determined by ELISA (see Fig. 2.8).

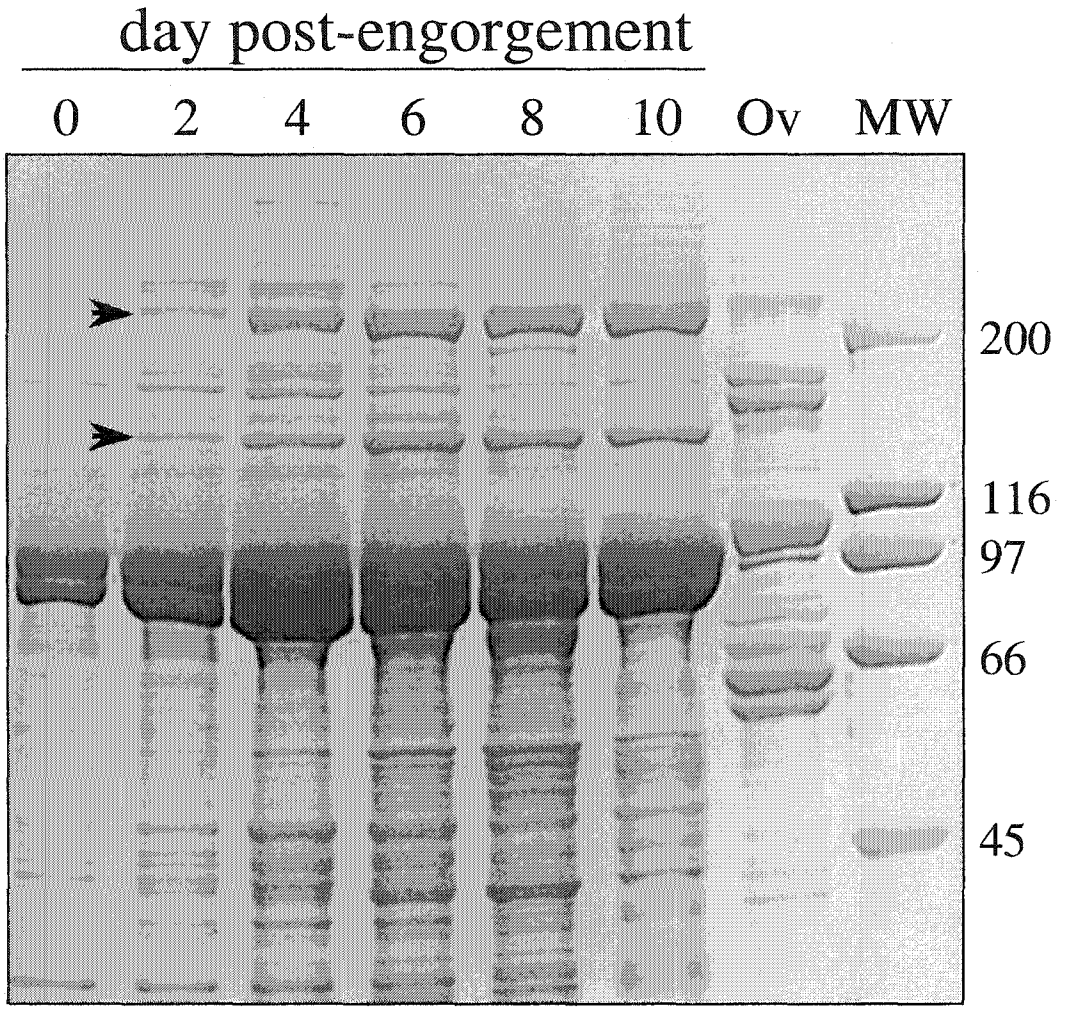
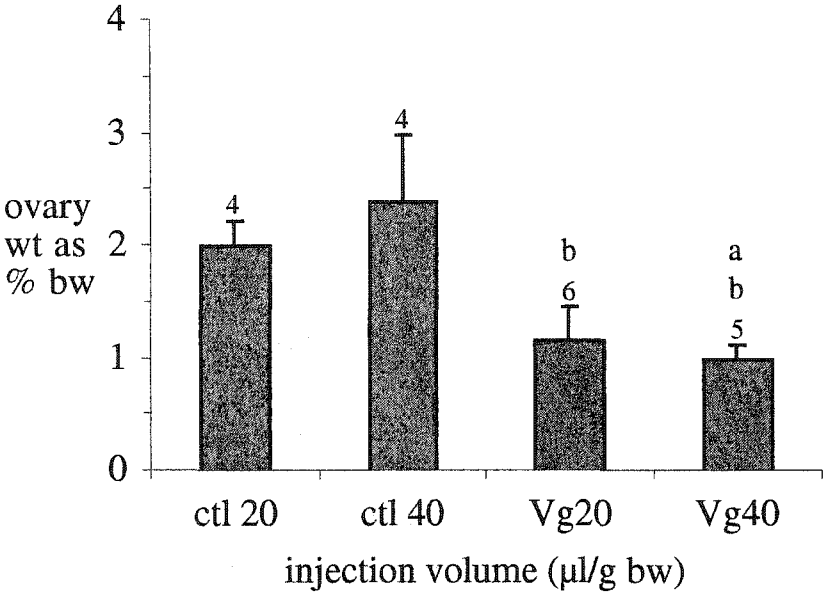


Figure 2.6. Effect of anti-Vg211 and anti-Vg148 antibodies on engorged ticks 10 days post-engorgement. Ticks received 20 µl (Vg20) or 40 µl (Vg40) injections of antisera. (A) mean ovary weight and (B) mean length of 8 of the longest oocytes. Significant differences from 20 µl and 40 µl preimmune serum controls (ctl 20 and ctl 40) indicated by 'a' and 'b', respectively.



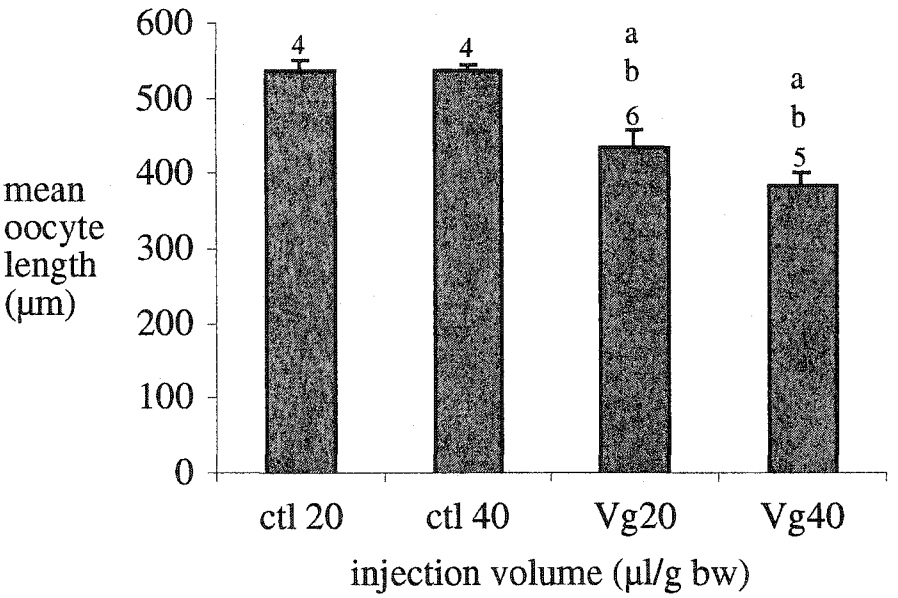


Figure 2.7. Immunoblot showing specificity of anti-Vg antibodies for polypeptides from day 10 ovaries (ov.) and haemolymph from day 0 and day 10 engorged female (d0eng and d10eng), and day 10 partially fed female (d10pf) ticks. Strips stained with a) Coomassie brilliant blue; b) anti-Vg211; c) anti-Vg148. Arrows indicate two polypeptides (Vg211 and Vg148) which are present in both day 10 ovaries and day 10 engorged haemolymph, but not in day 0 engorged females. In this immunoblot, only trace amounts of Vg211 and Vg148 were detected in haemolymph of day 10 partially-fed females.

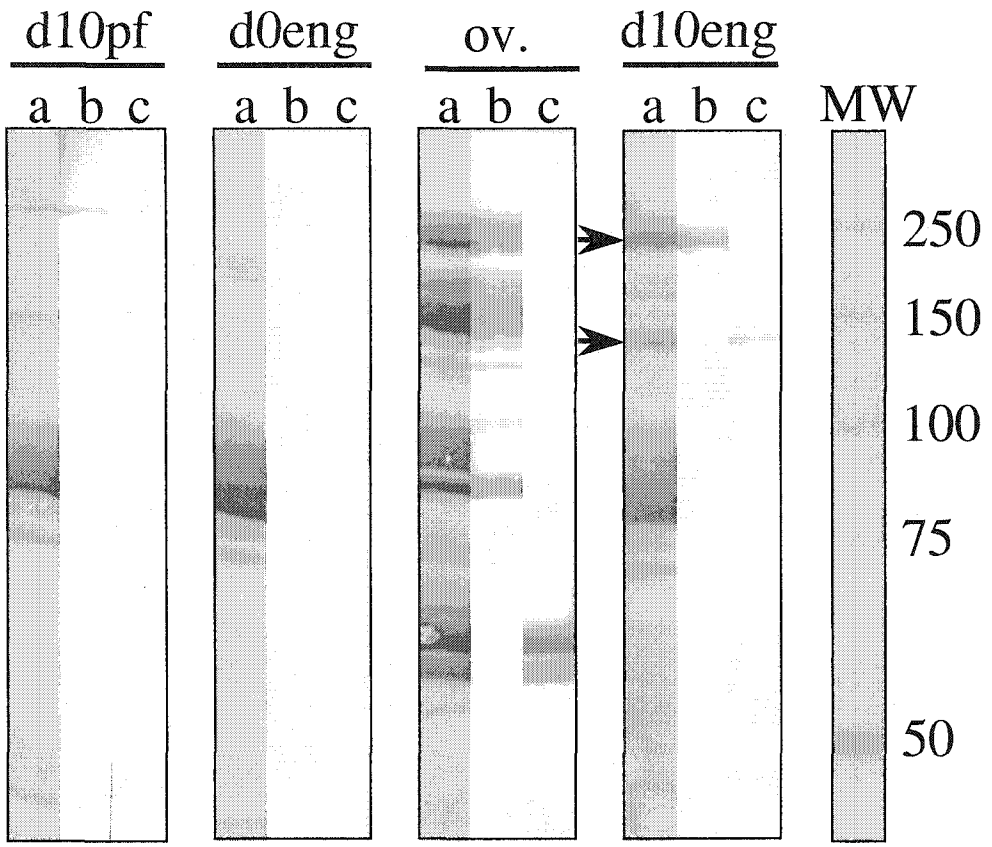


Figure 2.8. Profiles of haemolymph 20E-equivalents (open circles) and Vg-concentration (open diamonds) from engorged ticks over the first 20 days post-engorgement (n=5-15). Vg is reported as Vt-equivalents (see Materials and Methods). Vertical dotted line indicates onset of oviposition on day 10. The inset shows the data for days 0-4 on a magnified scale; note in the inset that the increase in Vg-concentration apparently lags behind that of 20E. SEMs are shown wherever they exceed the dimension of the symbols.

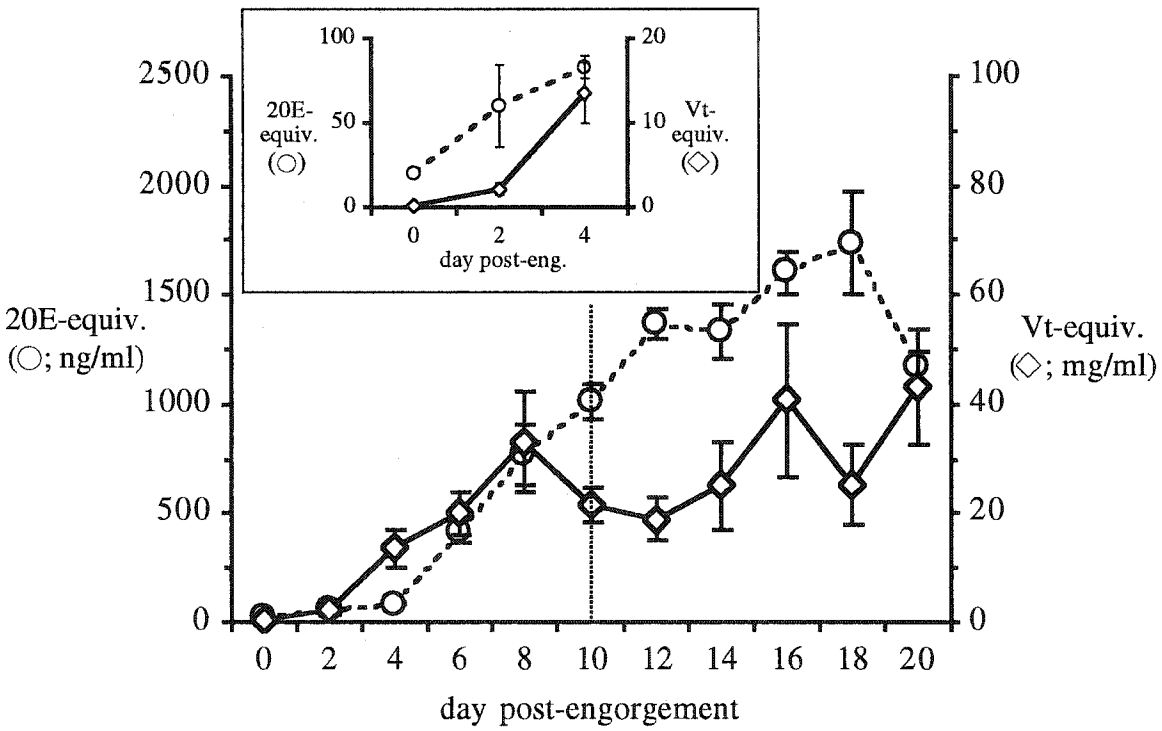
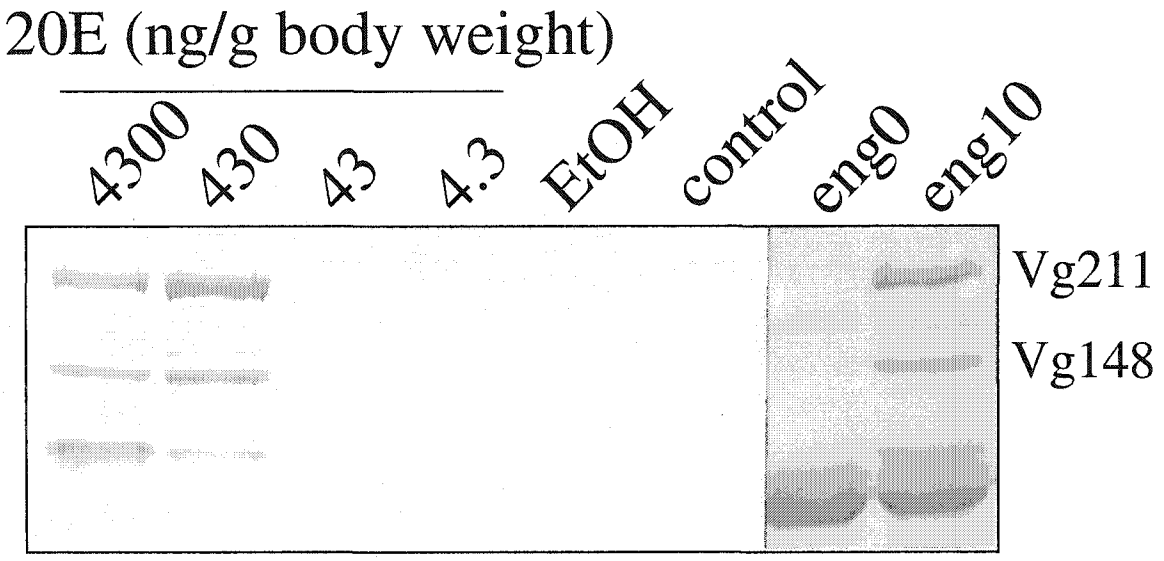


Figure 2.9. Immunoblot of haemolymph from partially fed females, below the 'critical weight' for Vg production, injected with 20E (see Materials and Methods). Vg211 and Vg148 increased dramatically 10 days after bolus injections of 430 or 4300 ng 20E/g body weight. Some additional polypeptides also reacted with this mixture of anti-Vg211and anti-Vg148 IgG, which may have resulted from the breakdown or processing of Vg. Ticks treated with lower concentrations of 20E and controls (ethanol- or non-injected) showed only traces of Vg. Normal day 0 (eng0) and day 10 (eng10) engorged haemolymph polypeptides were stained for protein only with Coomassie brilliant blue.



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Chapter 3

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*[†]

Introduction

The hormones that control yolk protein synthesis and egg development in insects are well characterized. Synthesis of vitellogenin (Vg) is regulated by the juvenile hormones (JHs), and/or the ecdysteroids (reviewed by Nijhout, 1994). Much less is known about vitellogenesis in ticks and other chelicerate arthropods (Kaufman, 1997). A series of experiments by Chinzei et al. (1992) indicates that Vg-synthesis is initiated by a neuropeptide from the synganglion ('vitellogenesis inducing factor'; VIF). VIF acts on some unknown tissue to cause the release of a vitellogenic hormone ('fat body stimulating factor'; FSF). FSF is the tick vitellogenic hormone, stimulating the synthesis of Vg and its release into the haemolymph. Here, we attempt to further characterize the vitellogenic hormone in the ixodid tick, *Amblyomma hebraeum*.

The weight of current evidence is in favour of an ecdysteroid being the vitellogenic hormone in ixodid ticks:

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

[†] A version of this chapter has been submitted for publication.

- Injections of 20E into small partially-fed ticks (*A. hebraeum*) stimulates enlargement of fat body, presumably reflecting accelerated yolk-synthesis (Lunke and Kaufman, 1993). However, exogenous 20E in partially-fed ticks does not stimulated yolk-uptake by oocytes.

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

Numerous neurosecretory centers have been identified histologically in the synganglion (=CNS) of ticks (reviewed by Binnington and Obenchain, 1982). Neurosecretory activity in general seems to increase shortly after a bloodmeal, and then decline 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 neuropeptide from the synganglion stimulates ecdysteroid-synthesis in *A. hebraeum.* While no specific neurosecretory products have been isolated from ticks, antibodies against bovine insulin bound to three neurosecretory regions in the syngangion of *Ornithodoros parkeri* (Zhu et al., 1991). Vertebrate insulin has up to 68% nucleotide sequence homology to bombyxin, an ecdysteroidogenic peptide originally isolated from the silkworm, *Bombyx mori* (Iwami et al., 1989). Both, bovine and porcine insulins, acting through insulin receptors, can stimulate ecdysteroid-synthesis in isolated ovaries of the mosquito, *Aedes aegypti* (Graf et al., 1997; Riehle and Brown, 1999). The effects of insulin have never been tested in ticks.

There is also evidence in ticks of a role for JH in egg production. Engorged argasid ticks treated with the JH blocker, precocene, produced and laid fewer eggs than untreated engorged ticks, an effect which was partially reversed by JH treatment (Pound and Oliver, 1979). In addition, 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 was not able to stimulate Vg-synthesis in unfed, virgin argasid ticks, even though such ticks do undergo vitellogenesis when treated with pyrethroid insecticides. In addition, although extracts of *Boophilus microplus* have JH-like bioactivity (Connat, 1987), no known JH has yet been identified in ticks by gas chromatography/mass spectrometry (Connat, 1987; Neese et al., 2000).

The latter studies suggest that whereas JH may not be the vitellogenic hormone in argasid ticks, a JH-like compound may play a role in ovulation/oviposition. The vertebrate hormone, thyroxine, is known to have physiological effects in insects, causing changes to protein and amino acid content of organs related to vitellogenesis (Chaudhuri and Medda, 1987). Both thyroxine, and its more potent derivative, 3,5,3'-triiodothyronine (T_3), bind to a putative JH-receptor in *Rhodnius prolixus* and *Locusta migratoria*, and thereby stimulate follicle cell patency in the same manner as JH (Davey and Gordon, 1996; Kim et al., 1999). If an unknown JH-like molecule is present in ticks, T_3 may mimic its effects.

In this study, the effects of 20E, insulin, JH-III, and T_3 on tick vitellogenesis were examined. As shown previously, 20E stimulated vitellogenesis but not yolk-uptake in partially-fed ticks. The possibility arises that up-regulation of a Vg-receptor (VgR) in the ovary is controlled by a distinct factor which is absent in partially-fed ticks. So in this study we also attempted to identify a putative VgR and determine when it appears during the feeding cycle.

Materials and Methods

Ticks

Our colony of *A. hebraeum* was kept in darkness at 27°C and >95% humidity. Tick feeding occurred on rabbits as described by Kaufman and Phillips (1973). Ticks were allowed to engorge and detach spontaneously, or were forcibly removed from the host below the 'critical weight' necessary to begin vitellogenesis (Kaufman and Lomas, 1996; Weiss and Kaufman, 2001). In this study engorged weight ranged from 900 to 3500 mg, and partially-fed females (below critical weight) ranged from 100-250 mg. Ticks were cleaned with water, weighed, and stored individually in gauze-covered glass vials until needed for dissection and haemolymph collection.

Hormone treatments

For injection of hormones, stock solutions were made as follows: 20E (Sigma) 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; 3,5,3'-triiodothyronine (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) 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 an AGLA micrometer syringe unit (Wellcome Reagents Ltd.). Final doses of these hormones were 0.0043-43 ng 20E/g bw, 20-2000 mU insulin/g bw, and 100-200 ng T_3/g bw. Vehicle-injected and/or non-treated controls were run in parallel. Injections were repeated on days 2 and 5. Ticks were kept under colony conditions until their haemolymph and tissues were collected on day 10 (see below).

Juvenile hormone III (JH-III; Sigma) was dissolved in acetone to 10 mg/ml; final concentrations for treatments were 2, 5, and 10 mg/ml. On day 0, 2, and 5 post-removal, partially-fed ticks received a dose of 1 µl/100 mg bw JH-III solution applied to the dorsal cuticle. 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 haemolymph and tissue samples were collected (see below).

