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Hormonal regulation of metamorphosis and reproduction in ticks

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TABLE OF CONTENTS

1. ABSTRACT

The presence of a "status quo" hormone like JH has not been found in ticks. The most advanced understanding of tick endocrinology is associated with female reproduction, where the sequence of the first messages for storage proteins (vitellogenin (Vg) and carrier protein), the Vg receptor, and male peptidic pheromones were recently reported. The current consensus model suggests that ecdysteroids from the epidermis regulated by a putative peptidic ecdysiotrophic hormone from the synganlion initiates the expression of the Vg messages in fat body and midgut. Vg protein, secreted into the hemolymph, requires an ovary Vg receptor to be absorbed by oocytes. Male pheromones transferred into the female genital tract during mating initiate blood feeding to repletion and vitellogenesis. The work so far on tick endocrinology is limited by the paucity of identified hormones and the small number of studies on a few tick models. The role of storage proteins in the evolution of hematophagy is discussed.

2. INTRODUCTION

Much of what we know about the molecular endocrinology of the Arthropoda has been based on studies of insects. To a lesser extent, but still significant, has been the work on the Crustacea, making the Mandibulata by far the main research focus. The emphasis on the Insecta has been based on a number of practical considerations which include their importance as a pest of agriculture, their significance in the transmission of animal and human diseases, the ease in working with insects in the laboratory including their relative large size, the separation of the body into clearly delineated segments (i.e., head, thorax and abdomen), and the availability of research funding. Insects are also interesting because they represent the apex of arthropod phylogeny and are a highly diverse group of animals that have developed many different life strategies. Because of the advances that have been made in understanding the endocrinology of insect larval development, metamorphosis and reproduction and the apparent similarities in these mechanisms between insects and the Crustacea, the most common working hypothesis

has been that the regulatory mechanisms of Arthropods in general including mites, ticks, and other Chelicerata must be the same as the Insecta. This conclusion, however, is based on similarities in the morphology of the Arthropoda; the fact that they feed, grow and shed their cuticle in developmental stages and instars; they undergo metamorphosis from a juvenile to an adult stage; and the adults typically mate and lay eggs. As we will demonstrate, this conclusion is probably incorrect. This chapter will address what we know about the molecular endocrinology of tick development and reproduction in the brief context of the well studied Mandibulata. With the recent release of the first tick genome from *Ixodes scapularis* and developing new sequencing technologies like 454 sequencing and related, our understanding of tick endocrinology will likely change rapidly in the next few years.

Ticks are ancient acarines comprised of two major families, the Ixodidae (hard ticks) and Argasidae (soft ticks), which appear to have evolved during the Cretaceous period. The class Arachnida which includes mites and ticks diverged from the Insecta in the Paleozoic Era. Considering the substantial evolutionary time after the divergence of insects, substantial differences might be expected between these two groups. Ticks also are obligate blood feeding ectoparasites. In each case where the evolution of hematophagy has been studied in the Animalia, it appears the adaptation to blood feeding has been a unique event peculiar to the group from which it was adapted. The evolution of hematophagy in ticks may have occurred prior to the divergence of their chelicerate and mandibulate ancestors, which would suggest that ticks may have a number of atypical adaptations distinct from the better-known insects, maybe even for fundamental regulatory processes. In contrast to blood feeding insects, ticks also undergo long periods of starvation between blood meals, often extending months and even years. How ticks deal with these challenges appears to be unique compared to blood feeding insects. This chapter will highlight these unique tick mechanisms and show that ticks are not merely "another kind of insect" in how they regulate their development and reproduction.

Significant strides in the advancement of our knowledge of tick endocrinology have been recent and are represented by a relative few number of studies. Although some aspects of development like reproduction appear to be known with some certainty but admittedly on only a few select model systems, others like the regulation of development and metamorphosis are a black box and we have much more to learn. Included in this discussion will be a consideration of whether ticks have juvenile hormone and what we know about the regulation of tick metamorphosis. Also recent advances in the role of male protein pheromones and ecdysteroids in the regulation of blood feeding and reproduction will be discussed and will include recent research on the molecular biology of the tick vitellogenin receptor and storage proteins, the latter of which appear to be critical to hematophagy and reproduction.

3. TICK ENDOCRINE SYSTEM

3.1. Insects

Our most complete understanding of the arthropod endocrine system and the role of hormones in the regulation of growth, molting, metamorphosis and reproduction is that for insects. Two major hormones regulate these developmental processes— (a) the ecdysteroids which are synthesized from dietary steroids and (b) the juvenile hormones (JHs), which are represented by a number of different types (JH 0, JH I, JH II, JH III, JH III bisepoxide, etc.) and are synthesized *de novo* (1-6).

Ecdysteroids titers are regulated by the peptidic hormone, PTTH (prothoracicotropic hormone). PTTH is synthesized by neurosecretory cells in the insect brain and released from the corpora allata (CA) into circulating hemolymph. The CA is a neurohemal organ and part of the stomatogastric nervous system located posterior to the brain in the insect head. The appearance of circulating PTTH in the hemolymph promotes the release of the penta-hydroxylated prohormone, ecdysone, from the insect prothoracic gland. Ecdysone is then further oxidized to 20-hydroxyecdysone (20-HE) by peripheral tissues, and 20-HE initiates molting and regulates a variety of other insect functions.

JH is synthesized and released from the CA. The synthesis of JH is regulated by peptidic hormones, the allatotropins and allatostatins, synthesized in the insect brain. Insect systems are unique in that the concentration of JH in the hemolymph is regulated by dynamic changes in both the rate of JH synthesis and release from the CA and hemolymph degradation by a specific JH esterase (7-9). The degradation process is controlled by JH sequestration to highly specific hemolymph JH binding protein in the Lepidoptera and other insects (7-12), which protects the hormone from degradation by non-specific esterases, epoxide hydrolases, monooxygenases and glutathione transferases. The balance between the rate of synthesis and degradation of JH allows for the appearance and rapid disappearance of the hormone at precise times relative to food availability, photoperiod, and changing seasonal conditions. The precise regulation of development is critical to insect survival and reproduction but at the same time provides maximum physiological and developmental plasticity.

Our best understanding of the role of hormones in the regulation of arthropod development is without question in the Insecta and especially within the Lepidoptera (the moths). This was made possible because of research contributions by many different laboratories and at least a generation and a half of scientific effort which is continuing today. JH is known as the "status quo" hormone (3-4), because its presence during larval development prevents metamorphosis to the adult stage. The regulation of larval-larval molting and the timing for metamorphosis is regulated by a balance between the levels of JH and the concentration of ecdysteroids (1-2, 6, 9). Ecdysteroids in the presence of JH triggers molting from one larval instar to the next (the maintenance of "status

quo"), while ecdysteroids in the absence of JH during the last larval stadium, triggers molting to the adult stage in the case of hemimetabolous insects or molting to the pupa for the holometabola. High levels of JH esterase activity during the first half of the last stadium and after the cessation of JH synthesis removes all traces of JH from the insect; then the presence of ecdysteroids in the absence of JH initiates cellular reprogramming which leads to metamorphosis (7-9, 13).

In most insects that have been studied, juvenile hormone also appears to be the female gonadotropic hormone (7-9). The synthesis and release of JH in the female adult, initiates the synthesis of vitellogenin, a glycolipostorage protein that is secreted into the insect hemolymph from the fat body and absorbed as vitellin in developing oocytes. One exception to this model for the regulation of insect egg development is that of mosquitoes where vitellogenesis is regulated by ecdysteroids (14). After adult eclosion, JH from the CA promotes host seeking behavior in female mosquitoes, promotes ovary development to the resting stage and allows the fat body to be responsive to ecdysteroids. Once a blood meal is taken, ovarian ecdysteroidogenic hormone (OEH) (15) is released from the brain, which stimulates the ovary to synthesize ecdysteroids. Ecdysteroid in turn initiates the production of vitellogenin by the fat body.

3.2. Crustacea

The Crustacea in many ways are like insects in respect to their regulation of development and reproduction but with a few exceptions. For example, it appears that the Crustacea are unable to synthesize the C10,11 epoxide found in JH (16-21). In this case, methyl farnesoate (MF) has the approximate function of JH in the Crustacea. MF promotes the production of ecdysone, reduces the duration of the intermolt period and may be involved in the regulation of metamorphosis in the Crustacea, although the exact understanding of this process is not as advanced as in the case for insects. Methyl farnesoate as in insects regulates the process of vitellogenin synthesis and yolk deposition.

3.3. Ticks

Our knowledge of the acarine endocrine system is in its infancy as compared to that for the mandibulata and in comparison to what we know about ticks, we know essentially nothing about mites. One of the historical problems with tick endocrinology research and this is also true for mites, is that research often has been misdirected by the assumption that ticks regulate their larval, nymphal and adult development by the same hormones and with the same mechanisms as that of insects. It appears that these assumptions may have been a mistake, at least in part.

Our knowledge of the neuroendocrine system of ticks is based mostly on histological investigations (reviewed by Sonenhine (22)). The prominent aspect of the central nervous system of the tick is the synganglion, homologous to the brain and ventral nerve cord of insects. The synganglion is located in the center of the body in the tick hemocoel. Pedal nerves from the synganglion innervate the walking legs. The retrocerebral organ complex (RCO) and associated synganglion neurosecretory cells of the tick are most likely the counterpart to the corpora cardiaca (CC)-CA complex of the insect stomatogastric nervous system. However, the function of the RCO in ticks is unknown, and no neuroendocrine products that play a direct role in the regulation of molting, metamorphosis and reproduction have been definitively identified from this complex. It has also been hypothesized that the synganglion perinerium can serve as a neurohemal organ. In addition, lateral segmental organs located in the lateral nerve plexus between the pedal nerves have been assumed to be the site of juvenile hormone biosynthesis, primarily based on the abundance of smooth endoplasmic reticulum in these glands and the histological similarity of these organs to the insect corpora allata. The evidence is clear that ticks can synthesize 20-HE in the epidermis (23-26), and this hormone like in insects can regulate molting. It also appears that the tick synganglion like the case with the insect brain, produces a peptidic hormone that regulates ecdysteroid biosynthesis in the epidermis, although the most definitive proof of this is limited to a single scientific paper and tick species and the protein sequence has not been determined (24).

4. DO TICKS HAVE JUVENILE HORMONE

The general assumption has been that ticks have JH and regulate their development, metamorphosis and reproduction with JH like in insects (reviewed by Obenchain and Galun (22) and Sonenshine (27)). Venkatesh *et al.* (28) and Roe *et al.* (29) measured low levels of JH esterase and JH epoxide hydrolase activity during *D. variabilis* nymphal and adult development, and Kulcsár *et al. (*30), Sonenshine *et al.* (31) and Lomas *et al.* (32) described proteins in *D. variabilis* and *Amblyomma hebraeum* that bound JH. However, these studies were equivocal in answering the question, do ticks have juvenile hormone. Enzymes that are capable of metabolizing the epoxide or methyl ester of JH but are not necessarily JH specific can be found in most if not all biological systems. What has been needed to prove that ticks have JH is the direct identification of the hormone. Connat (33) discovered JH-positive immunoreactive material in ticks but was unable to definitively identify JH using HPLC-coupled radioimmunoassays in hemolymph from *Rhipicephalus (Boophilus*) *microplus*. Surprisingly until recently, little effort was made to determine whether ticks had JH using direct methods of detection.

The current view is that ticks do not have JH. However, this is based on a single study to find juvenile hormone in a hard and soft tick species (*D. variabilis* and *Ornithodorus parkeri*, respectively), using three different direct detection methods. Each approach showing no JH in ticks will be discussed. Research examining the effects of JH, JH analogue, and anti-JH treatments on ticks will be discussed later and further support the view that ticks do not have JH.