Collection of tissue and haemolymph samples

Under our conditions for maintaining the tick colony, oviposition begins on day 10-12. For collection of haemolymph 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 haemolymph (Kaufman, 1991). A small incision was made in the cuticle, and haemolymph 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 in a laboratory freezer and all other samples were stored at -70°C until analyzed.

After collection of haemolymph, 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), 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. Ovaries were dissected out, and the length of the long axis of the 8 apparently largest ovoid oocytes was measured using an ocular micrometer fitted to

a compound microscope. The mean value for the 8 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), centrifuged at 13,000 X g for 5 min and the pellet discarded. Supernatants were stored at -70°C for further analysis.

Assay for salivary gland degeneration

As 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 ducts ligated using very fine strands of silk thread (Dermalon). 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 weighed. As dopamine stimulates salivary fluid secretion (Harris and Kaufman, 1984), the net weight increase is a direct measure of fluid secretory competence; loss of fluid secretory competence compared to controls is thus a quantitative measure of salivary gland degeneration.

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, ovary homogenate was passed through a gel filtration column (Superose 6B, Pharmacia) followed by an ion exchange column (DEAE-Sephacel, Pharmacia). Tick Vg contains a haem moiety (Sonenshine, 1991), so the fractions eluted from each column which contained large amounts of both haem and protein (determined by spectrophotometry; Kaufman et al, 1986) were pooled and concentrated. The protein concentration of the resulting extract was measured using the

Bradford assay (Bradford, 1976; Bradford reagent kit; Sigma), and the extract frozen at -70°C in 20 µl aliquots.

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 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 gels. Electrophoresis was performed using mini-Protean equipment (Bio-Rad). All gels consisted of a 3% stacking gel, and a 7.5% continuous 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 haemolymph polypeptides, Vg211 and Vg148 (Friesen and Kaufman, 2002). Membranes containing transferred proteins were first kept overnight in blocking buffer (5% skim milk, 2% bovine serum albumin (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 3 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 3 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 previous authors (Dhadialla et al., 1992; Sappington et al., 1995; Ramano and Limatola, 2000). Ligand blotting was performed as follows: briefly, tissue 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 block potential Vt binding sites (Dhadialla et al., 1992; Sappington et al., 1995). Following the overnight incubation, the membrane was washed and then treated with anti-Vg antibodies, as described above for immunoblots.

Immunoblots and ligand blots were converted to digital format using a benchtop scanner. Mobilities of polypeptide bands were then measured using NIH Image (National Institute of Health, U.S.A.) software, and the relative mobility of each band calculated in relation to the molecular weight standards.

RIA for ecdysteroid

Ecdysteroid-concentration in tick haemolymph was determined by the RIA procedure described by Kaufman (1990). Briefly, a methanol extract of each haemolymph 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 (NEN) per tube and an ecdysone antiserum (E-22-succinyl-thyroglobulin; supplied by Dr. L.I. Gilbert, University of North Carolina, or 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'.

ELISA for Vg and Vt

Haemolymph 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, and a mixture of anti-Vg antibodies, plus either known concentrations of Vt or unknown samples, 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 colour substrate kit (p-Nitrophenylphosphate and diethanolamine kit; BioRad). The colour reaction was quantified by measuring the absorbance of each well at 405 nm using a microtitre plate reader (Bio-Tek). 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 is approximately 5 ng of Vt-equivalents

Statistical analysis

Results are reported as mean ± SEM (n). Statistical analysis was done using Statview 4.02. Differences among treatments were analyzed using a 1-way analysis of

variance (ANOVA). Statistical significance is indicated as follow: (*) $0.01<P<0.05$; (**) $0.001<P<0.01$; (***) $P<0.001$.

Results

Effect of 20E

Injection of 20E had no significant effect on ovary weight (Fig. 3.1A) or oocyte growth (Fig. 3.1B) in partially-fed ticks by 5 or 10 days post-treatment. However, 20E treatments caused a significant increase in haemolymph Vg-concentration (Fig. 3.1C). Vg-concentration peaked at 18.3 ± 3.8 mg/ml on day 5 (approximately 30 times the day 5 EtOH control) and 35.0 ± 5.5 mg/ml on day 10 (approximately 20 times the day 10 EtOH control), following 3 bolus injections of 4,300 ng 20E/g bw. On both day 5 and day 10, ticks treated with 43,000 ng 20E/g bw had significantly lower haemolymph Vg-concentrations than those treated with 4,300 ng 20E/g bw ($P<0.0001$). Day 10 ticks treated with the highest dose of 20E also appeared "unhealthy"; during dissection, we noticed that the hypodermis had separated from the surrounding cuticle, indicating that 20E had induced apolysis. However, the 20E used in this study was reported by the manufacturer (Sigma) to be 99% pure, therefore it I cannot exclude the possibility that the observed "toxic" effects at the high 20E dose may be due to contaminants retained following commercial purification.

Salivary gland weight and salivary gland fluid secretory competence decreased in 20E-treated partially-fed ticks (Fig. 3.2). Compared to untreated controls, EtOH caused a significant increase in salivary gland fluid secretory competence by day 10 ($P<0.009$; Fig. 3.2B). However, at both day 5 and day 10, salivary gland fluid secretory competence was significantly lower at the highest dose of 20E than both untreated and EtOH controls (Fig. 3.2B).

Anti-Vg211 antibody (Friesen and Kaufman, 2002) bound to a 219 kDa polypeptide as well as a much fainter, 209 kDa polypeptide in the fat body of day 10 engorged ticks (Fig. 3.3). Anti-Vg148 antibody bound to three polypeptides of 211, 169, and 78 kDa, in fat body from engorged ticks. The latter three polypeptides were also present in fat body from 20E injected partially-fed ticks. Fat body of 20E-treated ticks also contained a single polypeptide of 134 kDa that was not present in fat body from day 10 engorged ticks, and that was specific to anti-Vg211 antibody. No polypeptides from Day 0 engorged and EtOH-injected partially-fed tick fat bodies were specific to anti-Vg antibodies (Fig 3.3).

Effect of insulin

Compared to vehicle-injected controls, multiple injections of bovine insulin into partially-fed ticks had no significant effect on ovary weight, salivary gland fluid secretory competence, haemolymph Vg-concentration, or haemolymph 20E-concentration (Fig. 3.4). The data for Vg was highly variable, however (Fig. 3.4C), and the HCl vehicle seemed to increase salivary gland fluid secretory competence relative to untreated ticks, although the difference was not significant (P=0.056; Fig. 3.4D).

Effects of JH-III and T_3

Multiple treatments of partially-fed ticks with JH-III had no significant effect on ovary weight (Fig. 3.5A), or haemolymph 20E- and Vg-concentrations (Fig. 3.5B) by day 6 post-treatment. Salivary glands of ticks treated with 10 µg JH/g bw weighed significantly less than acetone-treated controls (1.88 ± 0.09 mg in JH-treated glands and 2.64 ± 0.19 mg in control glands; Fig. 3.5C). 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); this effect was not observed at higher doses, however (Fig. 3.4D).

By day 5, a dose of 200 ngT_3/g bw caused a slight increase in ovary weight in partially-fed ticks (Fig. 3.6A). However, ovary weights of T_3 control ticks were noticeably smaller (0.43±0.04 % bw) compared to vehicle controls for 20E-treated ticks (0.63 ± 0.11 % bw; Fig. 3.1A), vehicle controls for insulin-treated ticks (0.96 ± 0.03 % bw; Fig. 3.4A), and controls for JH-III-treated ticks (day 6: 0.76 ± 0.10 % bw; Fig. 3.5A). In addition, there were no apparent effects of T_3 treatment on ovary weight by day 10, nor were there changes in haemolymph 20E-concentration (Fig. 3.6B), haemolymph Vg-concentration (Fig. 3.6C), or salivary gland fluid secretory competence (Fig. 3.6D).

Identification of a Vt-binding protein

Immunoblots of days 2-4 engorged ovary homogenate were incubated with partially purified Vt from day 10 engorged ovaries to test for Vt-binding proteins. In addition to Vt already present in the eggs, day 2-4 ovary homogenates contained a single 86 kDa polypeptide that bound to Vt (Fig. 3.7B). Subjecting ovary homogenates to reducing conditions did not affect the size or Vt-binding function of this polypeptide (Fig. 3.7A). Vt-binding to the 86 kDa polypeptide was blocked by 5 mM suramin (Fig. 3.8). Moreover, this polypeptide was not present in tissues other than ovaries (Fig. 3.9).

The 86 kDa binding protein was detected by ligand blotting in ovaries of engorged ticks up to day 4, but not on day 8 (Fig. 3.10). 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.

Discussion

Fat body and midgut cells are the primary 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 result in yolk-uptake by oocytes; however, fat body of 20E-treated ticks proliferated, suggesting the synthesis of Vg. Here, the fat body of engorged *A. hebraeum* 10 days post-engorgement contains at least 4 polypeptides which specifically bind antibodies raised against two haemolymph Vgs (Vg211 and Vg148; Friesen and Kaufman, 2002). Interestingly, both anti-Vg211 and anti-Vg148 bound to polypeptides in fat body that appeared larger than their corresponding haemolymph proteins (219 kDa and 211 kDa, respectively; Fig. 3.3). We do not yet know the changes occurring between yolk-synthesis in the fat body and its release into the haemolymph that would 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 engorged ticks was not detected in 20E-treated partially-fed ticks (Fig. 3.3), which may be explained by the experimental treatment as follows: Vg-synthesis in normal day 10 engorged ticks is maintained by the continual presence of high 20E-concentrations in the haemolymph (Kaufman, 1991; Friesen and Kaufman, 2002). However, 20E is rapidly metabolized/secreted following injection into partially-fed *A. hebraeum* (Weiss and Kaufman, 2001). Since fat body of partially-fed ticks was collected on day 10 (i.e. 5 days following the final 20E treatment on day 5; see Materials and Methods), Vg-synthesis may have ceased in the meantime, thus explaining the absence of the 211 polypeptide.

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

followed by a decrease at the highest concentration tested (Fig. 3.1C). However, as seen in Figure 3.1, and previously by Lunke and Kaufman (1993), no ovary or oocyte growth occurred in 20E-treated partially-fed ticks. During normal vitellogenesis, ovaries from engorged ticks begin to accumulate Vg by day 4-5 post-engorgement (Friesen and Kaufman, 2002). By day 10 post-engorgement, the ovaries weigh approximately 5% of the engorged body weight (Friesen and Kaufman, 2002), an increase that is largely due to yolk uptake by the oocytes (Diehl et al., 1982). The lack of yolk-uptake observed in this study indicates that a signal other than the vitellogenic hormone is needed for normal yolk-uptake to occur. This signal is present in engorged ticks, because ovaries from partially-fed ticks accumulate yolk when placed in the haemocoel of an engorged tick (Lunke and Kaufman, 1993).

In oviparous animals, Vg is specifically taken up by oocytes via a Vg-receptor (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. 3.7), which was not present in other tissues (Fig. 3.9), and which may be the VgR in this tick. Previous studies have indicated that the effective binding of Vg to VgR is inhibited when VgR is subjected to reducing conditions using disulfide reducing agents such as 2-mercaptoethanol (Sappington et al, 1995; Stifani et al., 1990). However, the *A. hebraeum* 86 kDa Vt-binding protein was not affected by treatments with 2-mercaptoethanol (Fig. 3.7A).

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), possibly by competing for positively charged ligand

binding sites (Brown et al., 1997). Binding of partially-purified Vt to the 86 kDa protein in *A. hebraeum* was inhibited by adding 5 mM suramin, which further suggests that this protein may be the VgR in this tick. However, the 86 kDa protein is also present in ovaries of small partially-fed ticks (100 mg and above), suggesting that lack of yolk-uptake by these ovaries in 20E-treated ticks was not for lack of a VgR; the additional factor which is necessary for yolk uptake in *A. hebraeum* thus remains unknown.

Injections of bovine insulin into partially-fed ticks did not stimulate 20E- or Vg-synthesis, suggesting that it is not a mimic for a putative VIF similar to that described by Chinzei and Taylor (1990). However, reports of insulin ecdysteroidogenic activity in mosquitoes (Graf et al., 1997; Riehle and Brown, 1999), 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 identification of several insulin-like peptides from *Drosophila* neurons (Rulifson et al., 2002), suggest that the insulin family of peptides is highly conserved. In addition, the fact that antibodies to bovine insulin bind to neurohaemal organs in *O. moubata* synganglia (described by Zhu and Oliver, 1991) suggest that an insulin-like molecule may also be present in ticks. What physiological role such a molecule may have remains to be determined.

Whereas a stimulating effect of JH in tick oogenesis has been suggested by other authors (Pound and Oliver, 1979; Connat et al., 1983), we have demonstrated that neither JH-III, nor the JH-mimic, T_3, stimulate 20E-synthesis nor Vg-sythesis in *A. hebraeum* (Figs. 3.5B, 3.6B, and 3.6C). 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 (Figs. 3.5C, 3.5D, and 3.6D). On the contrary, a significant increase in salivary gland fluid secretion occurred at a dose of 2 µg JH-III/g bw, an effect that was not observed at higher doses (Fig. 3.5D).

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). In conclusion, whereas 20E is very likely the vitellogenic hormone in *A. hebraeum*, an additional factor is necessary for yolk-uptake. Need for an additional factor is surprising since a major barrier to yolk-uptake in insects (the follicular epithelium) is absent in ticks (Kaufman, 1997). One function of the vitellogenic hormone in insects is to cause patency in the follicular epithelium, allowing Vg to access the surface of the oocyte (reviewed in Wyatt and Davey, 1996). Evidence from this study (Fig. 3.10) suggests that the tick 'Vg-uptake factor' does not act primarily by up-regulating the VgR.

Figure 3.1. Effect of 20E injections on partially-fed ticks. (A) ovary weight, (B) oocyte length, (C) haemolymph Vg-concentration on days 5 and 10 post-treatment. 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.

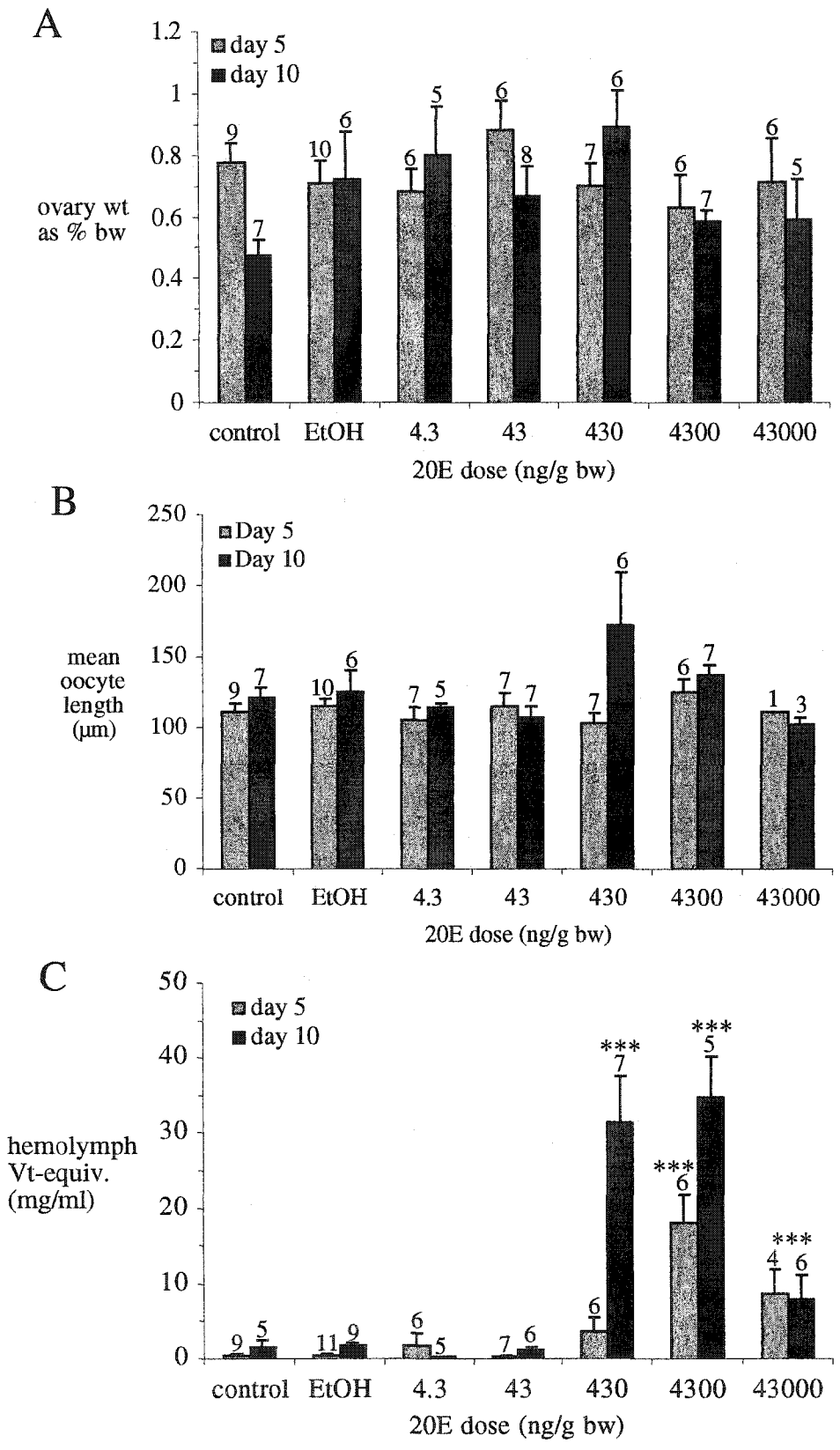
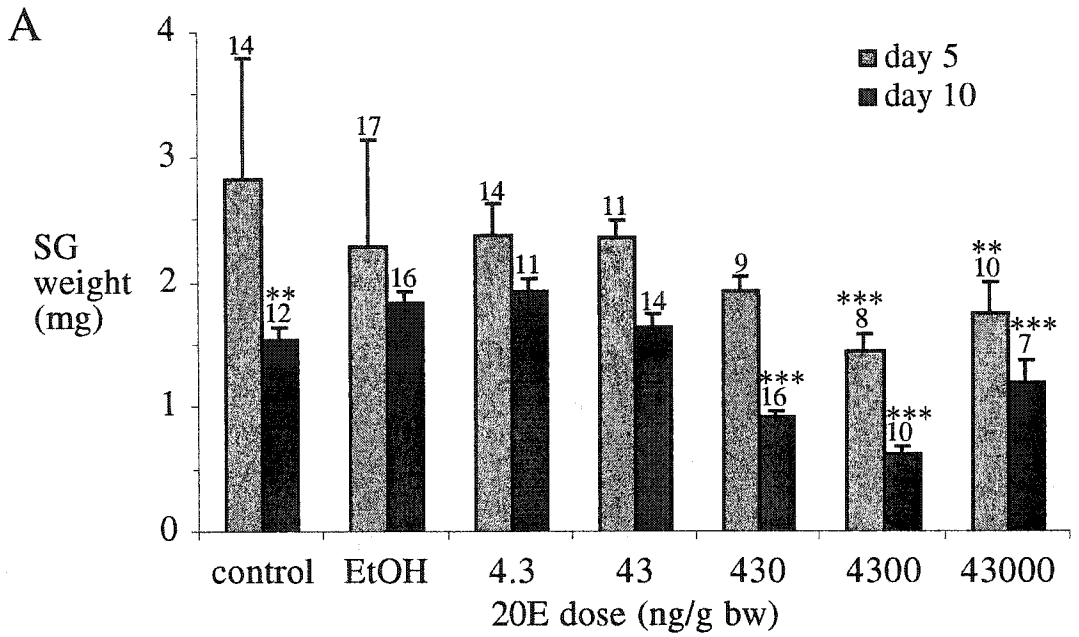


Figure 3.2. Effect of 20E injections on partially-fed ticks. (A) salivary gland weight, (B) salivary gland fluid uptake on days 5 and 10 post-treatment.



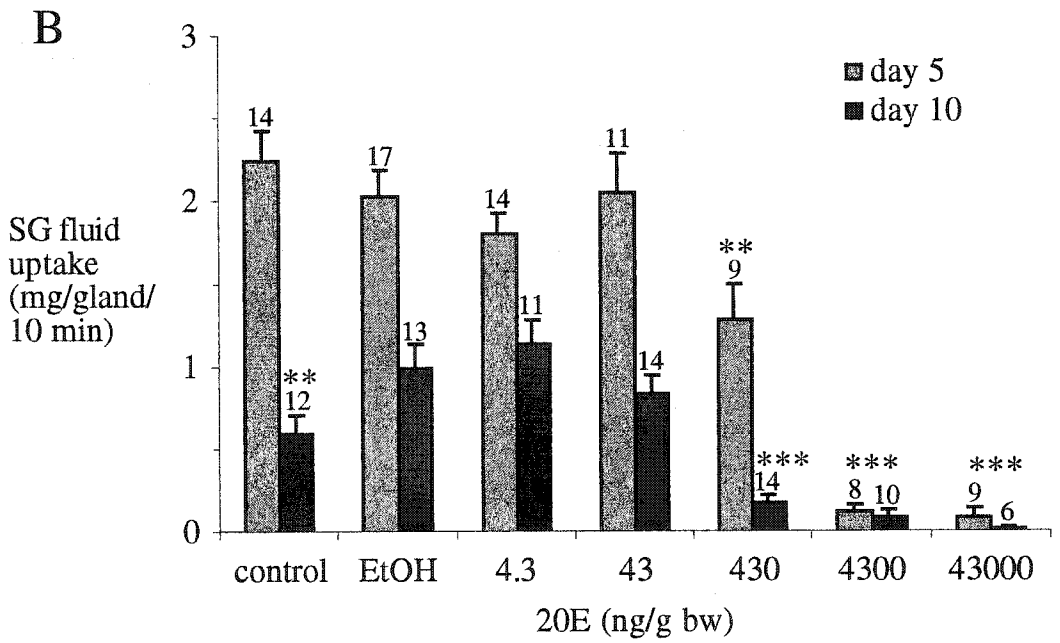


Figure 3.3. Immunoblot of fat body homogenates of days 0 and 10 engorged, and of 20E-injected partially-fed ticks. Each lane of the immunoblot was cut into 3 strips, which were treated as follows: (a) Coomassie blue, (b) Anti-Vg148 antibody, (c) Anti-Vg211 antibody. Vg polypeptides specific for anti-Vg148 or anti-Vg211 antibodies indicated by letter 'b' or 'c', respectively.

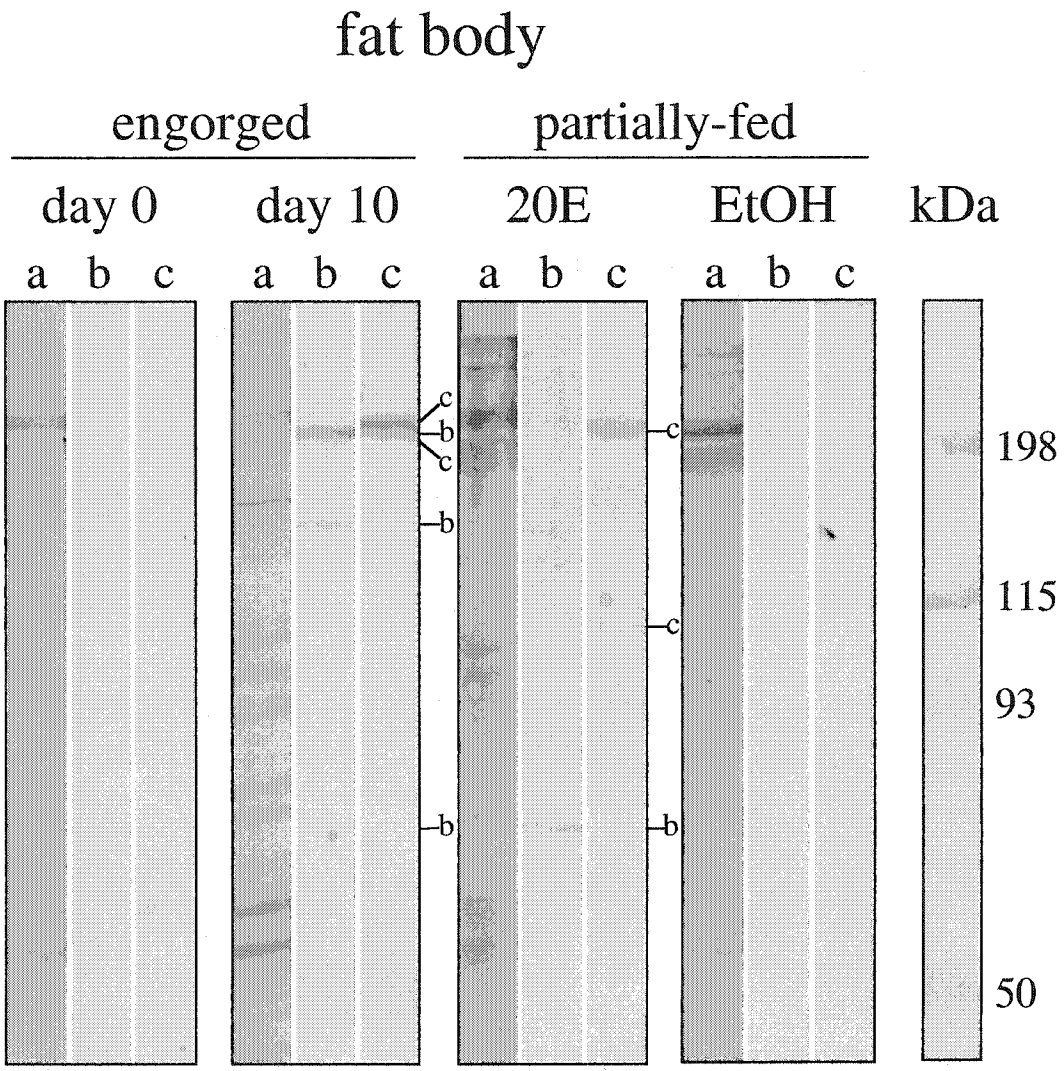
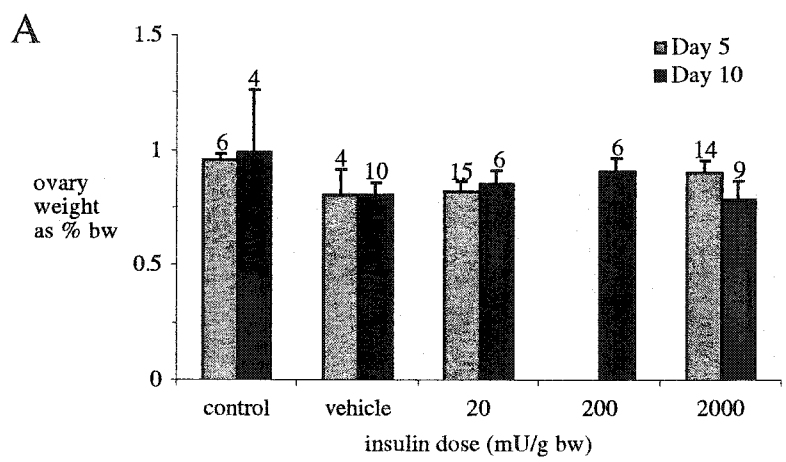
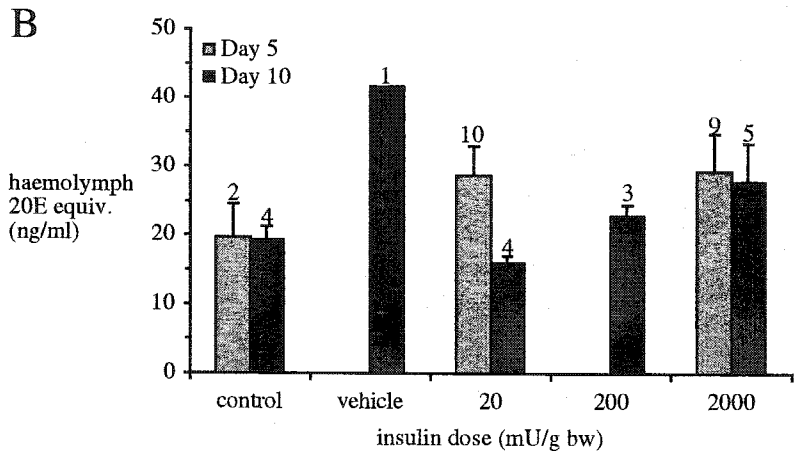
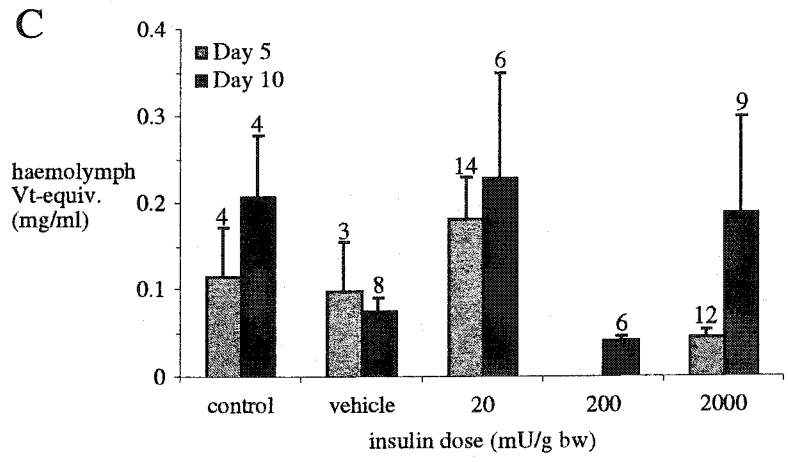


Figure 3.4. Effect of insulin injections on partially-fed ticks. (A) ovary weight, (B) haemolymph 20E-concentration, (C) haemolymph Vg-concentration, (D) salivary gland fluid uptake on days 5 and 10 post-treatment. Vehicle: HCl-injected control. Vg-concentration and 20E-concentration are reported as 'equivalents' for reasons described in Materials and Methods.







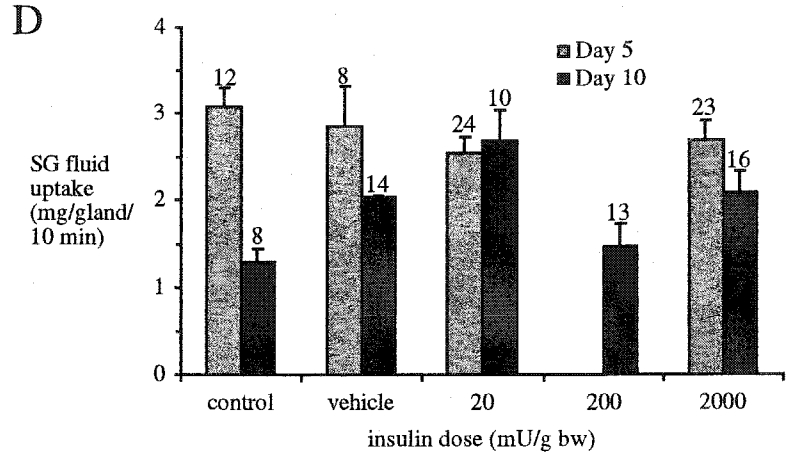
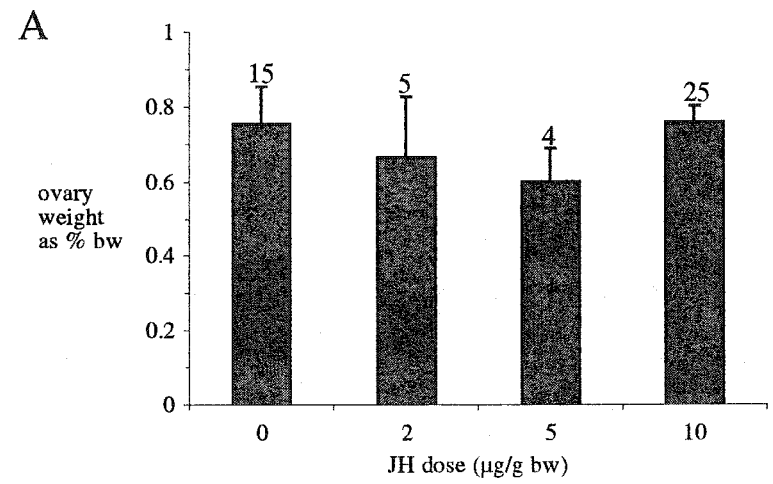
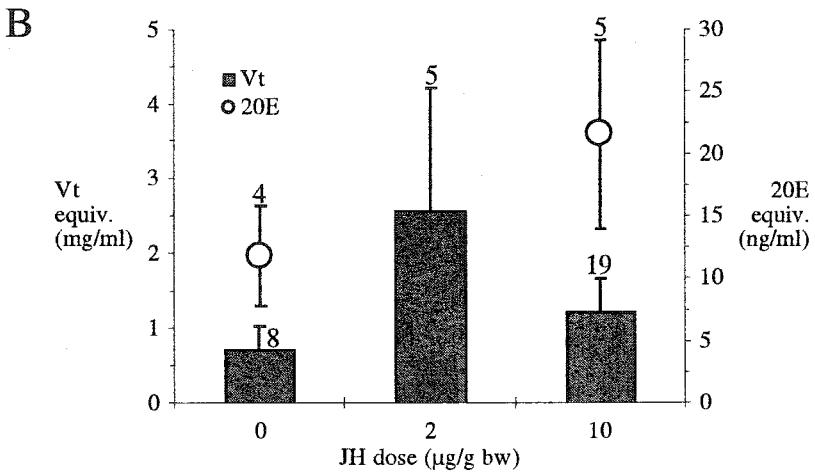
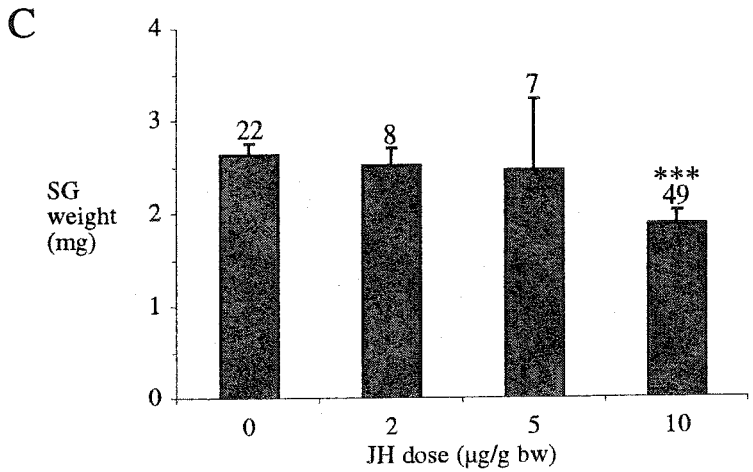


Figure 3.5. Effect of topical applications of JH-III on partially-fed ticks. (A) ovary weight, (B) haemolymph 20E-concentration (open circles) and Vg-concentration (grey bars), (C) salivary gland weight, (D) salivary gland fluid uptake on day 6 post-treatment. Vg-concentration and 20E-concentration are reported as 'equivalents' for reasons described in Materials and Methods.







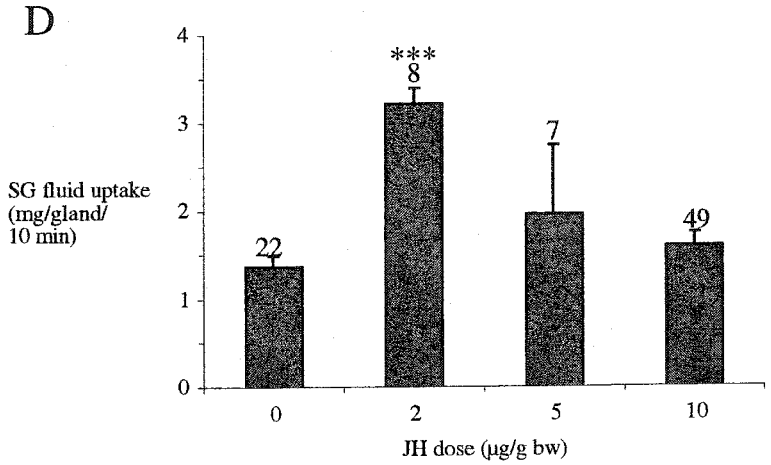


Figure 3.6. Effect of T_3 injections on partially-fed ticks. (A) ovary weight, (B) haemolymph 20E-concentration, (C) haemolymph Vg-concentration, (D) salivary gland fluid uptake on days 5 and 10 post-treatment. Control: NaOH-injected vehicle control. Vg-concentration and 20E-concentration are reported as 'equivalents' for reasons described in Materials and Methods.

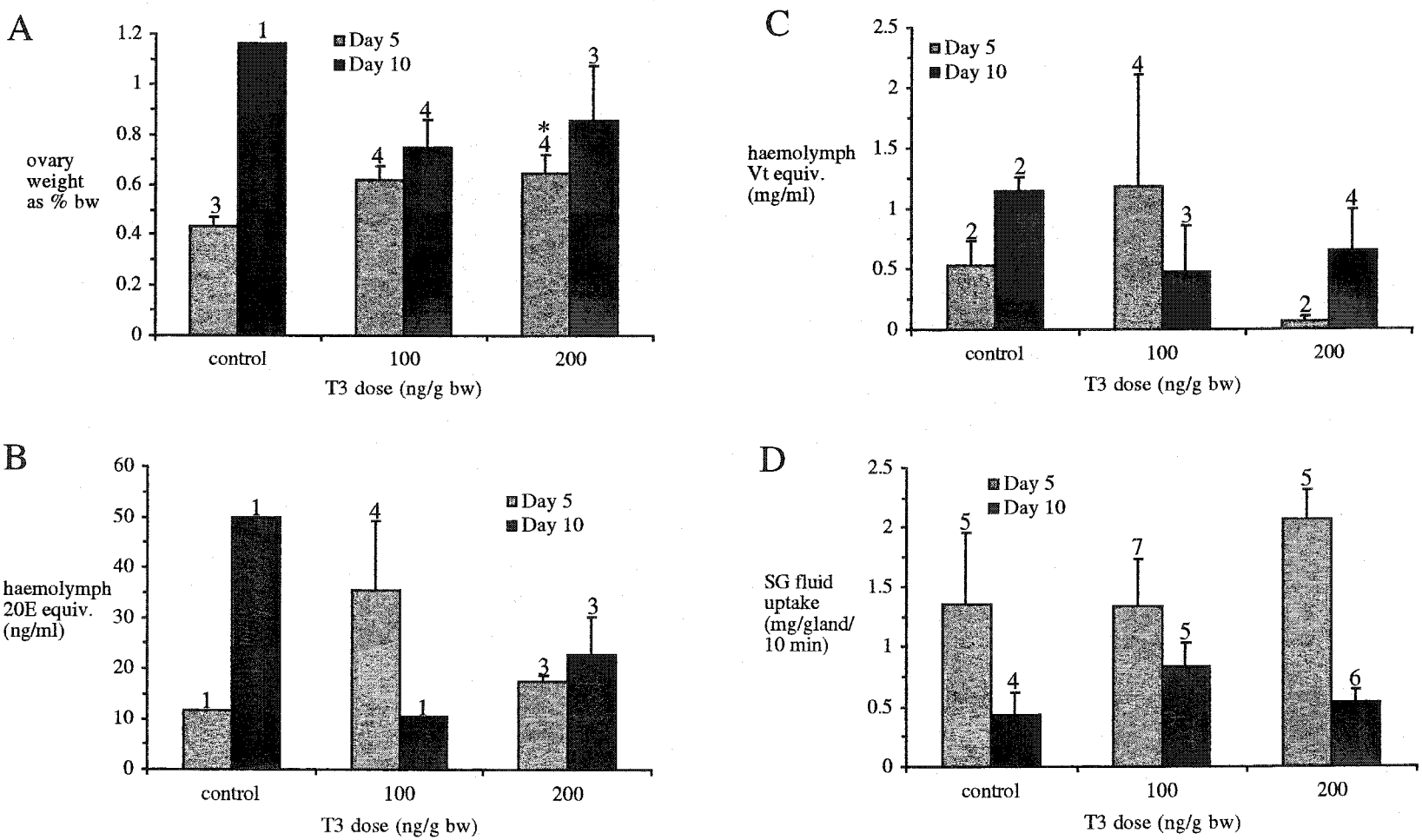


Figure 3.7. Ligand blot demonstrating the binding of Vt to an 86 kDa polypeptide from day 2-4 engorged ovary homogenates. A) reducing, denaturing SDS-PAGE; B) non-reducing, SDS-PAGE. Strips were treated as follows: (C.b.) Coomassie blue stain; (+Vt) with ligand; (-Vt) without ligand; (kDa) molecular weight markers.

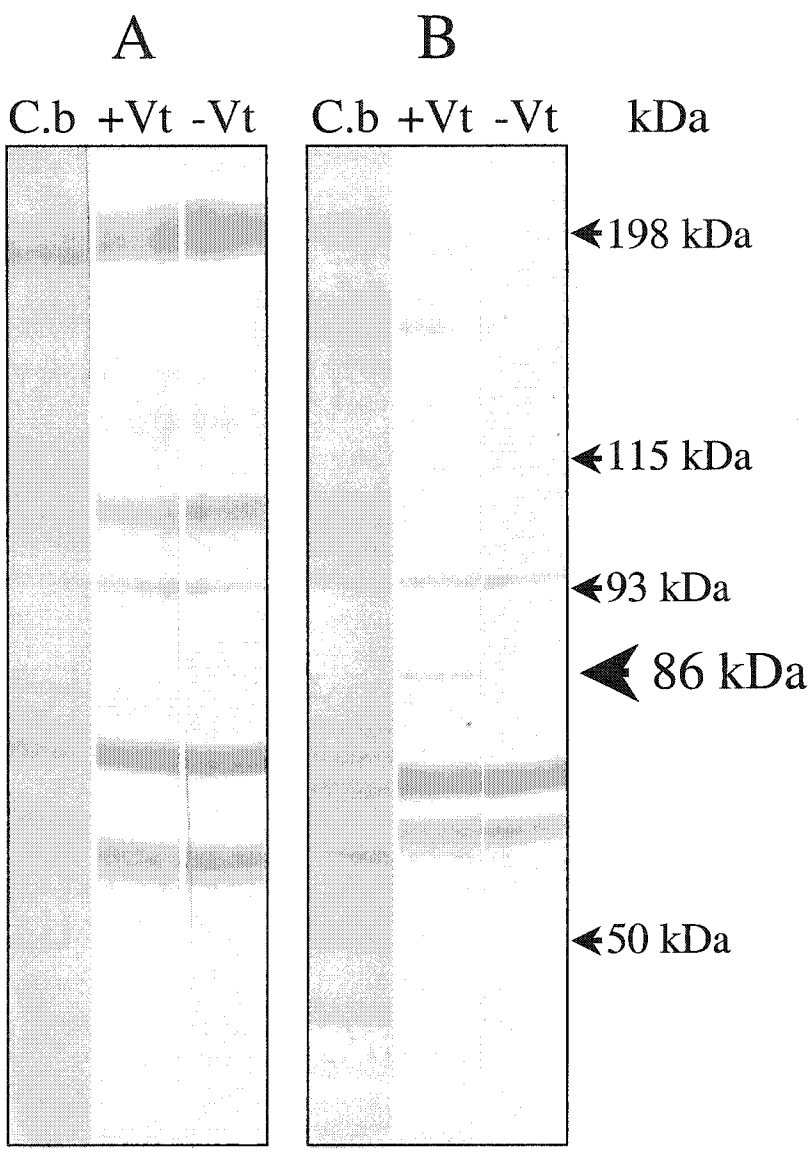


Figure 3.8. Ligand blot demonstrating the effect of suramin on Vt-binding to the 86 kDa ovary polypeptide. Strips were treated as follows: (C.b.) Coomassie blue stain; (-Vt) without ligand; (+Vt,+Sur) with ligand and 5 mM suramin (+Vt) with ligand.