4.1. JH biosynthesis in ticks

Pratt and Tobe (34) developed a highly sensitive assay to detect JH synthesis in insects. The assay monitors

the addition of a radiolabeled methyl group from methionine to farnesoic acid (a precursor of juvenile hormone) which produces a tritium labeled methyl ester on methyl farnesoate (MF). MF is further metabolized by a P450 to produce JH. This assay is typically conducted by incubating the CA in tissue culture media with radiolabeled methionine, the media extracted with an organic solvent, and the radiolabeled products resolved by chromatography. Using high specific activity, tritium labeled methionine, biosynthesis can be detected at extremely low levels. Neese *et al.* (35) were the first to apply this approach to determine whether ticks could synthesize juvenile hormone. When synganglia, salivary gland, midgut, ovary, fat body and muscle alone and in different combinations from *D. variabilis* and *O. parkeri* were incubated *in vitro* in separate experiments with high specific activity L- (*methyl*-^{3}H)methionine and farnesoic acid or with (1-^{14}C)acetate (another JH precursor), and the hexane-soluble products analyzed by radioHPLC, any biosynthesis of JH or its synthesis from farnesoic acid or acetate should be detectable by the appearance of radiolabled farnesol, MF or JH. Multiple life stages were examined in the studies by Neese *et al.* (35). These included for *D. variabilis*, 3 and 72 h old (after ecdysis) unfed nymphs, partially fed nymphs (18 and 72 h after attachment to the host), fully engorged nymphs (2 d after detachment from the host), 3 and 72 h old (after eclosion) unfed females, partially fed unmated females (12-168 h after attachment to the host) and mated replete females (2 d after detachment from the host). The life stages from *O. parkeri* were third and fourth stadium nymphs and females, 1-2 d after detachment. Positive controls were also conducted using the corpora allata from *Diploptera punctata*, *Periplaneta americana* or *Gromphadorina portentosa*. To prevent any possible degradation of the tritiated methyl ester of JH by either non-specific or JH specific esterases, the assays were conducted in the presence of the highly potent JH esterase inhibitor, octylthio-1,1,1-trifluoropropan-2-one (OTFP). No synthesis of farnesol, methyl farnesoate, JH I, JH II, JH III or JH III bisepoxide was detected from any tissues or at any developmental life stage examined in both tick species studied while JH III, methyl farnesoate and farnesol were detected in the positive controls. In many of the tick incubations, farnesoic acid was provided so ticks only had to synthesize the methyl ester and/or the C10,11 epoxide and yet no MF or JH was found. The synganglion preparations in all of the studies included the lateral segmental organs that were hypothesized to be the site of JH biosynthesis in ticks (reviewed by Sonenshine (22)). The lower limit of detection for (*methyl*-^{3}H)-JH and MF in these assays were also very low, 1.27 fmol per 10 tick equivalents of tissue after a 3 h incubation. *In vivo* radiobiosynthesis studies were also conducted with positive insect controls which further demonstrated that ticks did not make the common insect JHs at the detection limit of 1.27 fmol.

4.2. Can tick extracts juvenilize insects

DeLoof and Van Loon (36) developed a sensitive insect bioassay for juvenile hormone, known as the *Galleria* pupal cuticle bioassay. In this assay, an organic insect extract is mixed into oil and molten wax and then applied to a small area of the pupal epidermis of the wax moth after removal of the cuticle. The wax is allowed to harden and the insect to develop to the pharate adult stage. As discussed before, JH in insects is a "status quo" hormone. Therefore, the presence of JH on the epidermis of the pupa should result in the retention of darkened wrinkled pupal cuticle in the pharate adult. The interesting aspect of this assay is that any hormone that might produce a juvenilizing effect on insect cuticle irrespective of the structure would be detected by this method. Using the *Galleria* bioassay, no compounds with juvenilizing activity was detected in 10 and 15 d old eggs, unfed larvae, unfed nymphs and partially fed adult females of the American dog tick, *D. variabilis (*35). The lower limits of detection were 28 pg for the detection of JH I, 28 pg for JH II and 980 pg for JH III per gram of tick tissue. Neese *et al.* (35) also found that synganglion from blood-fed second instar nymphs when transplanted into fed last instar nymphs of *O. parkeri*, did not inhibit tick metamorphosis, further arguing that regardless of the structure, ticks did not have a "status quo" hormone. The ticks molted normally to the adult stage and the adult females bore a genital pore not found in nymphs.

4.3. EI-GC-MS identification of JH in ticks

One of the most definitive methods used in insects to indentify juvenile hormone, has been selective ion, capillary gas chromatography-coupled mass spectrometery (EI GC-MS). In this method, JH is usually partially purified from insect hemolymph and chemically derivatized to produce an ethyl-d_3–methoxyhydrin. Using the separation power of capillary gas chromatography coupled with selective ion monitoring, the insect JHs can be identified with a high degree of certainty and with lower detection limits that permit the detection of JH at levels normally found in insect hemolymph or other tissues. This same approach was used to determine whether the common insect JHs were present in the hemolymph from partially fed, unmated (forcibly detached 7 d after attachment) *D. variabilis*; mated, replete (allowed to drop naturally) *D. variabilis* females; and on mated, replete (1-2 d after detachment) *O. parkeri* females. These life stages were chosen since it was hypothesized that JH in ticks initiated egg development at this time; and because large quantities of hemolymph could be obtained from these stages. Nymphal ticks were too small to collect enough hemolymph for this analysis. The internal standard, JHIII-ethyl-d_3–methoxyhydrin (*m*/*z* 76 and 239) was detected in the tick assays at a retention time of 10.32 min. In the analyses of tick hemolymph, no JH I, JH II, JH III, JH III bisepoxide, farnesol or methyl farnesoate were detected. None of the tick samples contained any ions diagnostic of the common insect JHs, farnesol or methyl farnesoate where the MS sensitivity was 1.6. pg in the scan mode from 40 to 300 AMU and 750 fg in the SIM mode for fragments with *m*/*z* ratios of 76 and 225.

4.4. Conclusion

It is difficult to prove the negative and the number of studies to look for the insect JHs in ticks is minimal with only a single comprehensive effort using direct detection. However, the work of Neese *et al.* (35)

was unable to find any of the common insect JHs in both hard and soft ticks by radiobiosynthesis and EI GC-MS. In addition, the *Galleria* bioassay showed that regardless of structure, there were no compounds in ticks that would juvenilize insect epidermis. Since JH so far has only been identified in insects and MF in the Crustacea, the existence of the insect juvenile hormones in ticks is in question. Certainly, more research is needed to address the question of whether ticks can synthesize JH or its precursors, and recent efforts in tick genomics and advances in metabolomics may eventually provide new approaches to address this issue.

5. REGULATION OF TICK METAMORPHOSIS

As discussed before, the regulation of larval-larval molting in insects and the initiation of metamorphosis is well understood and is simply a balance between two hormones. Ecdysteroids in the presence of JH, initiates molting from one larval instar to the next while the presence of ecdysteroids in the absence of JH, initiates metamorphosis. The most convincing evidence in insects used to demonstrate the larval "status quo" function of JH involves the topical application of JH or JH analogues to demonstrate delayed metamorphosis and/or supernumerary molting; and the rescue with JH or JH analogs of anti-JH effects on molting and metamorphosis. These same experiments have been conducted by a number of investigators (37-40) in three species of ticks. The results have mostly been negative. The only study to show any effect from JH treatments was that of Khalil *et al.* (39) where JH I delayed nymphal molting by 9-13% but did not produce supernumerary molts when applied on the day of engorgement in the camel tick, *Hyalomma dromedarii.* These results are far from compelling in arguing for a role for JH in the regulation of metamorphosis. Leahy and Booth (39) could not rescue precocene induced nymphal molt-mortality with JH in the tick, *Argas persicus*. As discussed before, Neese *et al.* (35) also could not juvenilize last instar nymphs of *O. parkeri* when they were the recipient of transplanted brains including putative endocrine glands from next to last instar nymphs. With no direct evidence of JH in ticks in general and no clear indication of a "status quo" effect from the topical application of JH, the possible role of hormones in the regulation of larval/nymphal development and metamorphosis is unknown. It also is difficult to develop a plausible hypothesis for how ticks might regulate their larval and nymphal development. Some possibilities might include a fixed genetic programming triggered simply by the presence of ecdysteroids and without the plasticity and developmental polymorphisms demonstrated in larval insects. Alternatively, development may be regulated by different levels of ecdysteroids or "status quo" hormones in ticks that have not yet been identified and that may or may not be produced by the same synthetic pathway as that for the insect JHs. Because of the small size of larval and nymphal ticks and the central location of the synganglion, delineating the developmental mechanism for growth and metamorphosis using ligation and transplantation, parabiosis and homogenate injection experiments will be challenging. Recent advances in genomics will most likely be our best hope in understanding the tick system.

6. REGULATION OF TICK FEMALE REPRODUCTION

6.1. Historical perspective

Prior to the work of Neese *et al.* (35) discussed earlier, the strongest evidence suggesting that ticks might have JH was the studies of Pound and Oliver (41). In this research, the application of precocene, an anti-JH compound, inhibited egg production in adult females of the soft tick, *O. parkeri*. When the ticks were subsequently treated with 1 microgram of JH III, the effects of the precocene were reversed and egg development was re-started. These results suggested that JH regulated egg production in ticks. However, the experiments were not entirely clear. For example, the application of juvenile hormone greater than 1 microgram in a dose dependent manner in these same rescue experiments decreased egg production. JH is not an especially toxic compound, and increasing levels of JH are usually positively correlated with increased levels of yolk deposition in insects, not a decrease. Also, when similar experiments were conducted in three other tick species, *Argas persicus* (38), *R. microplus* (42) and another species of *Ornithodoros* (*O. moubata* (43-44)), JH was unsuccessful in rescuing anti-JH effects using both precocene and another anti-JH, fluoromevalonate.

There was also other evidence that JH was not involved in female reproduction in ticks. Taylor *et al.* (45) and Chinzei *et al.* (46) found that the topical application in acetone and the injection in mineral oil of JH homologs, JH acid and JH analogs did not initiate vitellogenesis over that of the carrier while the pyrethroid, cypermethrin, induced yolk synthesis. It appeared that the synthetic pyrethroid insecticide in some way was able to initiate female reproduction, presumably by action on the nervous system and not because it mimics a tick gonadotrophic hormone. Khalil *et al.* (39) also were unable to initiate reproduction with JH I while Connat *et al.* (47) found that JH and JH analogs increased egg laying. Dees *et al.* (48), Khalil *et al.* (39) and Abdelmonem *et al.* (40) found that JH, JH analogs and precocenes had no effect on sexual attraction. Solomon and Evans (49), Mansing and Rawlins (50) and Teel *et al.* (51) found that JH analogs inhibited vitellogenesis or oviposition. These contradictory results along with no definitive reports of the direct detection of JH in ticks or evidence of a "status quo" function for juvenile hormone, suggest that egg development must be controlled by another hormone or mechanism.

6.2. Proof for the role of ecdysteroids in the regulation of egg development

At about the same time that Neese *et al.* (35) was reporting that JH could not be found in ticks, Sankhon *et al.* (52) found that ecdysteroids added to fat body organ culture from the American dog tick was able to increase the levels of the yolk protein, Vg. Although the ecdysteroid regulation of vitellogenesis is not common in insects, there is one well studied example in mosquitoes where ecdysteroids have been shown to initiate yolk protein synthesis and egg maturation (14). Friesen and Kaufman (53) later found that ecdysteroids injected into non

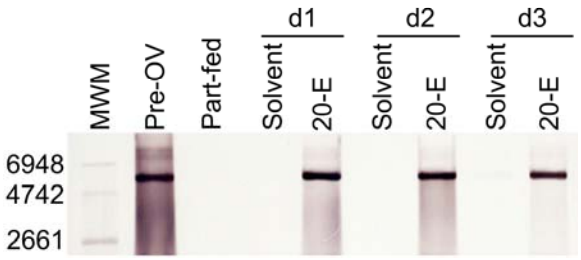

Figure 1. Northern analysis for vitellogenin from whole body of the American dog tick, *Dermacentor variabilis* on different days after injection with 20-hydroxyecdysone. The major band in the Pre-Ov lane is the Vg message. Note that the Vg message is not found in partially fed female adults or in the solvent injected controls but was present each day after the injection of ecdysteroid. MWM, molecular weight markers (units in number of nucleotides); Part-fed, RNA from partially fed, virgin females; Pre-Ov, RNA from pre-ovipositional, mated replete (vitellogenic) females; 20E, RNA from partially fed (virgin) females injected with 20-hydroxyecdysone.