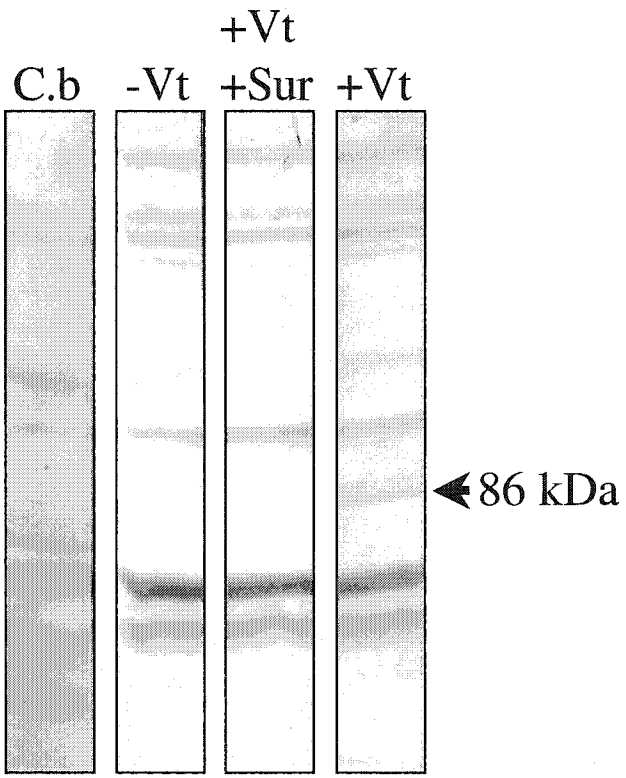


Figure 3.9. Ligand blot demonstrating the absence of the 86 kDa Vt-binding polypeptide in various tissue homogenates from ticks 2-4 days post-engorgement. (Ov) ovary, (RT) reproductive tract (seminal receptical, uterus, accessory glands), (Gut) midgut, (SG) salivary gland, (Mt) Malpighian tubule, (Trach) trachea. (-Vt) without ligand, (+Vt) with ligand.

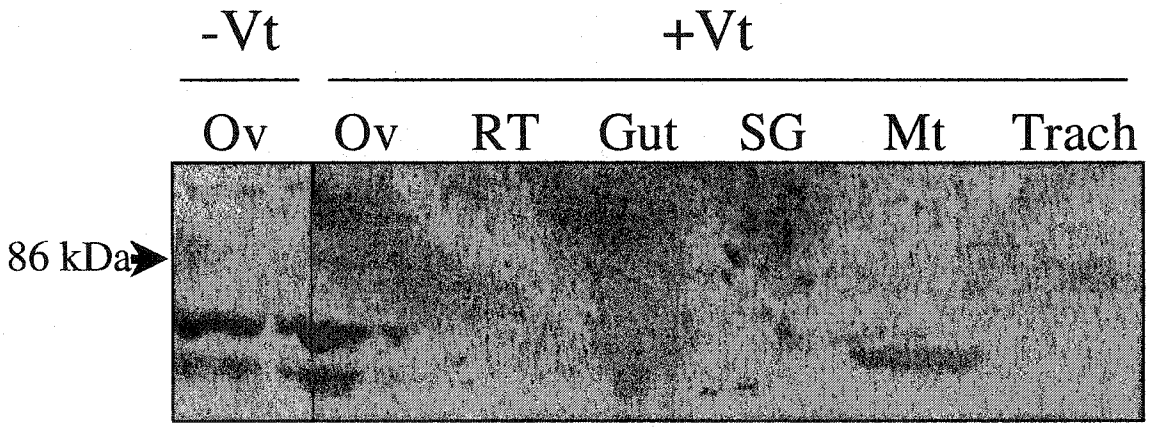
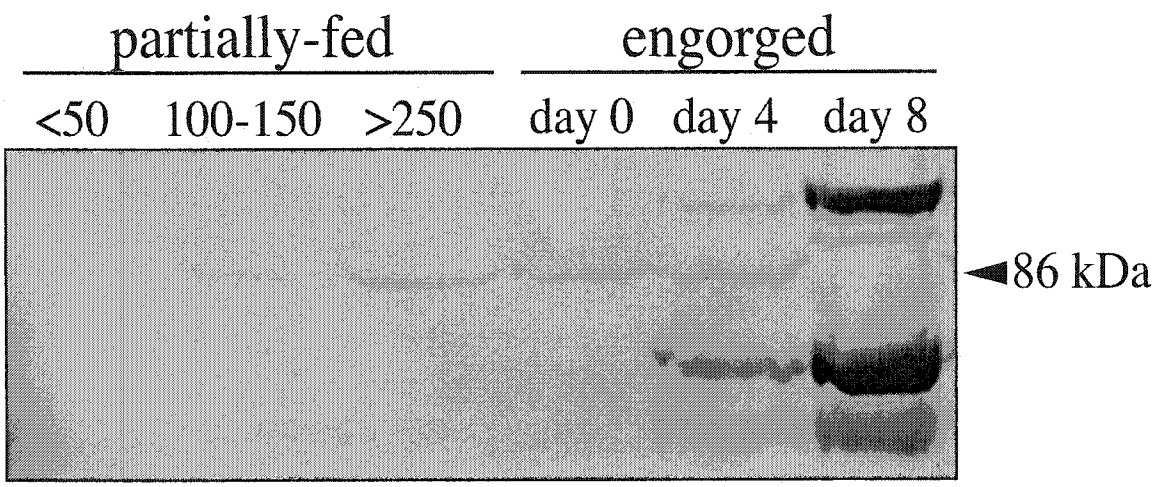


Figure 3.10. Profile of the 86 kDa Vt-binding protein at different stages of feeding, as demonstrated by ligand blot. Partially-fed ticks separated by weight range (mg).



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Chapter 4

Effects of the Avermectin, MK-243, on Ovary Development and Salivary Gland Degeneration in the Ixodid Tick, *Amblyomma hebraeum*[†]

Introduction

In ixodid ticks, vitellogenesis begins within a few days of engorgement on blood. Until recently, little was known about the hormonal regulation of vitellogenesis in ticks. Using *Ornithodoros moubata*, Chinzei and Taylor (1990) determined that a peptide from the synganglion ('vitellogenesis inducing factor'; VIF) acts on some unknown tissue to stimulate release of 'fat body stimulating factor' (FSF); FSF is presumed to be the vitellogenic hormone. FSF was hypothesized originally to be either an ecdysteroid or a juvenile hormone (JH). Although there is evidence that JH has physiological activity in ticks (Pound and Oliver, 1979; Connat et al., 1983), no known JH or JH-analogue has been authenticated from ticks by gas chromatography/mass spectrometry (Connat 1987; Neese et al., 2000).

Lunke and Kaufman (1993) were not able to stimulate yolk-uptake by oocytes after treating partially-fed *Amblyomma hebraeum* with JH or 20-hydroxyecdysone (20E). However, these authors noted a marked proliferation of fat body in 20E-treated ticks, suggesting that vitellogenin (Vg)-synthesis may have been stimulated by 20E. Recently, Friesen and Kaufman (2002) confirmed this hypothesis using immunoblots and an enzyme-linked immunosorbent assay (ELISA) for tick vitellin. Although injections of 20E into partially-fed ticks stimulated synthesis of Vg and its release into the haemolymph, egg development did not follow (Chapter 3). It thus appears that 20E stimulates vitellogenesis, but on its own is not a sufficient signal to trigger yolk-uptake.

[†] A version of this chapter has been submitted for publication.

Avermectins (AVMs) are a group of broad-spectrum anti-parasitic compounds originally isolated from the bacterium *Streptomyces avermitilis* (reviewed in Strong and Brown, 1987). Early research suggested that AVMs act as potentiators of γ-aminobutyric acid (GABA) systems in nematodes and arthropods, possibly through an agonistic action on GABA-mediated chloride channels (reviewed in Campbell et al., 1983). However, more recent reports indicate that the main mode of action is to modulate glutamate-gated ion channels (Cully et al., 1994; Arena et al., 1995; Martin, 1996), channels which may also bind GABA (Ludmerer et al., 2002).

Although treatment of host animals with AVM does not kill or cause detachment of ticks, it does interfere with physiological functions such as moulting, feeding to engorgement, and reproduction in *Amblyomma americanum* (Lancaster et al., 1982). AVMs also inhibit oviposition when injected into *A. hebraeum* (Kaufman et al., 1986). The mechanisms behind the latter effects are not known. However, Lunke and Kaufman (1992) observed a marked reduction of haemolymph 20E-concentration and inhibition of ovarian development in engorged female *A. hebraeum* following injection of the water-soluble AVM-analogue, MK-243. The effect of MK-243 might have been due to any combination of the following inhibitions: (1) 20E-synthesis and/or release, (2) Vg-synthesis and/or release into the haemolymph and, (3) uptake of yolk by the ovary. The purpose of this study was to clarify the relative importance of these proposed mechanisms.

Materials and Methods

Ticks

A. hebraeum ticks were maintained in a laboratory colony at 27°C, >95% humidity and in darkness. Ticks were allowed to feed on rabbits, as described by Kaufman and Phillips (1973), until they engorged and spontaneously detached.

Injections of MK-243 and 20E

MK-243 was a gift from Merck Sharp and Dohme Research Laboratories. A stock solution of MK-243 (1 mg/ml) in 1.2% saline was stored at -20°C until needed. Just prior to injection, this stock was diluted to 5, 10, and 15 µg/ml in 1.2% NaCl (isosmotic to tick haemolymph). Injected at 1 µl/100 mg body weight (bw), these concentrations of MK-243 corresponded to doses of 50, 100, and 150 ng/g bw. On the day of detachment (day 0), ticks were weighed and MK-243 was injected into the haemocoel through the camerostomal fold (articulation between the scutum and capitulum), using an AGLA micrometer syringe apparatus (Wellcome Reagents Ltd). Ticks were isolated in individual gauze-covered glass vials and stored under colony conditions until 5 or 10 days after injection, at which time haemolymph and tissue samples were collected. Control ticks were injected with 1.2% saline.

To test whether injections of 20E could reverse the effect of MK-243, ticks were treated with 150 ng MK-243/g bw on day 0, and this was followed by 3 bolus injections of 20E (Simes, Milan) on days 1, 3 and 5. Multiple injections were considered necessary because of the rapid rate of catabolism of 20E (Weiss and Kaufman, 2001). 20E stock solutions of (A) 5 mg/ml and (B) 15 mg/ml were prepared in 70% ethanol (EtOH), and diluted to working concentration in 1.2% NaCl immediately prior to injection. The concentration of EtOH in the injected solution was 3%; after injection of 20 µl/g bw, the concentration of EtOH in the body of the tick was estimated to be approximately 0.06%. The resulting doses were 5 µg/g bw and 15 µg/g bw for each bolus injection; these concentrations of 20E are known to stimulate vitellogenesis in partially-fed *A. hebraeum* (Friesen and Kaufman, 2002). Between each injection, ticks were allowed to sit undisturbed under colony conditions. Ticks were dissected on day 10 post-engorgement, and samples of ovary (and haemolymph where possible) were collected.

Collection of haemolymph and ovary samples

Tick mortality was determined at the time of dissection. If, during haemolymph collection or dissection (see below), the gut was noticed to be ruptured, that tick was discarded.

On the day of dissection (day 5 or day 10 post-injection), ticks were secured ventrally to a petri dish with cyanoacrylate glue and refrigerated for 15 min. Cooling ticks prior to haemolymph collection inhibits gut contraction, thus reducing the chance of accidentally breaking the gut and contaminating the haemolymph (Kaufman, 1991). Incisions (1-2 mm long) were made in the integument with a microscalpel. The exuding haemolymph was collected with volumetric glass capillary tubes and diluted 1:4 in phosphate-buffered saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0). Haemolymph samples were stored at -70°C until further analyzed.

Following haemolymph collection, 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. Ovaries were dissected out, and length of the long axis of the 8 apparently largest ovoid oocytes was measured using an ocular micrometer fitted to a compound microscope. The mean value for the 8 oocytes was recorded for each tick. Ovaries were then gently blotted, weighed, and homogenized in 100 μl PBS per 30 mg ovary. Ovary homogenates were stored at -70°C until further analyzed.

ELISA for Vg and Vt

Haemolymph Vg and ovary vitellin (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 with1 μg

partially-purified Vt, and a mixture of anti-Vg antibodies, plus either known concentrations of Vt or unknown samples, 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 colour substrate kit (p-Nitrophenylphosphate and diethanolamine kit; BioRad). The colour reaction was quantified by measuring the absorbance of each well at 405 nm using a microtitre plate reader (Bio-Tek). 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 is approximately 5 ng of Vt-equivalents.

Assay for salivary gland degeneration

As 20E triggers salivary gland degeneration (Kaufman, 1991), and because MK-243 reduces haemolymph ES-concentration (Lunke and Kaufman, 1992), 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 ducts ligated using very fine strands of silk thread (Dermalon). 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 weighed. As dopamine stimulates salivary fluid secretion (Harris and Kaufman, 1984), the net weight increase is a direct measure of fluid secretory competence; loss of fluid secretory competence compared to controls is thus a quantitative measure of salivary gland degeneration.

Photography

Whole ticks, ovaries, and salivary glands were photographed using a Nikon DXM1200 digital camera attached to a dissecting microscope. Digital images were re-touched for publication using Adobe Photoshop 4.0 software.

Statistical analysis

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

Results

Effects of MK-243 on engorged ticks

Except for a single tick at 50 ng MK-243/g bw, the doses of MK-243 used in this study did not kill engorged ticks by day 5 (Table 4.1). By day 10 post-engorgement, mortality increased only slightly with dose of MK-243, peaking at 11% at the highest dose (150 ng MK-243/g bw). However, ticks treated with MK-243 appeared bloated, had splayed legs, and displayed much shallower dorsal ridges than normal healthy ticks (Fig. 4.1), suggesting that the major dorso-ventral muscles and leg muscles were paralyzed.

Ovary weight and oocyte growth were inhibited in a dose-dependent manner by MK-243 treatment. Ovary weight in control ticks was 1.1 ± 0.3% bw by day 5, growing to 5.2 ± 0.5% bw by day 10 post-engorgement (Fig. 4.2A, control). In contrast, ovary weights of ticks treated with 150 ng MK-243/g bw were significantly smaller, growing to only 0.35 ± 0.03% bw by day 5 (32% of control), and 1.0 ± 0.3% bw by day 10 (19% of control). On day 5, mean oocyte length of ticks treated with 150 ng MK-243/g bw was 60% of the control value (174 ± 6 μm for MK-243 treated versus 292 ± 20 μm for control

ticks; Fig. 4.2B). Likewise, on day 10, oocyte length of ticks treated with 150 ng MK-243/g bw was 51% of the control (277 ± 30 μm for MK-243 treated versus 544 ± 17 μm for control ticks).

Total Vt content of the ovary in day 10 ticks treated with 150 ng MK-243/g bw was reduced by 91% compared with saline injected control ticks (1.2 ± 0.9 mg Vt-equivalents for MK-243 treated versus 13.5 ± 2.9 mg Vt-equivalents for control ticks; Fig. 4.2C, bars). Vt as % ovary weight dropped significantly from 15 ± 3% in controls to 6 ± 2% at 100 ng MK-243/g bw, with no further decline at 150 ng MK-243/g bw (Fig. 4.2C, open circles). Haemolymph Vg-concentration on day 5 was 10.8 ± 3.4 mg Vt-equivalents/ml for controls and 5.3 ± 1.6 mg Vt-equivalents/ml in MK-243 (150 ng/g bw) treated ticks, a difference which was not statistically significant (Fig. 4.2D). Likewise, the difference on day 10 (22.5 ± 7.5 mg Vt-equivalents/ml for controls versus 17.0 ± 6.4 mg Vt-equivalents/ml for 150 ng MK-243/g bw treated ticks) was not statistically significant (Fig. 4.2D).

Effects of 20E on MK-243 treated ticks

Because MK-243 inhibits 20E haemolymph titres (Lunke and Kaufman, 1992), and because 20E is probably the vitellogenic hormone in *A. hebraeum* (Friesen and Kaufman, 2002), we tested whether injections of 20E could reverse the inhibitory effects of MK-243 on the reproductive system. In general, 20E (5 or 15 μg/g bw) was not able to reverse the inhibitory effect of MK-243 on ovary weight (Fig. 4.3A), mean oocyte length (Fig. 4.3B), or ovary Vt-content (Fig. 4.3C). Note, however, that the mortality of ticks injected with 20E was substantially higher than the mortality of ticks treated with MK-243 alone, reaching 40% at a dose of 15 μg 20E/g bw (Table 4.2).

Effects of MK-243 on salivary gland weight and salivary fluid secretory competence

There were no significant differences in salivary gland weight of ticks treated with MK-243 compared with control ticks on day 5 post-engorgement (Fig. 4.4A). However, doses of 100 and 150 ng MK-243/g bw increased salivary gland weight significantly by day 10 (1.1 ± 0.1 mg for control; 1.9 ± 0.1 mg for 100 ng MK-243/g bw; and 1.7 ± 0.1 mg for 150 ng MK-243/g bw). Because the salivary glands degenerate significantly over the first 4 days of engorgement (Harris and Kaufman, 1984), salivary gland fluid uptake was generally low in both day 5 and 10 control ticks (0.26 ± 0.05 mg/gland/10 min on day 5; 0.19 ± 0.05 mg/gland/10 min on day 10; Fig. 4.4B). Doses of 50 ng MK-243/g bw and 150 ng MK-243/g bw, caused day 5 salivary glands to take up significantly more fluid (0.47 ± 0.9 mg/gland/10 min and 0.43 ± 0.07 mg/gland/10 min respectively) than control ticks. This trend was not seen on day 10, however.

Salivary glands of MK-243 treated ticks generally appeared more robust than those of control ticks. Ten days after engorgement, salivary glands of controls were extremely fragile, and had a wispy appearance under the dissecting microscope compared to MK-243 treated tick salivary glands (Fig. 4.5).

Discussion

At the outset of this study, we knew that ecdysteroids play an important role in the control of Vg-synthesis in *A. hebraeum*, and that, among other effects, AVMs inhibit egg development, oviposition, and markedly reduce ecdysteroid-titres in the haemolymph (Kaufman et al., 1986; Lunke and Kaufman, 1992). As outlined in the Introduction to this chapter, the evidence to date was insufficient to determine which of several mechanisms might be responsible for the AVM effects. The experiments presented here indicate that a major mechanism involves inhibiting yolk accumulation in the oocytes,

and suggest that these inhibitions may not be the result of inhibiting ecdysteroid-synthesis. The evidence supporting this conclusion is as follows:

1) On day 10, Vt accounted for approximately 15% of the ovary weight in control ticks (Fig. 4.2C). The Vt content (as % ovary weight) was reduced by 60% and the ovaries weighed up to 80% less in MK-243 treated ticks compared with controls (Fig. 4.2A), indicating that MK-243 inhibited Vg-uptake, and hence ovary growth. This is further supported by analyzing day 5 ovary weights. The ovaries of ticks treated with 150 ng MK-243/g bw weighed 66% less than controls (Fig. 4.2A), while the Vt content (as % ovary weight) was reduced by approximately 75% (from 4% of ovary weight in controls to 1% in 150 ng MK-243 treated ticks; data not shown).

2) As we have shown previously that MK-243 significantly reduces 20E titres in the haemolymph (Lunke and Kaufman, 1992), MK-243 may inhibit Vg-accumulation in the oocytes, ultimately via an inhibition of Vg-synthesis. This conjecture, however, is not supported by the results shown in Fig. 4.2D (no significant difference in haemolymph Vg-concentration between MK-243 treated and controls on day 5 or 10). Moreover, 20E-injections were not able to reverse the effects of MK-243 on Vg-uptake (Fig. 4.3), even though the doses used here were sufficient to stimulate Vg-synthesis in small partially-fed *A. hebraeum* (Friesen and Kaufman, 2002). Recall that 20E does not stimulate yolk-uptake in small partially-fed ticks (Kaufman et al., 1986), whereas it does stimulate Vg-synthesis/release (Friesen and Kaufman, 2002). Thus, the results presented here suggest that the main action of MK-243 is to inhibit Vg-uptake by oocytes. However, it is not yet possible to exclude an effect of MK-243 on Vg-synthesis. For example, if synthesis and uptake of Vg were inhibited to a similar degree, this would result in little or no change in Vg-concentration in the haemolymph, which is also consistent with Fig.4.2D. On the other hand, treatment with 20E should then have resulted in an increase in Vg-concentration of the haemolymph, but this was not the case (results not shown). This

matter might be resolved by measuring the effect of MK-243, with and without 20E, on radiolabelled amino acid accumulation into Vg.

The effects of MK-243 on the ovary were much more noticeable on day 10 than day 5. The bulk of ovary growth is due to yolk uptake, which occurs between days 4 and 16 (Friesen and Kaufman, 2002). By day 5, most of oocyte growth is due to the late previtellogenic phase of development (Diehl et al., 1982). This may explain why MK-243 showed less effect by day 5 than day 10 (Fig. 4.2B). Ovaries of day 5 ticks treated with MK-243 showed numerous regions where oocytes were in the late previtellogenic growth phase, but in general the oocytes were smaller than those of controls, with few having entered the vitellogenic phase of oocyte growth (Fig. 4.6A and 4.6B). By day 10, MK-243 treated ovaries contained some oocytes at advanced stages of Vg-uptake (Fig. 4.6D), whereas Vg-uptake in day 10 controls occurred in oocytes along the entire length of the ovary (Fig. 4.6C). Similar results were observed in mosquitoes, where oocyte growth due to yolk-uptake was inhibited after ivermectin treatment (Mahmood et al., 1991).

Oocyte length was not an accurate measure for testing the effect of MK-243 in *A. hebraeum*. MK-243 treatments caused the Vt-content of ovaries to be reduced by up to 81% (Fig. 4.2C), whereas oocyte length was reduced by up to only 45% (Fig. 4.2B). Although it is clear from Fig. 4.6 that many fewer oocytes accumulated yolk in MK-243 treated ticks compared to control ticks, at least a few clusters of oocytes accumulated Vg even at the highest dose of MK-243. Thus the presence of a small number of large oocytes caused our measure of oocyte length in 8 of the largest oocytes to be less robust than other reproductive indices. It is interesting that those oocytes, which did accumulate yolk, seemed to be clustered, rather than randomly distributed (Fig. 4.6D). The reason for this is unknown, but might be that these oocytes were at a more advanced stage of development at the time of treatment or that, for some other reason, they escaped the effects of MK-243.