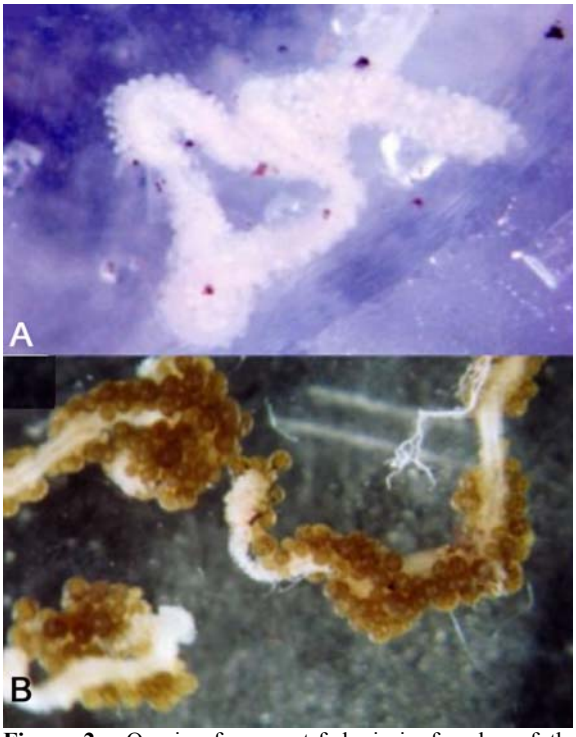

Figure 2. Ovaries from part-fed virgin females of the American dog tick, *Dermacentor variabilis*, after the ticks were injected with solvent (A) or 20-hydroxyecdysone (B) 4d after attachment to the host and then examined 4d after injection. Note that fully developed, brown (vitellogenic) eggs (45 X magnification) are present in the ticks injected with ecdysteroid (B) while those injected with solvent only (A, 37.5 X magnification) showed no development.

vitellogenic fed female adults of *Amblyomma hebraeum* forcible detached from the host, released yolk protein into the hemolymph. Recent advances have been made in the molecular biology of tick storage proteins including the female specific yolk protein, vitellogenin (54-55), which has led to the most convincing argument yet that ticks regulate egg development with ecdysteroids and not JH. Thompson *et al.* (54-55) were able to sequence vitellogenin (Vg) from the American dog tick and developed methods to monitor the expression of the Vg gene. In addition, Thompson *et al.* (54) developed a novel bioassay system whereby they could inject partially fed ticks still attached to a rabbit host and examine the effect of hormone injections on blood feeding, Vg gene expression, Vg protein synthesis and secretion into the hemolymph, and the development of oocytes in the ovary. In these studies, they found that the injection of 20-hydroxyedysone (20-HE) into partially fed (virgin), non-vitellogenic females of *D. variabilis* attached to the rabbit host at reasonable physiological levels initiated expression of the Vg gene 1-3 d after treatment. This was noted as an increase in Vg message in whole tick preparations (Figure 1), increased levels of Vg protein in the hemolymph as determined by electrophoresis (data not shown), and Vg uptake by the ovary producing fully developed eggs (Figure 2). The injection of 20-HE into part-fed virgin females produced ovaries similar in weight to that of a mated, replete female. Microscopic examination of the vitellogenic ovaries indicated that almost all of the eggs could be classified as category 5 on the Balashov scale (large and brown). Other eggs were category 3 or 4 and filled with yolk granules. In other experiments, they found the Vg message appeared only after mating and feeding to repletion and during oviposition and was positively correlated with increased hemolymph Vg protein levels, hemolymph ecdysteroid levels and egg maturation (23,56-57). Injection of partially fed (virgin) females of the American dog tick with 1,000 ng of JH III per tick in these studies had no effect on oocyte development. This was the most complete work to date showing that ecdysteroids and not JH are directly responsible for the initiation of Vg gene expression and the appearance of the Vg message, Vg protein synthesis and secretion into the hemolymph, and the uptake of Vg in the ovary to produce fully developed eggs and ovaries. It was interesting in the studies of Thompson *et al.* (55) that the Vg message was found by Northern analysis in fat body, midgut and ovary, suggesting multiple possible tissues sources for hemolymph and egg yolk protein. These studies also showed that ecdysteroids alone could produce fully developed eggs in partially fed, virgin females of *D. variabilis* attached to the host. However, elevated levels of 20-hydroxyecdysone did not initiate blood feeding to repletion, suggesting that other factors control feeding separate from vitellogenesis.

7. MOLECULAR BIOLOGY OF TICK STORAGE PROTEINS

Two different classes of storage proteins, carrier protein (CP) and vitellogenin (Vg), were recently sequenced in full for the first time in the American dog tick, *D. variabilis (*55, 58) (Table 1). CP is the predominant protein found in every stage of both male and female tick development except in the egg. The protein is especially predominant in hemolymph and salivary gland as characterized in female adults of the American dog tick

Table 1. Sequence analysis of Vg1, Vg2, CP and VgR from *Dermacentor variabilis*

Protein	nt Acc#	Bp	Protein Acc#	AA	MW[1]	Match %ID[2]
Vg1	AY885250	5744	AAW78557	1843	206,468	*Rhipicephalus microplus* partial Vg – 73.4%
Vg2[3]	NA	2186	NA	673	NA	*Rhipicephalus microplus* partial Vg – 18.0%
CP	DQ422963	4951	ABD83654	1547	176,087	*Caenorhabditis elegans Vg* – 14.3%
VgR	DQ103506	5673	AAZ31260	1798	196,732	*Periplaneta americanum VgR* - 33%

[1]Theoretical molecular weight calculated using the mature protein sequence. [2]Highest % identity found. Search with BlastP at NCBI; ClustalW alignment used to report % identity[3]Incomplete sequence.

and acts as a storage protein binding heme, lipids and carbohydrates (59-61). After mating in adult females, however, a replacement storage protein, vitellogenin, appears. This protein shares a common ancestor with CP (Figure 3) and both proteins are related to storage proteins from other arthropods. However, the tick vitellogenin and CP are unique within the Arthropoda in their ability to bind and transport heme. The importance of these two proteins in the evolution of hematophagy will be discussed. Vitellogenin is synthesized by the female fat body and midgut and possibly other tissues and becomes the major storage protein, vitellin, in eggs.

7.1. Tick vitellogenin

Our knowledge of the molecular biology of tick vitellogenins is limited to the sequence of a single full-length Vg1 message isolated from the fat body of adult females of *D. variabilis* (55) (Table 1). The Vg cDNA is 5744 nt in size with 19 residues in the poly-A tail. The 5' untranslated region is relatively short, only 42 nt while the 3' non-coding region extends 151 nt from the stop codon to the start of the poly-A tail. Conceptual translation of the nucleotide sequence for Vg yields 1843 aa (AAW78557) with a calculated molecular weight of 208 kD. Analysis by SignalP indicates that an 18 aa signal sequence is present, as would be expected for a protein which is secreted into hemolymph. Cleavage would yield a predicted molecular weight of 206 kD. From proteomics studies, the molecular weight of Vg1 from *D. variabilis* is in the range of 320-486 kD (54,59-62). Vitellin (Vn), Vg after deposition in the egg, has been reported in the range of 370-480 kD (63). Although there has been considerable variation in the reported sizes of native Vg and Vn in the American dog tick, it is apparent that in all of these reports, the molecular weight exceeds that predicted from translation of the actual Vg message. If *Dv* Vg exists as a dimer in hemolymph, the predicted molecular weight would be 412 kD (2 times 206 kD). This is in reasonable agreement with that for Vg and Vn previously reported in the literature for this species (54, 59-63). Although we cannot confirm the exact oligomeric form of Vg and Vn in *D. variabilis*, Vg is known to exist in oligomeric forms in its native state in other Arthropods (64), and the same seems to be true for the American dog tick. The complete coding sequence of Vg is not available from any other ticks. In *D. variabilis*, Vg1 demonstrated a high content of Leu and Val (12.9 and 12.1%, respectively) followed in decreasing abundance of Pro, Lys, Thr, Glu, Tyr and Ser (7.9, 7.8, 7.5, 7.2, 7.2 and 6.6%, respectively). The abundance of the remaining aa was 5.8% or less.

Sappington and Raikhel (64) noted that most insect vitellin proteins have a single cleavage site, which generates two protein subunits. The cleavage signal, RXXR, is found in *Dv* Vg at amino acids 465–468. The Vg monomers of most insects are composed of one large (>150 kD) and one small (≤65 kD) subunit (reviewed in (65-67)) derived from the cleavage of a single precursor in the fat body (68-73). Cleavage at the RXXR cleavage signal (aa 465-468) of *Dv* Vg1 would produce subunits with predicted molecular weights of 49.5. and 157 kD, consistent with that found in insects. Exceptions in the insects include the Vgs of higher Hymenoptera (suborder Apocrita) (72, 74-75) and two species of whitefly (Homoptera) (76), which are not cleaved. Vg in *Ixodes scapularis* contained 8 subunits with molecular weights from 48 to 145 kD (77) while 7 subunits were reported for Vg (62) in *D. variabilis*. While *Dv* Vg and Vn migrate as a single protein on non-denaturing PAGE, *Dv* Vn on SDS-PAGE migrated as seven major bands (54-55). The subunits were 210, 172, 157, 111, 76.2, 58.7, and 50.8 kD. The subunits with observed molecular weights of 50.8 and 157 kD were predicted (49.5 and 157 kD, respectively) based on the RXXR cleavage site at amino acids 465–468. A third 210 kD protein apparently arises from the uncleaved monomer which had a predicted molecular weight based on the sequence and translation of the cDNA of 206 kD (after removal of the signal sequence); uncleaved Vg protein also occurs in some insects (72, 74-76). Other known dibasic endoprotease recognition motifs of the sequence R/K X R/K R in addition to RXXR (78) were not found in *Dv* Vg1. However, 8 KXXK sites homologous to KFKKAN found in Vg of the bean bug, *Riptortus clavatus* (79), were present in *Dv* Vg1. These sites do not appear to explain the remaining Vg proteins found on SDS-PAGs, and the mechanism for their production is unknown.

Two domains of interest can be found in Vg1 from *D. variabilis*, a lipoprotein domain, which spans amino acids 34–721 and a von Willebrand factor type D domain near the carboxy-terminus of the protein from amino acids 1485–1655. Vitellogenins in general are predicted to contain both of these domains (80) in about the same positions as described for the native Vg protein of *Dv*. Among the insect Vg genes there is a highly conserved GL/ICG motif close to the C-terminus (81-82). Interestingly, the Vg partial sequence from *R. microplus* (accession number AAA92143) and that from *D. variabilis* contain a variant sequence, GLCS. The significance of this difference is not known.

Dv Vg1 aligns well with GP80 from *R. microplus*, having 81% identity at the nt level and 80.2% similarity and 73.4% identity at the aa level (Table 1). A radial dendrogram was developed using the full amino acid sequence of Vgs from representative insects and Crustacea (Figure 3). Included in the analysis was the Vg1 sequence from the American dog tick. In this analysis, there is a clear separation of the insect and crustacean groups as might be expected based on their known phylogeny. The