20E stimulates the salivary glands of ixodid ticks to degenerate following the bloodmeal (Kaufman, 1991). Because MK-243 reduces haemolymph ecdysteroid-concentration (Lunke and Kaufman, 1992), the effects of MK-243 on reversing the normal course of salivary gland degeneration was tested in this study also. There was a slight, yet significant, increase in salivary fluid secretory competence on day 5 in ticks treated with 50 and 150 ng MK-243/g; no such trend was evident by day 10. Note, however, that healthy salivary glands from partially-fed *A. hebraeum*, 5-10 days post-removal, secrete about 3 mg/gland /10 min (Kaufman et al., 1986; Lomas and Kaufman, 1992). The average rate of secretion by salivary glands of engorged ticks treated with MK-243 in this study was only 0.48 mg/gland/10 min (Fig. 4.4B), or approx. 20% of the value reported by previous authors for small, partially-fed ticks. Salivary gland weights of day 10 (but not day 5) MK-243 treated ticks were also significantly greater than controls (Fig. 4.4A), indicating that somewhat less degeneration than normal had occurred. Likewise, the appearance of salivary glands of MK-243 treated ticks seemed more robust than those of control ticks (Fig. 4.5). Lunke and Kaufman (1992) reported no effect of MK-243 on salivary gland function, and they hypothesized that while the haemolymph 20E-concentration was decreased to 50 ng/ml in these ticks (approximately a 10-fold reduction), it may still have been high enough to trigger salivary gland degeneration, the threshold for salivary gland degeneration being about 30 ng 20E/ml (Harris and Kaufman, 1985).

MK-243 had low toxicity at the doses used in this study. At the highest dose of MK-243, tick survival was 100% on day 5 and 90% on day 10 (Table 4.1). However, MK-243 inhibited tone of the dorso-ventral muscles, with MK-243 treated ticks being rounder, having splayed legs, and having less pronounced dorsal ridges than controls (Fig. 4.1). While Gration et al. (1986) also report that GABA acts to inhibit contraction

of the dorso-ventral muscles of *A. hebraeum*, we don't know whether this effect of MK-243 on muscle tone in ticks is mediated by GABA and/or glutamate.

In summary, this study demonstrates that MK-243 inhibits ovary development and decreases Vt-content in mature ovaries, without having a substantial effect on Vg-concentration in the haemolymph of engorged females. The data suggest that the effects of MK-243 are not primarily due to inhibition of 20E-mediated vitellogenesis. Rather, MK-243 seems to act primarily by inhibiting yolk-uptake by a mechanism that remains to be determined.

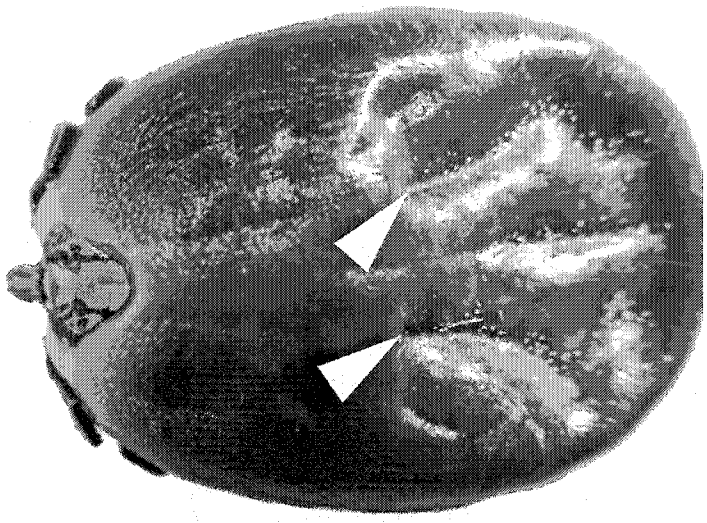
Table 4.1. Mortality of MK-243 treated engorged female *A. hebraeum* on day 5 or 10 post-engorgement.

	[MK-243] ng/g bw							
	0		50		100		150	
	% mortality	n	% mortality	n	% mortality	n	% mortality	n
Day 5	**0**	36	**7.1**	14	**0**	34	**0**	71
Day 10	**3.7**	27	**6.4**	47	**7.9**	38	**10.9**	55

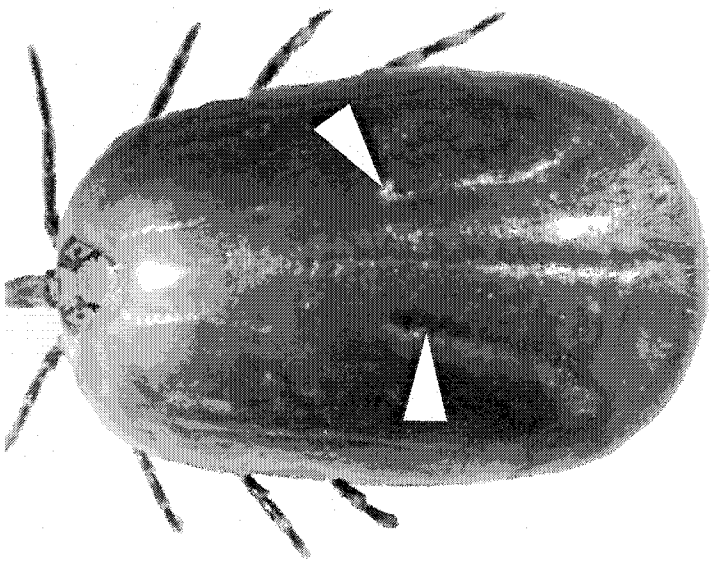
Table 4.2 Mortality of MK-243/20E treated engorged female *A. hebraeum* on day 10 post-engorgement.

Treatment									
untreated		EtOH		MK-243 (150 ng/g bw)		MK-243 + 20E (5 µg/g bw)		MK-243 + 20E (15 µg/g bw)	
% mortality	n	% mortality	n	% mortality	n	% mortality	n	% mortality	n
0	16	**0**	21	**0**	20	**14.8**	27	**40**	10

Figure 4.1. Appearance of engorged female *A. hebraeum* 10 days after injection of (A) 1.2% NaCl or (B) 150 ng MK-243/g bw. The cuticular ridges (white arrowheads) are caused by the contraction of the dorso-ventral muscles. Note that the ridges are much shallower in the MK-243 treated tick and that the legs are splayed (indices of paralysis) compared to the control.



A) Day 10 control



B) Day 10 MK-243 (150 ng/g bw)

Figure 4.2. Effect MK-243 on ovary development in *A. hebraeum*, 5 days (light grey bars) or 10 days (dark grey bars) post-engorgement. MK-243 was injected on the day of engorgement (day 0). (A) Ovary weight as % engorged body weight on day 0; (B) mean length of 8 of the largest oocytes; (C) total Vt-content of ovary; (D) haemolymph Vg-concentration (reported as 'Vt-equivalents', see Materials and Methods). For all panels, data are reported as mean ± SEM (n). Statistical significance is indicated as follow: (*) $0.01<P<0.05$; (**) $0.001<P<0.01$; (***) $P<0.001$.

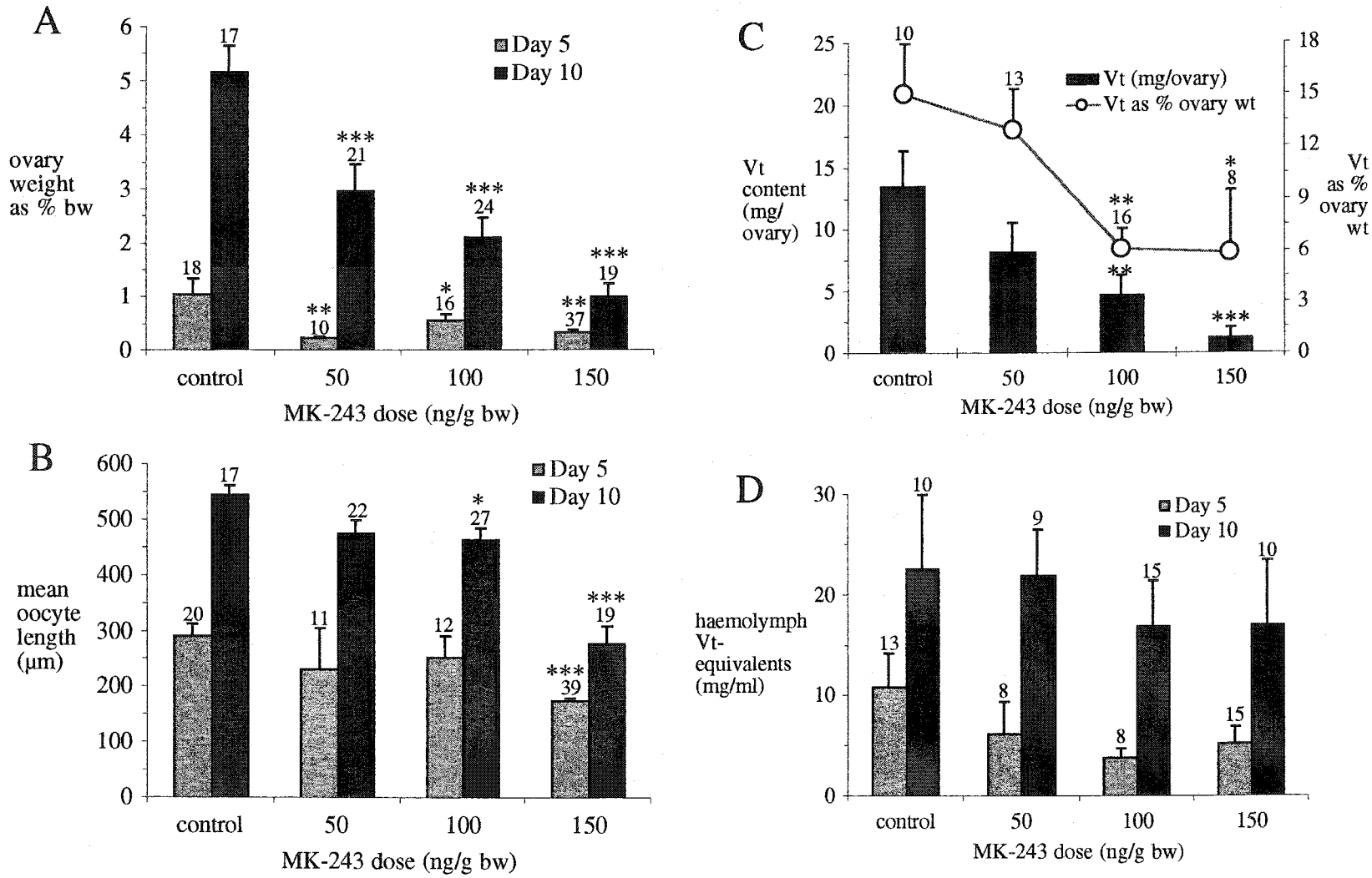


Figure 4.3. Inability of 20E to reverse the effects of MK-243 in engorged *A. hebraeum*. MK-243 (150 ng/g bw) or saline control was injected on day 0. Bolus injections of 20E were given on days 1, 3, and 5 post-engorgement, and ovaries collected on day 10. (Control): ticks that received no injection, (EtOH): ticks that received EtOH (final concentration 0.06%) in 1.2% NaCl on days 1, 3, and 5, (MK-243): ticks that received MK-243 followed by ethanol/saline injections (days 1, 3, and 5). (A) Ovary weight as % bw; (B) mean length of 8 of the longest oocytes; (C) total Vt-content of the ovary. Data are reported as mean ± SEM (n). Significant differences are indicated as in the legend to Fig. 2.

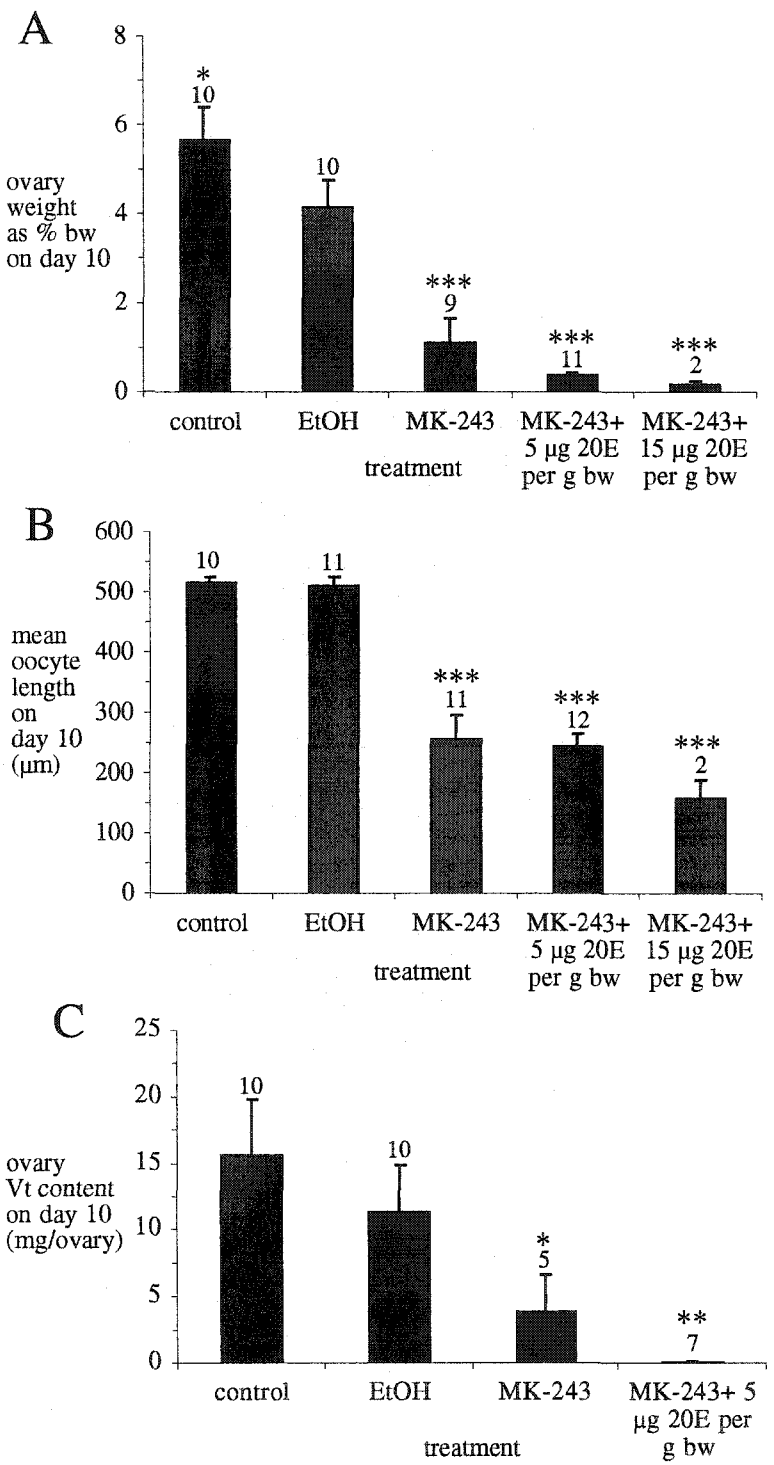


Figure 4.4. Effect of MK-243 on salivary gland (SG) weight (A) and fluid secretory competence (B) 5 and 10 days post-engorgement. Ticks received a single injection of 1.2% NaCl (control) or MK-243 (50, 100, or 150 ng/g bw) on the day of engorgement. Data are reported as mean ± SEM (n). Significant differences are indicated as in the legend to Fig. 2.

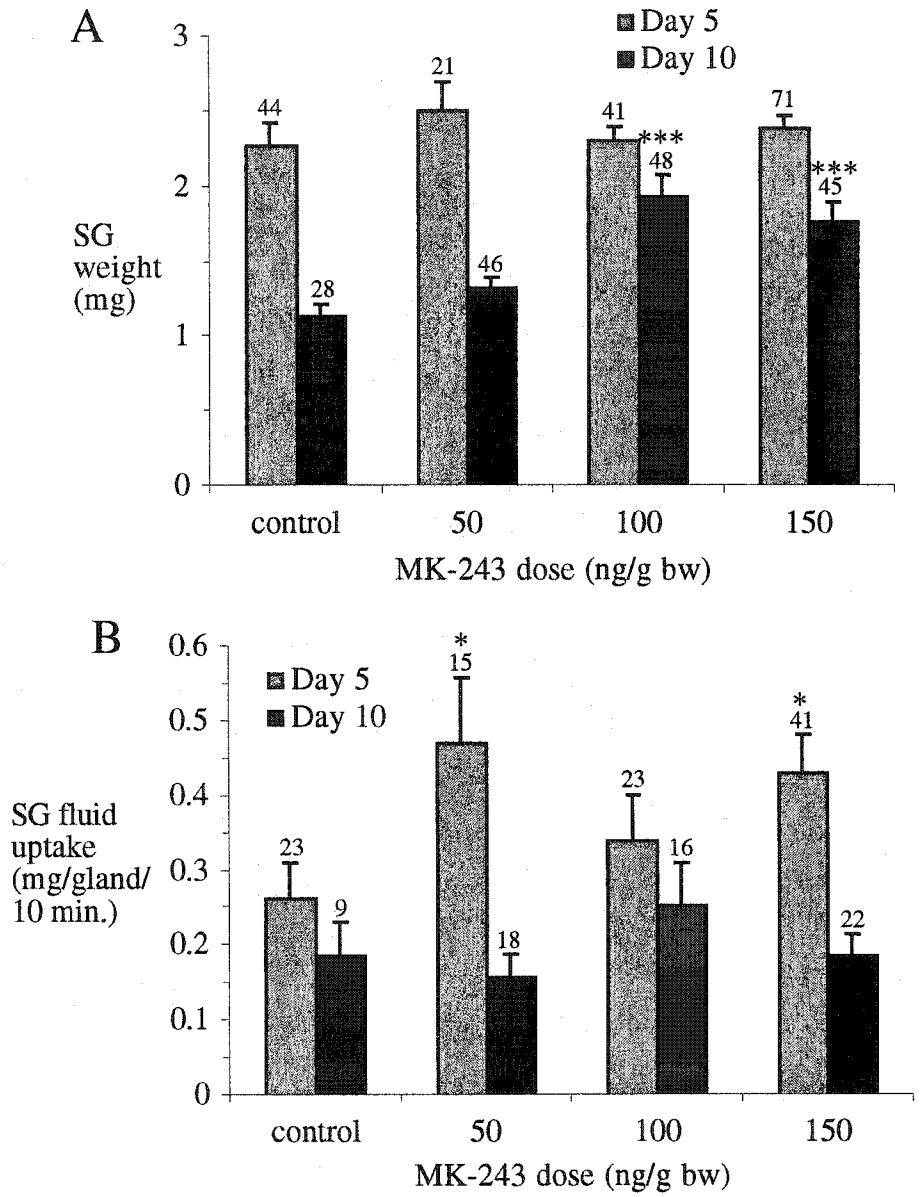
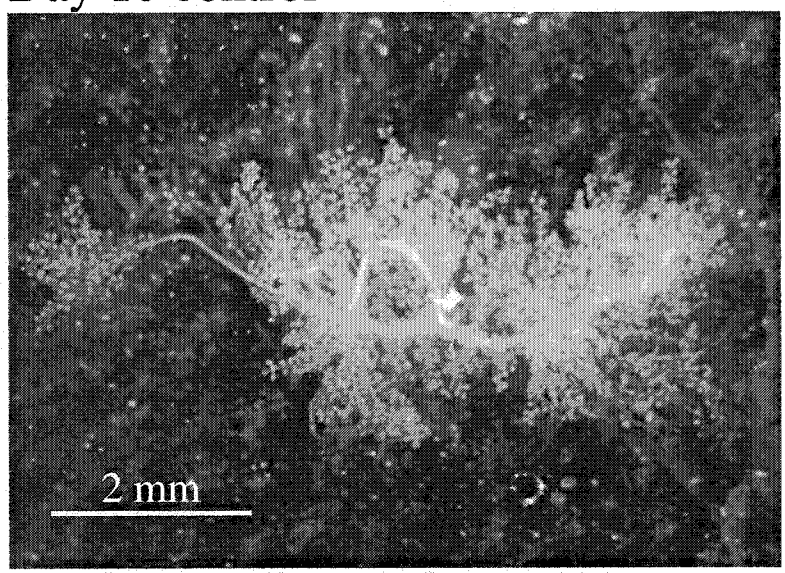


Figure 4.5. Effect of saline injection (A), or 150 ng MK-243g bw (B), on appearance of salivary glands of *A. hebraeum* 10 days post-engorgement. Note the wispy appearance of the control salivary gland versus the more robust appearance of the MK-243 treated gland.