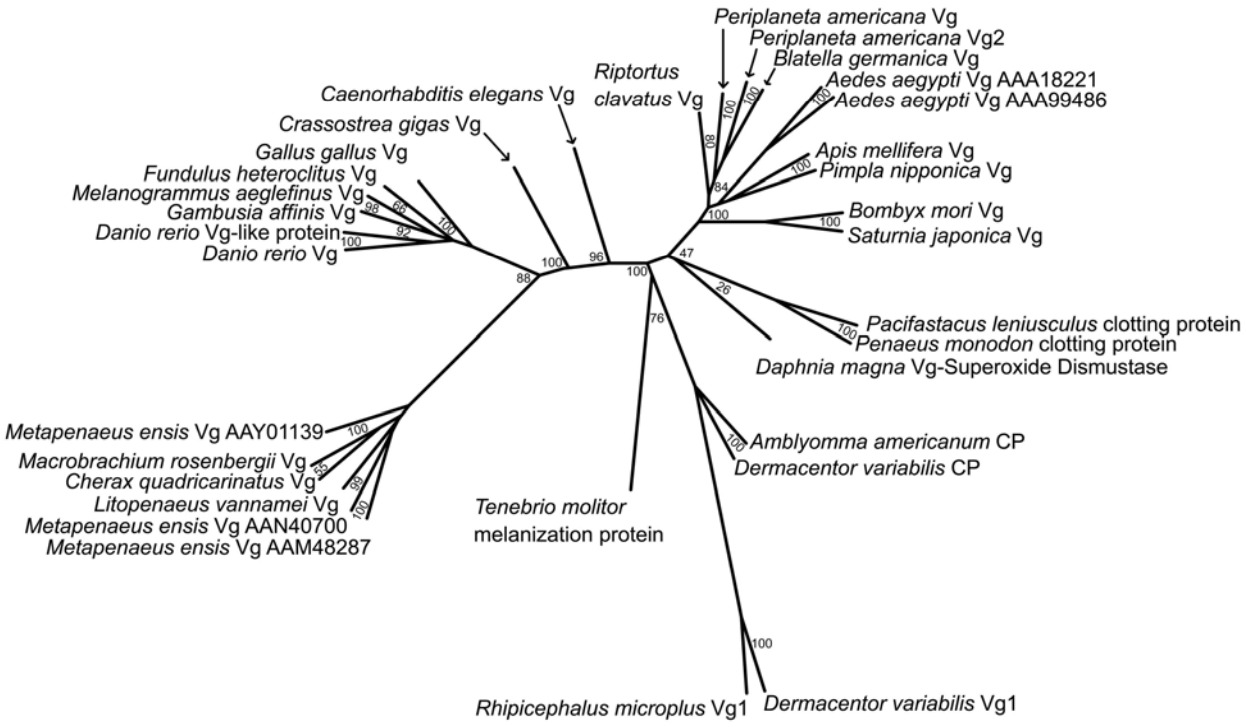

Figure 3. Unrooted phylogenetic tree of vitellogenins and Vg-similar proteins. The tree was constructed using maximum likelihood analysis; numbers at each node indicate bootstrap values. Species for which multiple Vg's are available that have not been given a distinct number (i.e., Vg1, V2, etc.), accession numbers are shown in the figure. Accession numbers for the above taxa are *Aedes aegypti* Vg AAA18221 and AAA99486, *Amblyomma americanum* CP ABK40086, *Apis mellifera* Vg NP_001011578, *Blatella germanica* Vg CAA06379, *Bombyx mori* BAA06397, *Caenorhabditis elegans* Vg BAB69831, *Cherax quadricarinatus* Vg AAG17936, *Crassostrea gigas* Vg BAC22716, *Danio rerio* Vg NP_739573, *Danio rerio* Vg-like protein CAK03619, *Daphia magna* Vg-Superoxide Dismustase BAE94323, *Dermacentor variabilis* CP ABD83654, *Dermacentor variabilis* Vg1 AAW78557, *Fundulus heteroclitus* Vg AAA93123, *Gallus gallus* Vg BAA13973, *Gambusia affinis* Vg BAD93698, *Litopenaeus vannamei* Vg AAP76571, *Macrobrachium rosenbergii* Vg BAB69831, *Melanogrammus aeglefinus* Vg AAK151157, *Metapenaeus ensis* Vg AAY01139, AAN40700 and AAM48287, *Pacifastacus leniusculus* clotting protein AAD16454, *Penaeus monodon* clotting protein AAF19002, *Periplaneta americana* Vg BAA86656, *Periplaneta americana* Vg2 Q9BPS0, *Pimpla nipponica* AAC32024, *Rhipicephalus microplus* Vg AAA92143, *Riptortus clavatus* Vg AAB72001, *Saturnia japonica* Vg BAD91195 and *Tenebrio molitor* melanization protein BAB03250.

full-length protein from *D. variabilis* as might be expected based on phylogeny sort into a separate node from the Mandibulata. Interestingly, there are 6 repeats of P (T/P)HH (K/E) (Y/P) in GP80 from *R. microplus*. At the carboxy-terminus of Vg1 from the American dog tick, there were 11 similar repeats of P (T/S)HH (K/E)Y. None of these repeats were found in the other Vg proteins examined outside of ticks. Vg is a heme binding protein in ticks (reviewed by Gudderra *et al.* (61)) but not in other animals. The mechanism of association of heme with tick Vg is not known. Even though the P (T/P)HH (K/E) (Y/P) and P (T/S)HH (K/E)Y repeats of the tick Vgs are so far exclusive to Vgs that bind heme, it appears they are not critical for heme binding. The heme binding protein, CP (discussed in more detail below), also found in *D. variabilis* hemolymph does not have these repeats. The function of the *Dv* Vg P (T/S)HH (K/E)Y repeats are currently unknown.