Day 10 control



Day 10 MK-243 (150 ng/g bw)

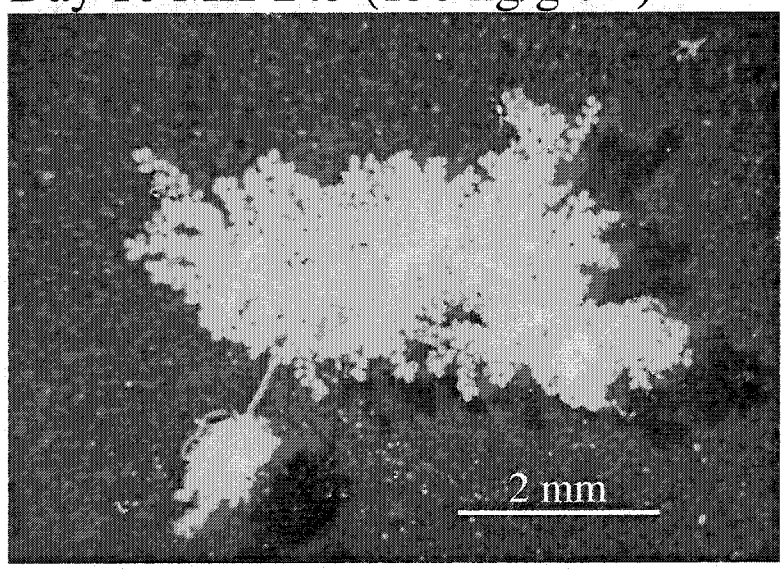
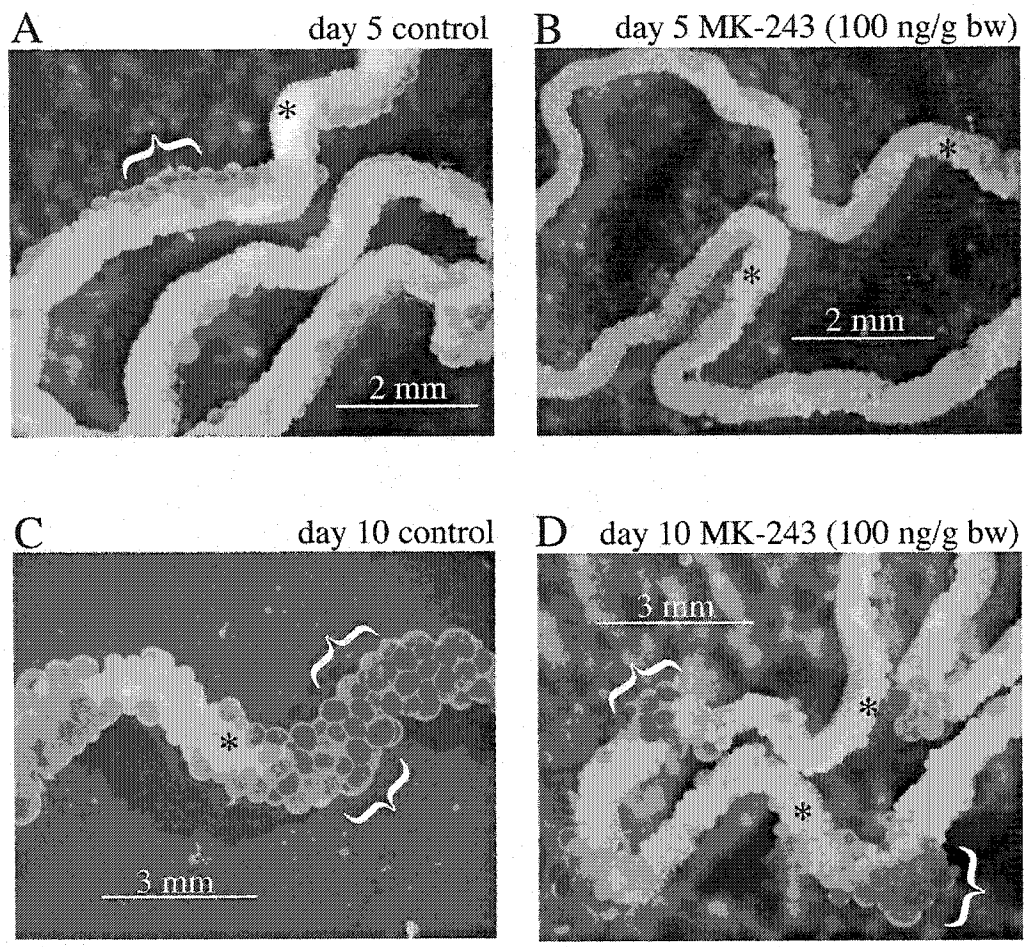


Figure 4.6. Effect of MK-243 on appearance of the ovary. Ovaries are from (A) day 5 post-engorgement saline injected control, (B) day 5, 100 ngMK-243/g bw, (C) day 10, saline injected control (D) day 10, 100 ng MK-243/g bw. Some of the regions containing vitellogenic oocytes are indicated by white brackets. Asterisks (*) indicate a few regions where oocytes are toward the end of the great cytoplasmic growth phase, but that have not yet accumulated much Vg (see Discussion).



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Chapter 5

Cypermethrin Inhibits Egg Development in the Ixodid Tick, *Amblyomma hebraeum*[†]

Introduction

The roles of juvenile hormone (JH) and 20-hydroxyecdysone (20E) during vitellogenesis are well understood in insects, and provide useful models for the study of vitellogenesis in other arthropods. Our detailed understanding of some insect systems serves to emphasize our rudimentary understanding of the hormonal control of vitellogenesis in most chelicerates, including ticks (Kaufman, 1997). For example, there is only circumstantial evidence for JH being the vitellogenic hormone in mites (Oliver et al, 1985) and for 20E being the vitellogenic hormone in spiders (Trabalon et al, 1992).

Vitellogenesis in the argasid tick, *Ornithodoros moubata*, is initiated by a peptide ('vitellogenesis inducing factor'; VIF) from the synganglion (Chinzei et al, 1992). This neuropeptide triggers the release of 'fat body stimulating factor' (FSF), which in turn stimulates yolk-synthesis in fat body tissue (Chinzei and Taylor, 1990). The identity of this second factor is unknown, although circumstantial evidence from several studies suggest that it may be JH (Pound and Oliver, 1979; Connat et al, 1983) or an ecdysteroid (Connat et al, 1985). So far, however, attempts to identify JH in ticks by gas chromatography/mass spectrometry have failed (Connat, 1987; Neese et al., 2000).

Evidence for an ecdysteroid stimulating vitellogenesis in ixodid ticks is mounting. In engorged *Amblyomma hebraeum*, haemolymph ecdysteroid-concentration increases in parallel with yolk-accumulation by ovaries (Kaufman, 1991) and with haemolymph vitellogenin (Vg)-concentration (Friesen and Kaufman, 2002). Likewise, an increase of Vg-synthesis by fat body corresponds to an increase of haemolymph ecdysteroid-

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[†] A version of this chapter has been submitted for publication.

concentration in female *Ixodes scapularis* (James et al, 1997). Sankhon et al. (1999) stimulated *in vitro* Vg-synthesis and release from fat body of *Dermacentor variabilis* with 20-hydroxyecdysone (20E), but not with the JH-mimic, methoprene. More recently, Friesen and Kaufman (2002) demonstrated that injection of partially-fed *A. hebraeum* females with 20E triggered a marked increase of haemolymph Vg-concentration.

Pyrethroid insecticides stimulate the continuous firing of action potentials in neurons of both the central and peripheral nervous systems of insects (Ruigt, 1985). Neurosecretory cells of both *Rhodnius prolixus* (Orchard, 1980) and the stick insect, *Carausius morosus* (Orchard and Osborne, 1979), show increased spontaneous activity after application of pyrethroid, a response which may result in increased secretion of neurohormones from these cells. Indeed, sublethal doses of CyM induced ovarian development in the ladybird beetle, *Henosepulachna vigintioctopunctata*, which the authors attribute to the release of neurosecretions (Kono and Ozeki, 1987). Similarly, CyM-treated, unfed, virgin female argasid ticks undergo vitellogenesis to the same degree as normal, fed, mated females (Taylor et al, 1991a), which the authors attribute to an ultimate release of VIF. The latter study indicates that even without a blood meal and mating, a female argasid tick can synthesize Vg and produce eggs if given an effective stimulus. These findings for argasid ticks led us to consider that, if CyM can stimulate egg development in ixodid ticks as well, it would provide evidence for neurosecretory control over vitellogenesis, and may prove to be a valuable pharmacological tool for identifying an ixodid tick VIF.

In this study, the effects of CyM on vitellogenesis were tested in the ixodid tick, *A. hebraeum*. In order to directly measure Vg in haemolymph or tissues in response to CyM treatments, an enzyme-linked immunosorbent assay (ELISA) was used (Friesen and Kaufman, 2002). Results obtained using this ELISA, along with other assays for vitellogenesis, indicate that the effects of CyM are not the same in *A. hebraeum* as in

argasid ticks (Chinzei et al, 1989; Taylor et al., 1991a). In addition, this study provides further evidence that an ecdysteroid may act as the vitellogenic hormone in *A. hebraeum.*

Materials and Methods

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, or were forcibly removed from the host below the 'critical weight' necessary to begin vitellogenesis (Kaufman and Lomas, 1996; Weiss and Kaufman, 2001). In this study weights of partially-fed females (below critical weight) ranged from 180-275 mg, and engorged females ranged from 900 to 3500 mg. After removal from the rabbit, ticks were cleaned with water, weighed, and stored individually in gauze-covered glass vials until needed for dissection and haemolymph collection.

Application of CyM

CyM was purchased from Chem Service Inc. (West Chester, PA) and Zeneca Ag Products (Calgary, AB), and was designated by the manufacturers as 92-93% pure. CyM was diluted in acetone to working concentration (0.05-30 mg/ml), and stored in darkness at room temperature in glass vials. Ticks were divided into groups of defined weight range. In all groups CyM was applied topically to the dorsal integument, just behind the scutum, via a 1 µl glass capillary tube or micropipettor. CyM doses ranged from 0.05-30 µg/tick in 1 µl acetone for partially fed ticks. Engorged ticks received 0.05-30 µg CyM/100 mg body weight. Controls received acetone alone, or nothing. After treatment, all ticks were held in individual glass vials in darkness at 27°C and >95% relative humidity. Each treatment group was stored in a separate, sealed, plastic container to

avoid inadvertent exposure to unintended doses of CyM. The containers were opened at least once every other day during a five or ten day incubation period to renew the air. At the end of the incubation period the ticks were re-weighed and the salivary glands, the ovary, and a sample of haemolymph collected.

Collection of haemolymph and tissue

Tick mortality was determined at the time of dissection. If, during haemolymph collection or dissection, the gut was noticed to be ruptured, that tick was discarded.

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 haemolymph (Kaufman, 1991). A small incision was made in the cuticle and haemolymph 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 ELISA. Samples for RIA were stored at -17°C and all other samples were stored at -70°C until analyzed.

After collection of haemolymph, ticks were flooded with 1.2% NaCl (approximately isosmotic with tick haemolymph), the dorsal cuticle removed using a microscalpel, and the salivary glands and ovaries dissected out for further analysis (see below).

Measurement of fluid uptake by salivary glands

After the removal of a salivary gland, the salivary duct was tied off with a fine strand of silk thread peeled from 8-0 Dermalon® (a gift from Davis and Geck Co., Pearl River, New York). Glands were gently blotted with filter paper to remove the adhering

extraglandular fluid, weighed to the nearest 10 μg, and suspended in a solution of 10 μM dopamine (Sigma Chemicals) in Tissue Culture Medium 199 (TC199; Gibco) for 10 min. Glands were then blotted and re-weighed. The net weight increase of a gland was used as a measure of its fluid secretory competence (see Harris and Kaufman (1984) for further details).

Assay for ovarian development

Ovarian development was assessed by three measures: ovary weight, oocyte size, and vitellin (Vt) content in ovary homogenates. After removal of an ovary, the length of the 8 largest oocytes was measured on the stage of a light microscope. The ovary was then weighed and homogenized in 3 ml distilled water. Homogenates were frozen at -17°C. At the time of analysis, frozen samples were thawed and centrifuged at 13,000X g for 5 minutes to remove insoluble material, and the supernatant used to quantify Vt content. Because of the haem-moiety attached to tick Vt, absorbance of ovary homogenates can be measured at a wavelength specific for haem (400 nm) from which is subtracted an absorbance non-specific for haem (500 nm). The difference between the two values provides a quantitative assay for Vt content (Kaufman et al., 1986).

RIA for 20E

20E-concentration in tick haemolymph was determined by the RIA procedure described by Kaufman (1990). Briefly, a methanol extract of each haemolymph sample was dried in a vacuum centrifuge 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 (NEN) per tube and an ecdysone antiserum (E-22-succinyl-thyroglobulin; supplied by Dr. L.I. Gilbert, University of North Carolina, or Dr. E. Chang, University of California, Davis). 20E standards (0-500 pg) were treated similarly. Antiserum-bound ligand was

precipitated with a crude suspension of 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), and radioactivity 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'.

ELISA for Vg

Vg and 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 with1 µg partially-purified Vt in 100 µl PBS (containing 0.02% sodium azide) and allowed to sit in a sealed container overnight at 4°C. Simultaneously, anti-Vg polyclonal antibodies were mixed with known concentrations of partially purified Vt (standard) or with haemolymph samples (diluted to 0.1 and 0.01 mg/ml total protein); equilibration occurred overnight at 4°C. The next day, the standard and sample mixtures were added to the wells, allowing the plate-bound Vt to compete for antibody binding with the Vt or Vg in solution. Two hours later, the wells were incubated with secondary antibody (alkaline phosphatase (AP)-goat anti-rabbit IgG; BioRad) for a further 2 hours and the color reaction performed using an AP substrate kit (p-Nitrophenylphosphate and diethanolamine kit; BioRad). Bound Vt-antibodies were quantified by measuring the absorbance of each well at 405 nm using a microtitre plate reader (Bio-Tek).

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 is approximately 5 ng of Vt-equivalents.

Statistical analysis

Statistical analyses were performed using Statview® 4.02 software (Abacus Concepts). Data are reported as Mean ± SEM (n). Significant differences were identified using one-way ANOVA at a 5% significance level. Statistical significance in figures is indicated as follow: (*) $0.01<P<0.05$; (**) $0.001<P<0.01$; (***) $P<0.001$.

Results

Effects of CyM on partially-fed ticks

Weight loss (as % initial body weight (bw)) of acetone-treated partially-fed control ticks increased significantly ($P<0.004$) from day 5 (12 ± 1.6% bw) to day 10 (19 ± 1.5% bw) post-treatment. Five days after treatment, partially fed ticks which received greater than 0.2 µg CyM had lost significantly more weight (up to 24 ± 1% loss of bw) than acetone-treated controls (Fig. 5.1A). Tick survival 5 days post-treatment dropped from 100% in control ticks to 40% in ticks treated with 20 µg CyM per tick (Fig. 5.1A). By day 10, ticks treated with 1 and 5 µg CyM/tick lost significantly more weight (25 ± 1.7% and 27± 4% bw, respectively), relative to acetone-treated control ticks. Beginning at 74% for controls, the percent of ticks surviving to day 10 generally decreased with dose, with no ticks surviving at doses of 10 or 20 µg per tick.

CyM had no dose-dependent effect on the ovary from partially-fed ticks on day 5 or 10. There were no significant differences in ovary weight (Fig. 5.2A), oocyte size (Fig. 5.2B), or ovary Vt content (Fig. 5.2C) across partially-fed treatment groups on day 5 or 10; a single exception was that oocyte length of 20 µg CyM-treated ticks on day 5

was significantly smaller than acetone-treated controls (95 ± 5 μm for CyM treated and 135 ± 18 for controls; Fig. 5.2B).

There were no differences in ovary weight (as % bw) between engorged and partially-fed ticks on day 5. However, by day 10, the ovary weights of engorged ticks were much higher than for partially-fed ticks (3.4 ± 1.0 % for acetone-treated engorged, and 1.0 ± 0.1 % for partially-fed acetone-treated controls; Fig. 5.2A). Oocytes were also significantly larger in acetone-treated engorged (256 ±12 μm on day 5; 425 ± 23 μm on day 10) compared to partially-fed acetone-treated control ticks (135 ± 18 μm on day 5; 162 ± 21 μm on day 10; fig. 5.2B). Ovaries contained more Vt in acetone treated engorged (11.7 ± 1.5 abs/g ovary on day 5; 19.5 ± 3.2 abs/g ovary on day 10) than partially-fed acetone-treated controls (4.7 ± 1.1 abs/g ovary on day 5; 7.0 ± 2.6 abs/g ovary on day 10; Fig. 5.2C). Acetone treatment had no significant effect on ovary weight, oocyte length, or ovary Vt content in engorged ticks on day 5 or day 10.

CyM (up to 20 μg/tick) had no significant effect on wet weight of salivary glands from partially fed ticks (Fig. 5.3A). However, salivary gland fluid uptake was significantly reduced compared to controls both on day 5 (from 1.69 ± 0.41 mg/gland/10 min for controls to 0.36 ± 0.16 mg/gland/10 min (10 μg CyM)) and day 10 (from 1.4 ± 0.2 for controls to 0.31 ± 0.07 mg/gland/10 min (5 μg CyM); Fig. 5.3B).

Effects of CyM on engorged ticks

Weight loss (compared to initial bw) increased significantly ($P<0.001$) from day 5 (8.7 ± 1.0%) to day 10 (12.5 ± 0.7%) in untreated engorged ticks (Fig. 5.4); however, there was no significant difference in weight loss in acetone-treated engorged ticks between day 5 and day 10.

There was no significant difference in weight loss across treatment groups by day 5 post-engorgement (Fig. 5.4A). Tick survival on day 5 dropped from 97% (acetone

controls) to 20% (30 µg CyM/g bw), and then recovered somewhat at the 100 and 300 µg CyM/g bw doses (Fig. 5.4A). A CyM dose of 30 µg/g caused a significant increase in weight loss in engorged ticks on day 10 [up to 15.1 ± 2.6% (30 µg CyM/g bw) from 10.8 ± 0.7 (acetone controls); Fig. 5.4A], but at 300 µg CyM/g bw, the weight loss (4.9 ± 0.96%) was significantly less than controls. The percent of ticks which survived to day 10 decreased with CyM dose; at doses of 30 and 100 µg/g only 33% of ticks survived compared with 96% for acetone treated controls (Fig. 5.4B). At 300 µg/g doses, day 10 tick survival on day 10 had recovered somewhat to 67%.

Although ovary weight in day 5 ticks was not significantly affected by CyM, CyM caused a significant reduction in oocyte length (from 238 ± 15 µm in acetone controls to 134 ± 31 µm in 100 µg CyM/g bw treated ticks; Fig. 5.5B). There was no significant change in yolk content, however, at any dose of CyM (Fig. 5.5C). On day 10, however, doses of 1-3 µg CyM/g bw inhibited all observed indices of ovary development (Fig. 5.5). Ovary weight was reduced to 0.67 ± 0.07 % bw (300 µg CyM/g bw) compared with 3.84 ± 0.42% bw in acetone treated controls (Fig. 5.5A). Oocyte length was reduced from 519 ± 15 µm (acetone controls) to 230 ± 24 µm (300 µg CyM/g bw; Fig. 5.5B); untreated control ticks (425 ± 23 µm) also had significantly smaller oocytes than acetone controls. Ovary Vt content was also reduced on day 10, by up to 80% in ticks treated with 300 µg CyM/g bw (from 37 ± 11 abs/g ovary for acetone controls to 7 ± 2 abs/g ovary for CyM treated ticks).

Salivary glands of day 5 engorged ticks treated with over 10 µg CyM/g bw were significantly heavier (up to 5.3 ± 0.3 mg) than those of acetone controls (2.7 ± 0.2 mg; Fig. 5.6A). By 10 days post-engorgement, salivary gland weight in acetone treated controls had fallen to 1.6 ± 0.1 mg, but there was a significant recovery of weight (4.83 ± 0.80 mg) at 30 µg CyM/g bw (Fig. 5.6A). Salivary gland fluid secretory competence on day 5 increased by up to 2.5-fold in ticks treated with 10 µg CyM/g bw, compared to

acetone treated controls (1.38 ± 0.14 mg/gland/10 min for CyM treated and 0.56 ± 0.10 mg/gland/10 min for controls; Fig. 5.6B). Higher doses of CyM did not have this effect, however (Fig. 5.6B). Likewise, on day 10, CyM at most doses caused significant increase in fluid secretory competence compared to controls (Fig. 5.6B).

Due to difficulty in obtaining haemolymph samples on days 5-10 post-engorgement, CyM treatment groups were pooled as follows: 'low dose' (1 and 3 μg CyM/g bw), 'medium dose' (10 and 30 μg CyM/g bw), and 'high dose' (100 and 300 μg/g bw). There was no significant effect of CyM treatment on haemolymph 20E- or Vg-concentrations by day 5 (Fig. 5.7). However, by day 10, haemolymph 20E-concentration was reduced from 837 ± 80 pg/μl in acetone treated controls to 123 ± 59 pg/μl in the high dose group (Fig. 5.7A). Likewise, on day 10, CyM treated ticks had haemolymph Vg-concentration as low as 2.3 ± 0.3 μg/μl (medium dose) relative to acetone controls (32.1 ± 8.2 μg/g bw; Fig. 5.7B).

Discussion

As described in the Introduction, sublethal doses of CyM can be used to stimulate the release of neurohormones and to trigger associated physiological events. Thus, Taylor et al. (1991a) applied CyM to unfed, virgin female *O. moubata*, and stimulated Vg-synthesis and ovarian development to levels characteristic for normal engorged ticks. CyM did not have similar effects in this study, however: in partially-fed ticks, CyM did not stimulate ovary development (Fig. 5.2). In engorged ticks, CyM inhibited ovary growth and egg development (Fig. 5.5), as well as 20E and Vg-synthesis (Fig. 5.7). Thus, at least part of CyM's inhibitory effect on Vg-synthesis is likely to be the result of inhibiting 20E-secretion (Fig. 5.7A). In *A. hebraeum*, ecdysteroids are synthesized in the epidermis in response to a neuropeptide from the synganglion (Lomas et al., 1997). We

do not know whether CyM inhibits 20E-synthesis by blocking the release of the ecdysteroidogenic neuropeptide, or by a direct action on the epidermis.

The effects of CyM on oocyte development were more pronounced on day 10 than day 5, likely because in *A. hebraeum* vitellogenesis has only begun by day 5, whereas it is almost complete by day 10 (Friesen and Kaufman, 2002). Although by day 5 there were no significant differences in ovary weight or ovary Vt content, a decreasing trend is apparent in engorged ticks treated with higher doses of CyM (Figs. 5.5A and 5.5C). Day 5 oocytes from engorged ticks were significantly smaller after CyM treatments (Fig. 5.5B), being approximately the same size as day 5 partially-fed oocytes (Fig. 5.2B). This suggests that oocyte growth had been delayed prior to or during the late previtellogenic growth phase. In addition, by day 10, all of the parameters of egg development were reduced by CyM treatment to the same levels as day 5 acetone treated controls. These results strongly suggest that CyM inhibits Vg uptake by oocytes.

Salivary gland degeneration in female ixodid ticks following engorgement is triggered by 20E (Kaufman, 1991). By day 10, high doses of CyM inhibited haemolymph 20E-concentration in engorged ticks by 85%. There was a similar trend by day 5, though the difference was not significant, perhaps because of the very small number of ticks available for this experiment (Fig. 5.7A). In partially-fed ticks removed from the host below the critical weight, haemolymph ecdysteroid-concentration does not rise (Lomas and Kaufman, 1992), and the salivary glands do not degenerate (Harris and Kaufman, 1984; Harris and Kaufman, 1985). CyM, perhaps due to a toxic effect, reduced salivary fluid transport (Fig. 5.3B). In engorged ticks, on the other hand, CyM significantly increased salivary gland weight and fluid transport (Fig. 5.6). At first sight, perhaps it was through its effect on reducing haemolymph ecdysteroid-concentration (Fig. 5.7A). But there are some problems with this explanation. The reduction of haemolymph ecdysteroid-concentration by CyM occurred only at 10 μg CyM/g and

above, whereas an increase in salivary gland weight and fluid transport began as low as 3 µg CyM/g on day 5, and 1 µg CyM/g on day 10 (Fig. 5.6B). Moreover, even at 10-100 µg/g, CyM reduced haemolymph ecdysteroid-concentration only to 100-200 ng/ml. At this level, ecdysteroid-concentration should still have been high enough to cause salivary gland degeneration (Harris and Kaufman, 1985; Lomas and Kaufman, 1992). Thus, the mechanism whereby CyM increases salivary gland weight and fluid transport remains an enigma. However, similar effects on salivary gland fluid secretion were observed in *A. hebraeum* using the avermectin, MK-243 (Chapter 4), which also causes a marked reduction of haemolymph ecdysteroid-concentration in this tick (Lunke and Kaufman, 1992).

In insects, pyrethroids cause paralysis and death by stimulating repetitive action potential firing in neurons (Ruigt, 1985, and Vais et al., 2001). While CyM was the most effective pyrethroid tested at stimulating ovarian development in the ladybird beetle, it was also the most toxic; 36-56% of animals treated with 0.05 µg CyM died (Kono and Ozeki, 1987). However, in ticks, Taylor et al. (1991a) found that >80% of mated, unfed *O. moubata* survived a dose of 20 µg CyM/tick until day 10 post-treatment, and CyM was the least toxic of the six pyrethroids tested. As reported here, CyM seemed to be more toxic in *A. hebraeum* than in *O. moubata* (see Figs. 5.1 and 5.4). CyM also appeared to be more toxic to partially-fed ticks than to engorged ticks; by day 10, a dose of 10 µg CyM/tick (~36-55 µg/g, corrected for body weight), caused 100% death in partially-fed ticks (Fig. 5.1B), compared to 66% death in engorged ticks at 100 µg CyM/g (Fig. 5.4B). The reason for this difference is obscure.

The effect of CyM on vitellogenesis in *A. hebraeum* is significantly different from that observed for *O. moubata* and *O. parkeri* (Chinzei et al., 1989; Taylor et al., 1991a, 1991b; Chinzei et al., 1992). In the latter, a compelling argument was made that CyM releases VIF, a neuropeptide from the synganglion, which then triggers apparently

normal vitellogenesis and egg development. In *A. hebraeum*, CyM exerts only inhibitory effects on the reproductive system, but the sites and mechanisms of action remain largely unknown.

Figure 5.1. Weight loss and mortality of CyM treated partially-fed ticks on (A) day 5 and (B) day 10 post-treatment. (E/ctl): untreated engorged ticks; (E/ac): acetone-treated engorged ticks. Mortality is reported as percent of ticks surviving treatment (n). Weight loss here, and data in subsequent figures, are reported as mean ± SEM (n). Statistically significant differences from partially-fed acetone-treated control (0 μg) are indicated as follows: (*): $0.01<P<0.05$, (**): $0.001<P<0.01$. (***): $P<0.001$.

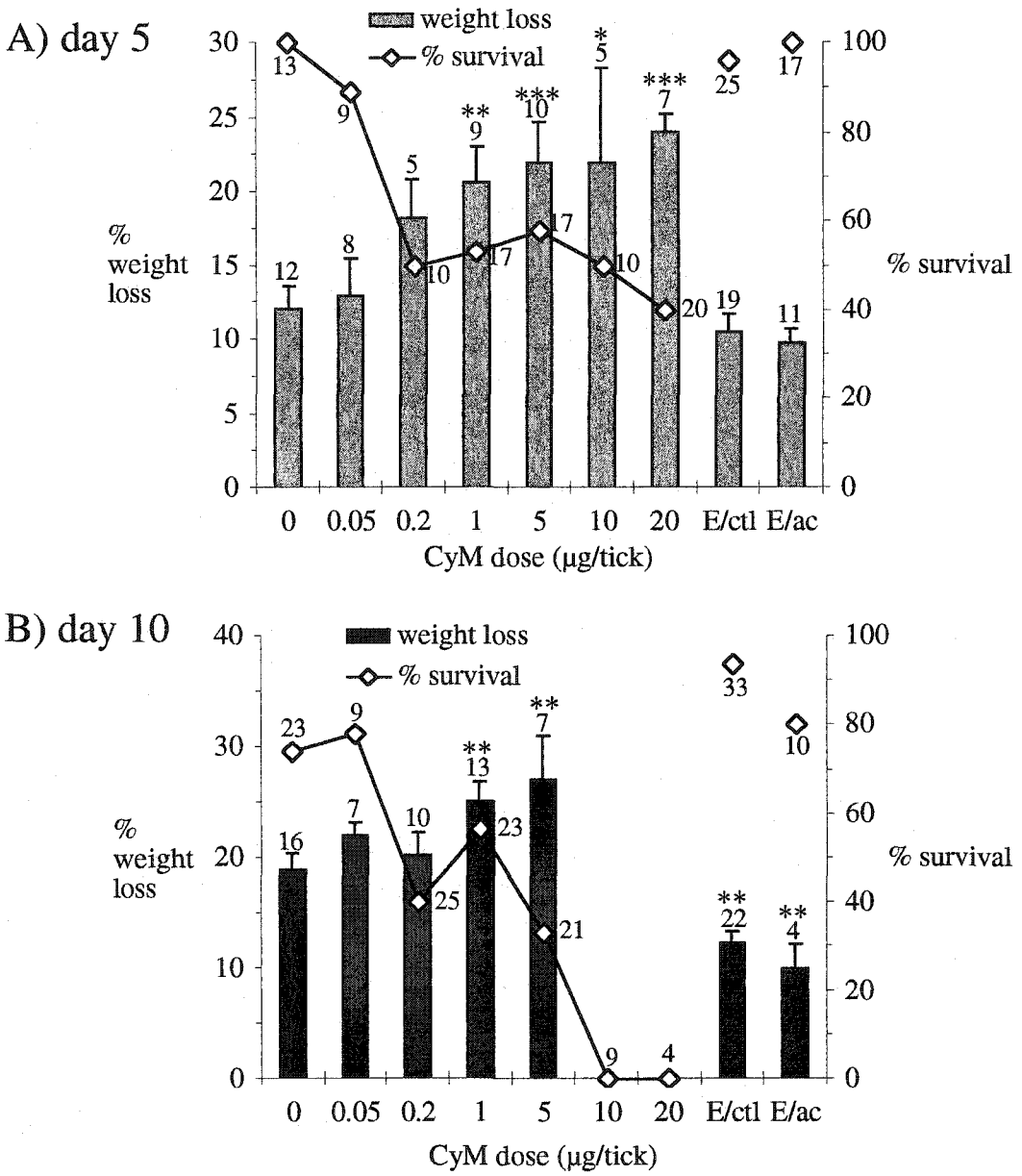
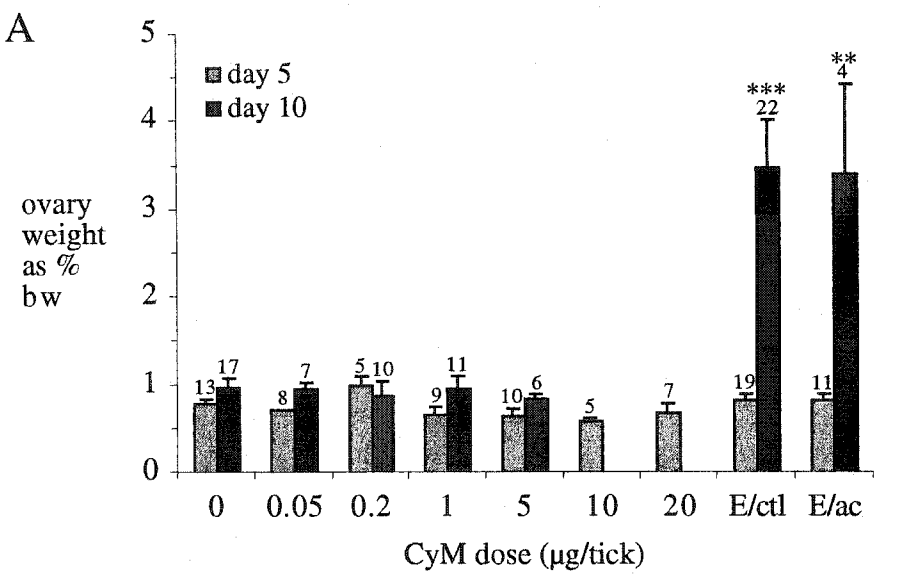
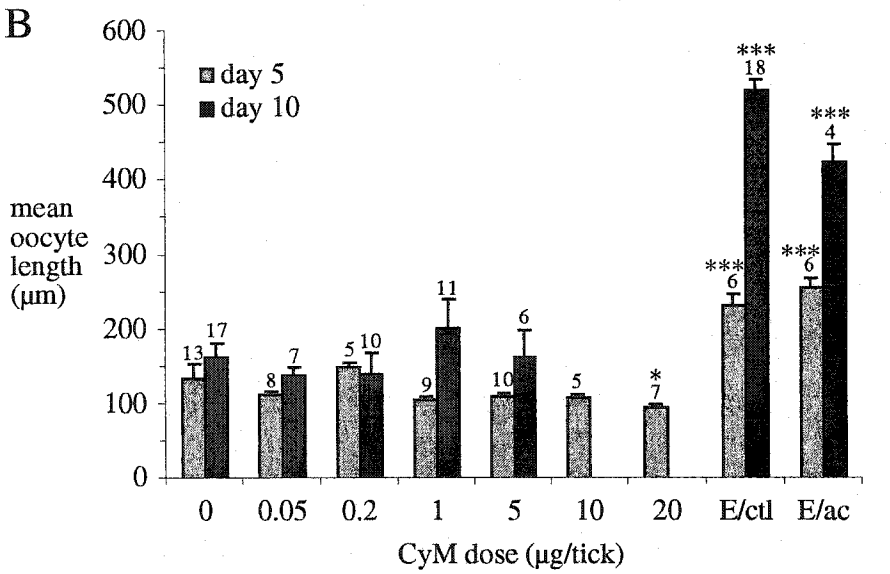


Figure 5.2. Effect of CyM treatment on ovary development in partially-fed ticks on day 5 (light grey bars) and 10 (dark-grey bars) post-treatment. (A) ovary weight; (B) mean length of 8 of the largest oocytes; (C) ovary Vt content, measured as absorbance per g ovary at haem-specific wavelength (400Δ500 nm; see Materials and Methods). Abbreviations and statistically significant differences from partially-fed control (0 μg) are indicated as for Fig. 5.1.





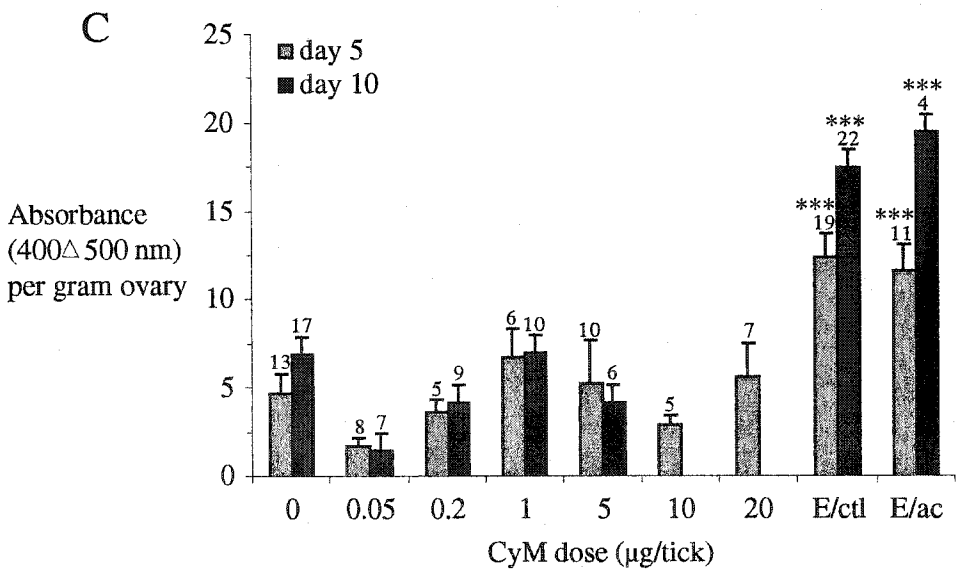


Figure 5.3. Effect of CyM treatment on (A) salivary gland (SG) weight and (B) salivary fluid secretory competence in partially-fed ticks on day 5 (light grey bars) and day 10 (dark grey bars) post-treatment. Abbreviations and statistically significant differences from partially-fed control (0 μg) are indicated as for Fig. 5.1.

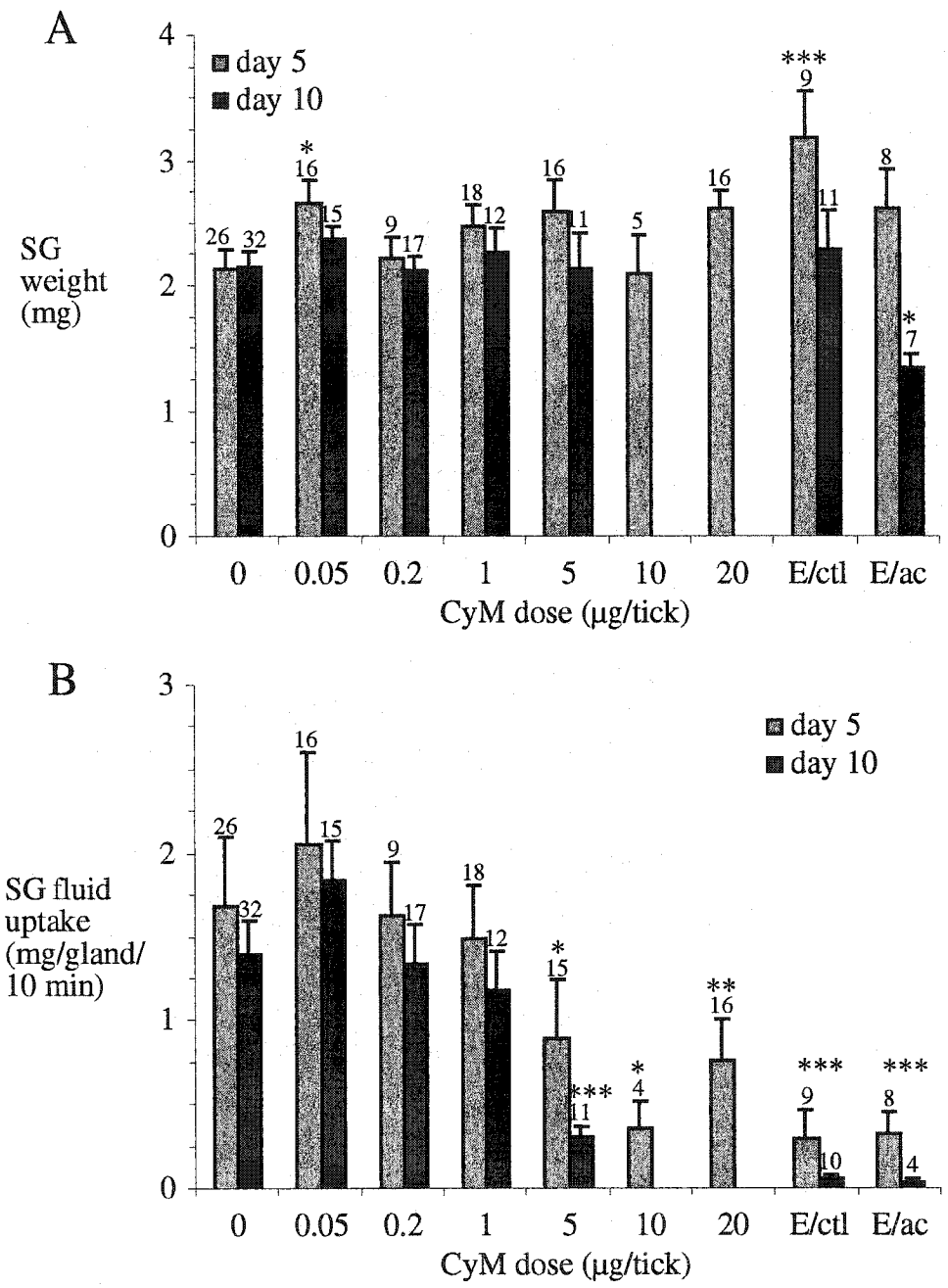
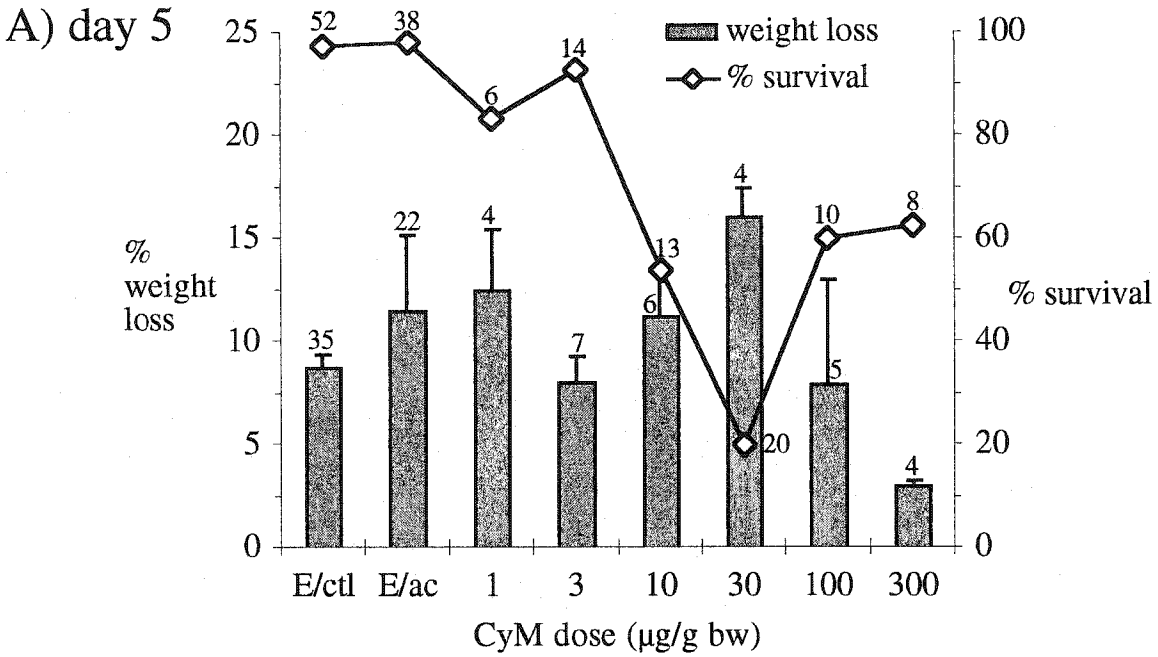


Figure 5.4. Weight loss and survival of CyM treated engorged ticks on (A) day 5 and (B) day 10 post-treatment. Mortality is reported as percent of ticks surviving treatment (n). Abbreviations and statistically significant differences from engorged acetone-treated control are indicated as in Fig. 5.1.



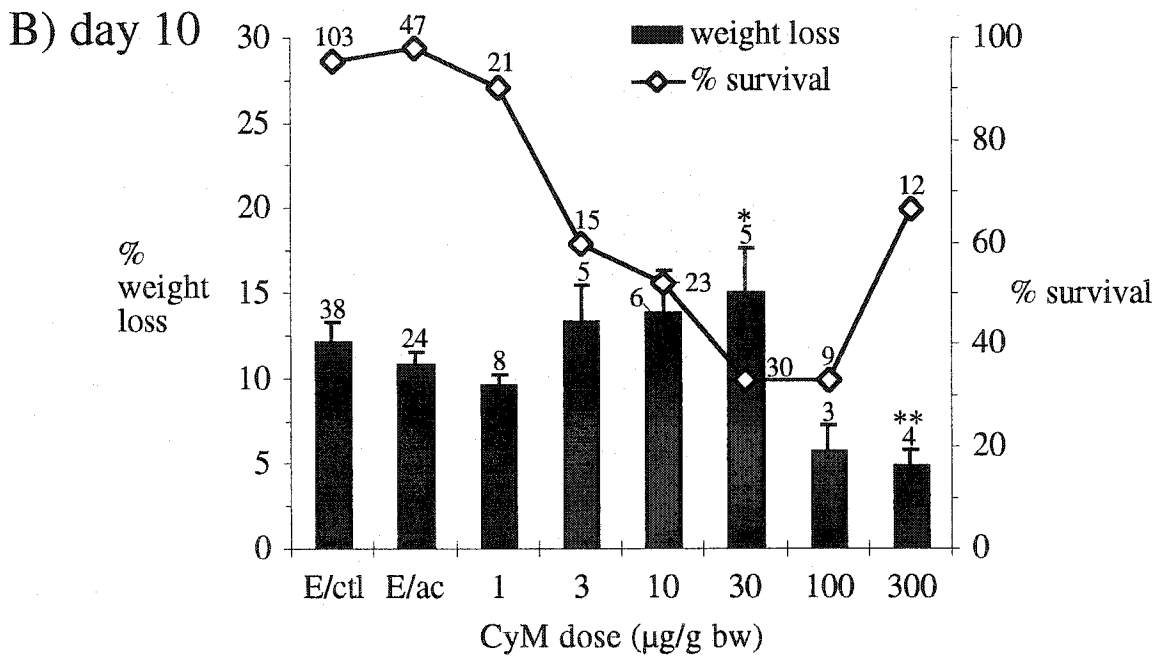


Figure 5.5. Effect of CyM treatment on ovary development in engorged ticks on day 5 (light grey bars) and 10 (dark-grey bars) post-treatment. (A) ovary weight; (B) mean length of 8 of the largest oocytes; (C) Vt content of ovary, measured as absorbance at haem-specific wavelength (400Δ500 nm; see Materials and Methods). Abbreviations and statistically significant differences from engorged acetone-treated control are indicated as in Fig. 5.1.

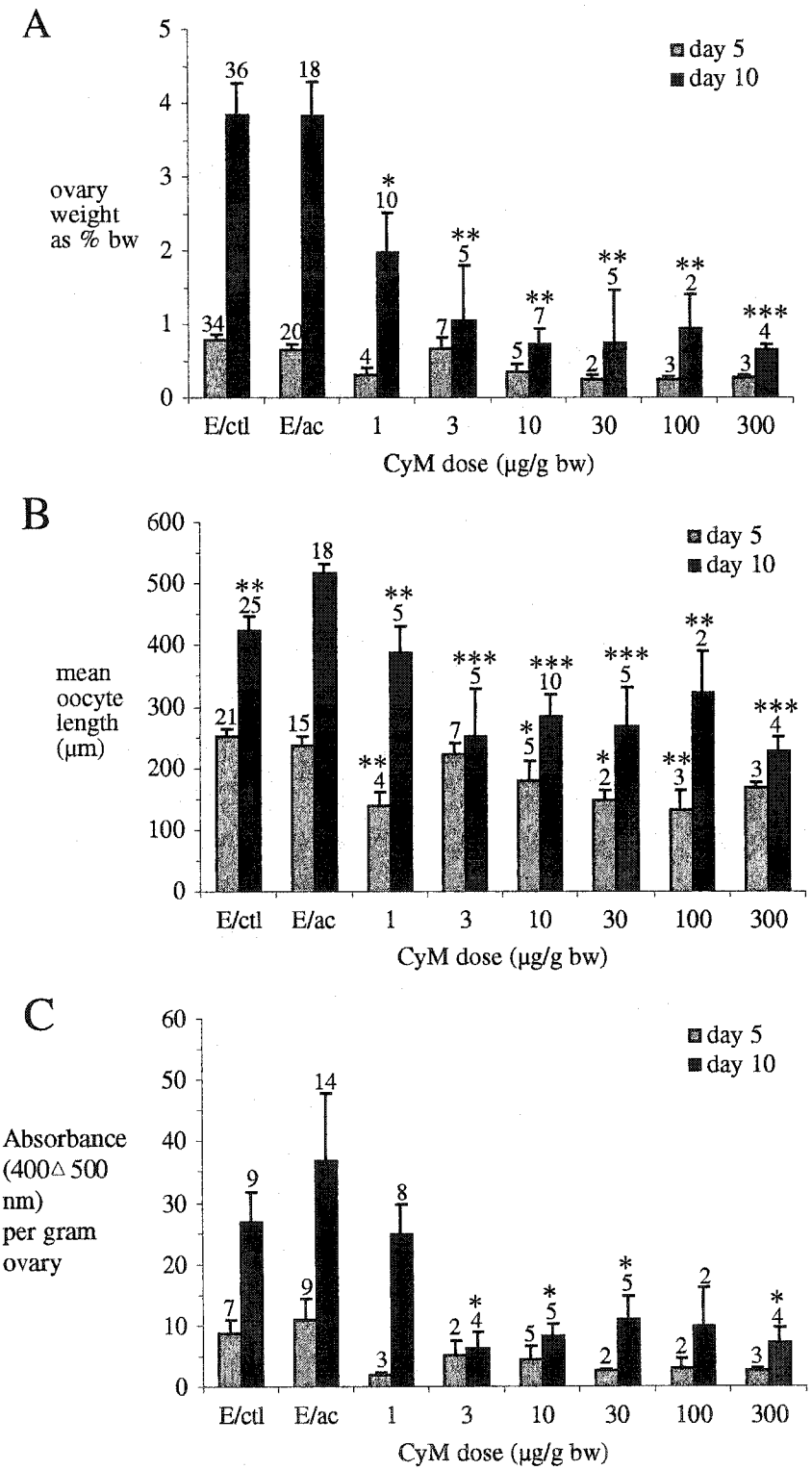


Figure 5.6. Effect of CyM treatment on (A) salivary gland (SG) weight and (B) salivary fluid secretory competence in engorged ticks on day 5 (light grey bars) and day 10 (dark grey bars) post-treatment. Abbreviations and statistically significant differences from engorged acetone-treated control are indicated as in Fig. 5.1.

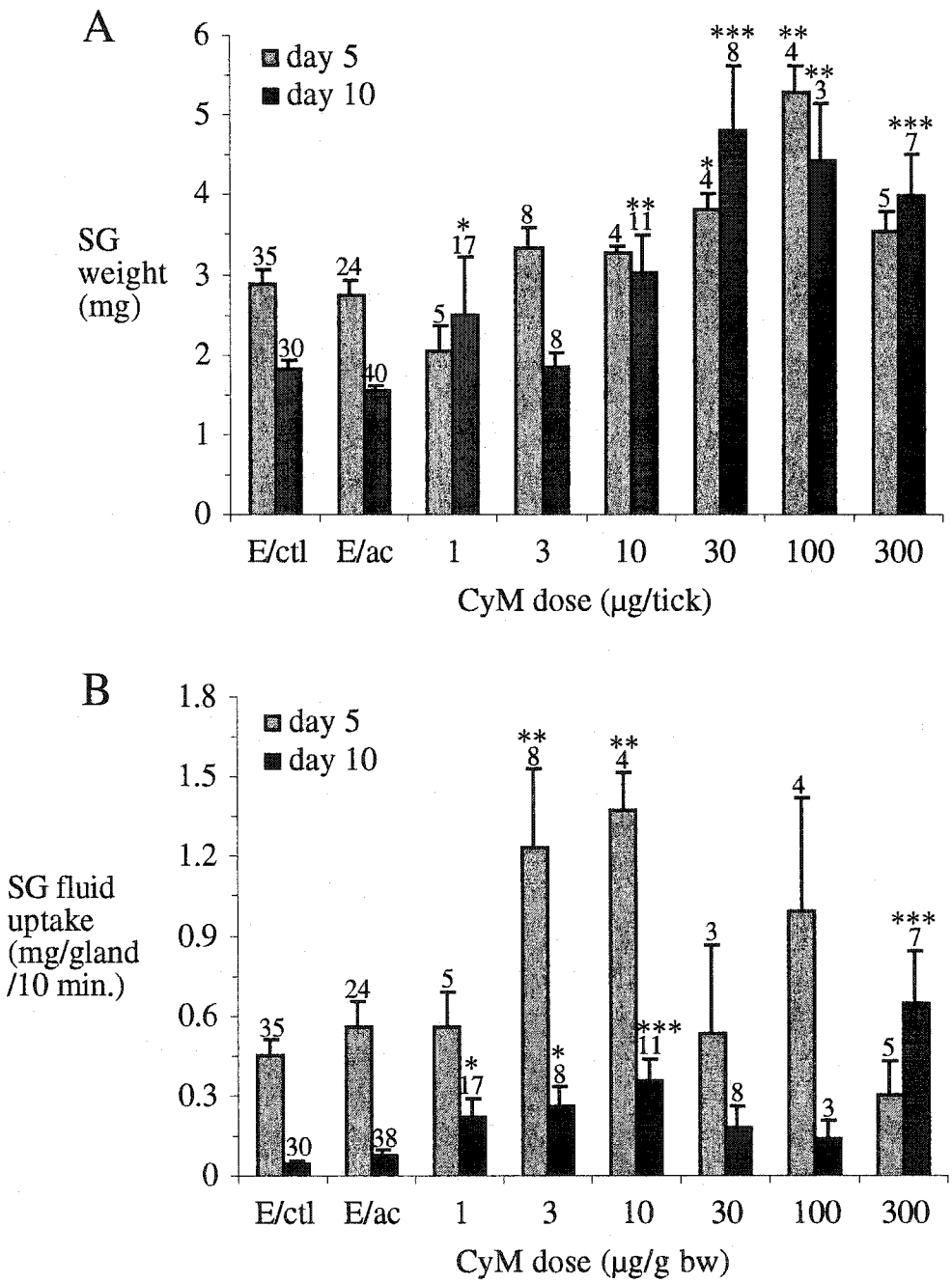
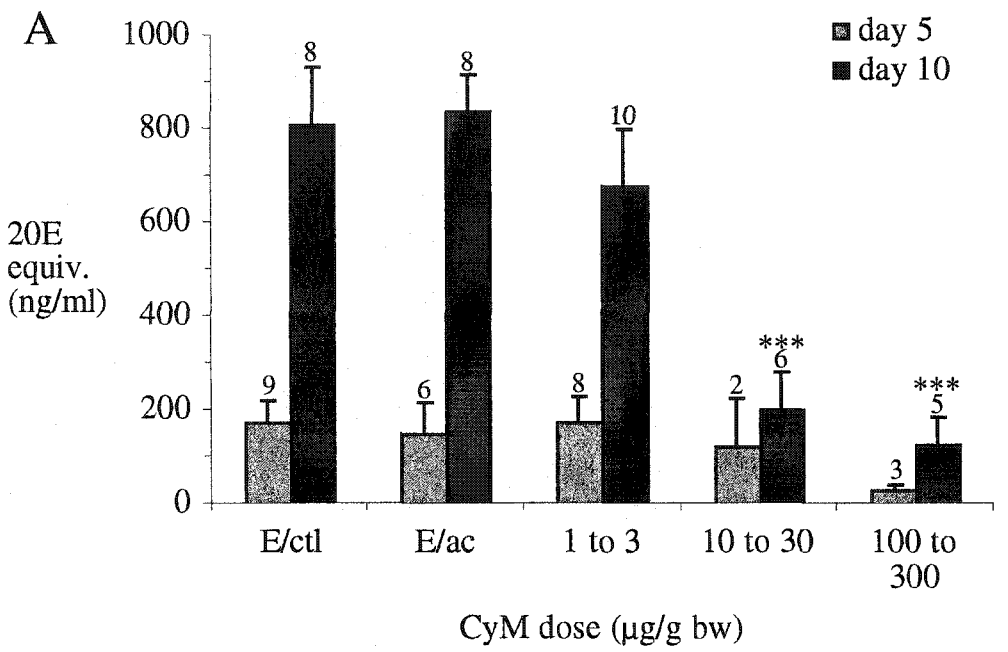
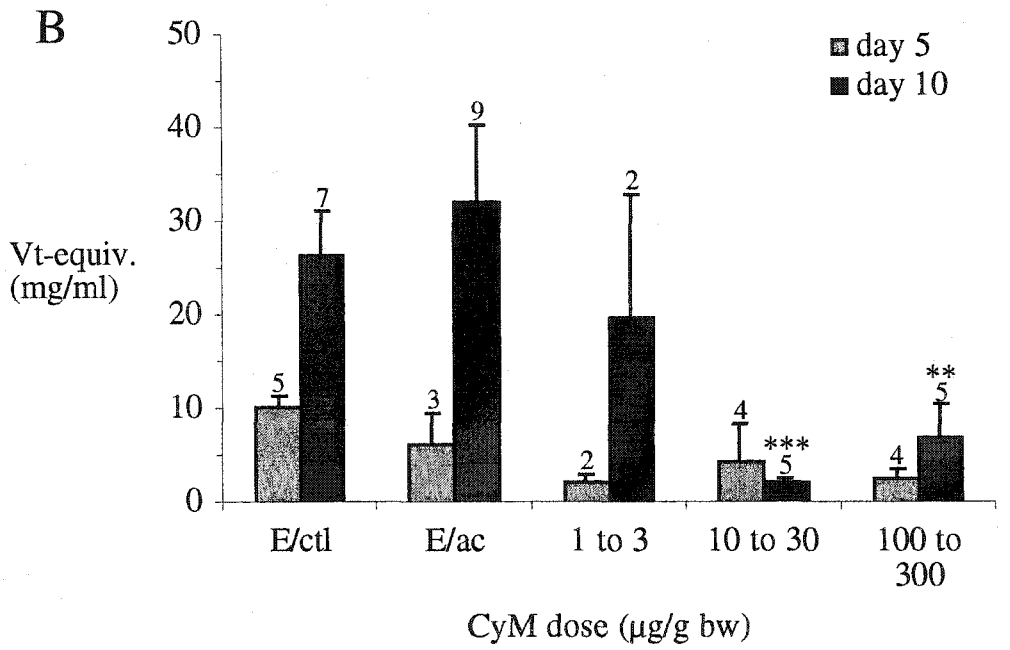


Figure 5.7. Effects of CyM on haemolymph (A) 20E-concentration and (B) Vg-concentration in engorged ticks on day 5 (light grey bars) and day 10 (dark grey bars) post-treatment. Abbreviations and statistically significant differences from engorged acetone-treated control are indicated as in Fig. 5.1.





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Chapter 6

General Discussion and Conclusions

As described in Chapters 2 and 3, until recently, our understanding of the hormone which regulates Vg-synthesis in ticks has been largely circumstantial. Here, the experimental evidence offers further support for the role of an ecdysteroid, if not 20E specifically, as the vitellogenic hormone in *A. hebraeum.*

While 20E stimulated Vg-synthesis in partially-fed ticks, yolk-uptake did not occur. All chelicerate arthropods lack a follicular cell layer that, in insects, serves as a physical barrier to yolk-uptake (Kaufman, 1997). Thus, uptake of Vg in ticks may be prevented in one of several ways:

(1) 20E-treated tick ovaries have not yet begun to synthesize the VgR, which is necessary for yolk-uptake. This explanation seems unlikely, as in Chapter 3 the VgR for *A. hebraeum* was detected in small partially-fed ticks, indicating that the potential for Vg-uptake is present.

(2) Some other cellular machinery necessary for endocytosis or sequestration of yolk, such as clathrin, has not been synthesized by the ovary. Oocytes in partially-fed ticks below the critical weight are only beginning the late previtellogenic growth phase, and a large increase in protein synthesis occurs only at the end of this phase with the proliferation of the rough endoplasmic reticulum (Diehl et al., 1982). Identifying the presence of clathrin by immunocytochemistry could indicate the level of competence of developing oocytes to begin yolk-uptake. Completing the blood meal may offer an additional signal to advance this stage of oocyte development, and thus allow additional endocytotic machinery to be synthesized. Indeed, in mosquitoes, JH exposure is required for previtellogenic oocytes to become competent to begin yolk-uptake; JH seems to stimulate clathrin synthesis in these oocytes

(Raikhel, 1992). While JH does not appear to have a similar function in ticks, another hormone released by replete female ticks may allow previtellogenic oocytes to become competent for yolk-uptake. Methods for identifying this 'egg development factor' are described below.

(3) The Vg synthesized by fat body of 20E-treated ticks is not recognized by the VgR. The profile of Vg polypeptides found in the fat body of 20E-treated partially-fed ticks was slightly different from that of engorged tick fat body (Chapter 3); in particular, the 211 kDa polypeptide (specific to anti-Vg148 antibodies) was not detected. Under the treatment conditions described here, it is possible that an incomplete form of Vg was synthesized in 20E-treated partially-fed ticks—one which was not recognized by the VgR. Analysis of post-translational modifications to the Vg molecule, such as changes to the size or conformation of the protein, or the addition of lipid, carbohydrate, phosphate, or haem components, may offer insight into this problem. In addition, if the VgR is further characterized (see below), the binding of the 20E-induced Vg to VgR may then be compared with the binding of Vg purified from engorged ticks to VgR.

As demonstrated by my research, and that of Lunke and Kaufman (1993), a signal other than the vitellogenic hormone seems to be necessary to stimulate yolk-uptake in *A. hebraeum*. Lunke and Kaufman (1993) transplanted partially-fed tick ovaries into the haemocoel of engorged ticks; eight days later, the partially-fed ovaries had accumulated yolk to the same degree as engorged tick ovaries, indicating that all of the required signals for complete vitellogenesis are (1) haemolymph-borne endocrine factors, and (2) present in engorged ticks. These authors also attempted to inject partially-fed ticks with haemolymph from engorged ticks undergoing vitellogenesis. Although there was no apparent effect on yolk-uptake in partially-fed ticks, multiple injections of engorged tick

haemolymph might be necessary by maintaining the concentrations of the hypothesized haemolymph-borne factors which allow yolk uptake to occur. If egg development can be induced in partially-fed ticks to the same degree as engorged ticks, further attempts could be performed to identify the hormones involved.

Although I have isolated a Vt-binding protein from tick ovaries (Chapter 3), whether this protein is the VgR remains uncertain. Further studies that involve the purification of this protein and more detailed analysis of its Vt-binding kinetics are necessary. Identification of any similarities that the 86 kDa protein may have with members of the LDL receptor family also remains an interesting prospect; if this is the *A. hebraeum* VgR, it is the smallest VgR reported to date. In addition, the 86 kDa protein does not lose its capacity to bind Vt after being treated with reducing agents, whereas members of the LDL receptor family are inactivated by reducing agents (Sappington and Raikhel, 1998). This could be a novel VgR.

Finally, the identity of the ecdysteroidogenic neuropeptide described by Lomas et al., (1997) from engorged *A. hebraeum* should be identified. Several authors have previously described the roles of neurohormones in tick vitellogenesis (Chinzei et al., 1989; Chinzei and Taylor, 1990; Shanbaky et al., 1990a, 1990b; Chinzei et al., 1991; Taylor et al., 1991a, 1991b; Chinzei et al., 1992). The experiments of Chinzei and Taylor (1990) and others demonstrated that the hormone from the synganglion of *O. moubata* that regulates vitellogenesis is a peptide; the neuropetide described by Lomas et al. (1997) may be homologous to the VIF of *Ornithodoros*.

Based on the results of experiments reported in this thesis, as well as past research, I propose the following mechanism for the control of vitellogenesis in *A. hebraeum* (Fig. 6.1):

A factor from the spermatophore ('engorgement factor'; Weiss and Kaufman, in preparation), transferred to the female's seminal receptical (SR) during mating, allows the female to feed past the critical weight and completely engorge (1). The site of action of this 'engorgement factor' is unknown, but it could act directly on the synganglion (SyG) of the female. Completion of the blood meal (2) causes the release of an endocrine factor, 'egg development factor' (EDF), which allows egg development to enter the late previtellogenic growth phase (3a); in addition, the blood meal stimulates the release of VIF from the synganglion (3b). VIF acts on the epidermal cells to initiate ecdysteroid (ES) synthesis. Ecdysteroids released from the epidermal cells then act on the fat body to stimulate Vg-synthesis (4). Vg travels through the haemolymph and is accumulated by oocytes that have reached the late previtellogenic stage of development (5).

In conclusion, the research presented here has added considerably to our understanding of the endocrinology of tick vitellogenesis. As suggested above, future research in this area should be directed at (1) identifying the ultimate hormone that stimulates release of the vitellogenic hormone, (2) elucidating the signal or signals required for complete egg maturation in engorged ticks, (3) characterizing the modifications of Vg that occur during its synthesis in the fat body, and (4) establishing the identity of the 86 kDa ovary polypeptide as the VgR *A. hebraeum.*

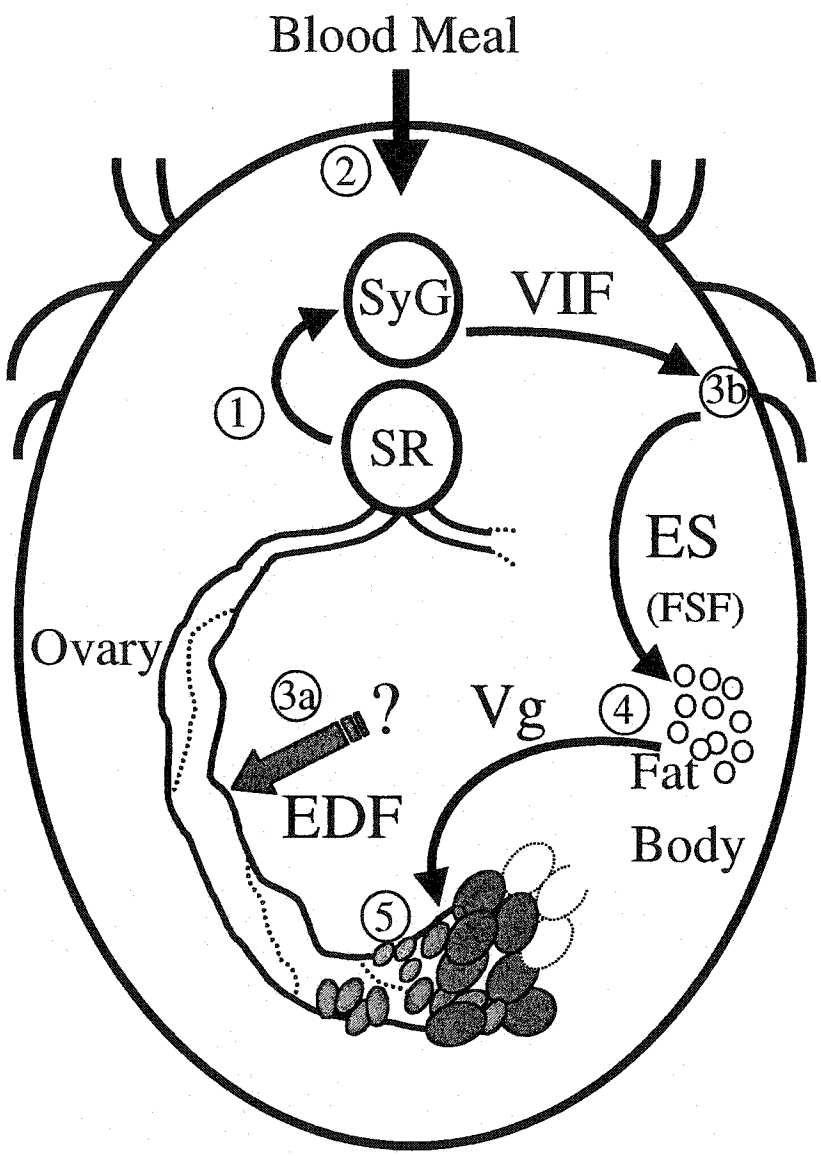

Figure 6.1. Proposed model for the hormonal control of tick vitellogenesis. See text for description of endocrine events (1-5). EDF) egg development factor, ES) ecdysteroid, FSF) fat body stimulating factor, SR) seminal receptical, SyG) synganglion, Vg) vitellogenin, VIF) vitellogenesis inducing factor.

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