Thompson *et al.* (54-55) were the first to observe that the subunits of Vn on SDS-PAGs appeared as dimers which suggest the presence of multiple Vgs in ticks. This would not be unexpected considering the multiplicity of vitellogenin genes in other organisms (Figure 3). Vg2 from *Dv* has been partially sequenced (Table 1; Figure 4). The Vg2 message was found in total RNA isolated from the whole body of pre-ovipositing and ovipositing, replete mated females but not in unfed males, fed males, unfed virgin females or 7 d part-fed virgin females (Figure 5) as would be expected for this protein. Ecdysteroid injections into part-fed females attached to the host as described before also increased the expression of the putative Vg2 message (data not shown) as was also the case for Vg1 (Figure 1). Tryptic digest LC MS sequencing of purified Vg protein from tick hemolymph produced a fragment (DAMALQVVLK) that was found in the conceptual translation of the putative Vg2 sequence but not in Vg1 (Figure 4). Also, protein blast results against the GenBank database showed that the partial Vg2 sequence contains the von Willebrand factor type D domain (Figure 4), which is typically present near the C- terminal end of all known Vg proteins. Based on this evidence and the fact that the

ELFXKGHRIPAVRAGXLRGPAQGRGGLEGRLVPQGRQREAPAPERXPTXTYAY
GLEFVVSALGPLVPKAVYGHFVYGTSFDKTLKLYQLYANKVDSEYEAVVNSAL
VKSPKPSPFKTYATKKDAHYLYATSTAEVFSYYGPLKYSVEFEASPSVEQLEY
SPLTVVNSAVKKLKTRRPHTIQSSLWRQHEGYDNLPYKKYATKMENVGAYSVP
VSELPWYYAKCQEDAEAGKELVSYACKKVHWHEHRLDKFVLKVTLPTQYPSLL
LNATHKLFQTAKVLLYDKSTTEYVESPVVTEGQFLVEAVLEDLYTGVTTANVT
VHTPHFEKVTFKRLPWLRPLRPTVAYDFTSQLVSAVRKGYPFPTCVVSPYYLK
TFDNVTYPLETLKKQVSHVLVRHAVDDPEFLVLLEQKSEDAMALQVVLKNQTL
VKLTPPKDQSTYEVEVNGTLLTVDPLKSHVLQYYHNYSSQVLLYVTVHPEVAP
TLWLKVRDSDFLLAYNGSSVLVTVRSPEYRGTFAGLCGDNNKEYQYEYVTPEK
CVVTDVEDFLNAYSLEPETVLKGHVFCPYGVTLKFGTTRYGSTSYNHSSYNHT
KYNTTKNVRTSKIGKIISKITKIRGHRVDEDEVVAQVTTPECITERRKIIYEE
GKVCMSLKKVTACKRGCKPSRLSHRSSSSSAWTXEPS

Figure 4. Conceptual aa sequence of Vg2 from the American dog tick, *Dermacentor variabilis*, showing the peptide fragment (in red, end of line 8) obtained from the tryptic digest LC MS analysis of Vg protein from the hemolymph of vitellogenic females of the same species. Also, the vWF type D domain is shadowed.

purified Vn protein subunits appear on SDS-PAGs as doublets, suggests that there are at least two Vg genes in the American dog tick.

7.2. Tick Carrier Protein

DvCP sequence**.** The only published description of a complete CP mRNA sequence is that from the American dog tick, GenBank accession number DQ422963 (58) (Table 1). The message is 4951 bp with a 5' untranslated region (UTR) from base pairs 1 to 43, a TGA stop codon at bp 4685 followed by the 3' UTR, an AATAAA polyadenylation signal and a 17-adenine tail. The 5' UTR is relatively short although similar in length to that of *Dv*Vg1. Comparison of the UTR's from *Dv*CP and *Dv*Vg1 do not produce a significant alignment, however (only 13.4% identity). The 5' UTR of the lone star tick, *Amblyomma americanum*, HeLp (EF050790) herein referred to as *Aa*CP, excluding the reported vector and SMART IIA oligo contamination, is 87% identical to that of *Dv*CP (data not shown).

The theoretical 4641 bp open reading frame for *Dv*CP encodes for a protein with 1547 amino acids that has been confirmed by tryptic digest LC tandem mass spectrometry (ABD83654) from purified tick CP protein. The protein precursor contains a 15 residue secretion signal predicted by the SignalP Server. In addition, a RXXR cleavage site common to storage proteins and vitellogenins is found immediately upstream of the n-terminus of the 98 kD CP subunit (amino acids 746 to 749). Tryptic digest LC-MS/MS analysis confirmed that the protein in the tick consists of a 92 kD subunit (residues 16 to 749; 734 amino acids total) and a 98 kD subunit (residues 750 to 1547; 798 amino acids total). Three motifs in the completed *Dv*CP protein sequence occurs—a lipoprotein n-terminal domain from residues 16 to 622, a domain of unknown function (DUF1943) from residues 667 to 945 and a von Willebrand factor type D domain at the carboxy-terminus from residues 1351 to 1522. Tryptic digestion LC-MS/MS analysis also identified several glycosylated peptidic fragments, and six potential N-linked glycosylation sites are present in *Dv*CP.

Identity and Function of CP. Since the *Dv*CP message has a low identity to the vitellogenin message from the American dog tick, was found in both male and female ticks unlike the female specific Vg messages from the same species (54-55) (Figure 5), and the increase in the *Dv*CP message occurred in response to blood feeding and not from mating like that for the *Dv*Vg message, Donohue *et al.* (58) concluded that CP was not the egg yolk protein. Gudderra *et al.* (59-61) found that hemolymph *Dv*CP which was the predominant whole tick protein in male and female adults was also predominant in nymphs and larvae of *D. variabilis*. Only trace amounts were found in eggs. Since the native protein in adult hemolymph contained lipids, carbohydrates, and heme and appeared to be the predominant protein in both males and females throughout development (except in eggs), Gudderra *et al.* (59-61) concluded that CP was the major tick storage protein. An analogous protein was found in the soft tick, *O. parkeri* (59). Donohue *et al.* (58) in the analysis of the CP message found a lipoprotein n-terminal domain which is common for proteins that bind lipids, carbohydrates and metals and further supports the putative function of CP as a storage protein. The DUF1943 domain that was discovered is found in proteins with several large open beta sheets but the exact function is unclear. The von Willebrand factor type D domain (83) found in CP is likely important in multimerization of the two CP subunits. It is clear that CP is not the major egg yolk protein, but there are obvious functional similarities between these proteins including their ability to both bind heme. The mechanism of heme-binding by *Dv*Vg or *Dv*CP is yet to be determined.

Nene *et al.* (84) reported several *R. appendiculatus* tentative consensus sequences, TC1551 and TC1839, which were homologous to the n-terminus of *R. microplus* apoHeLp-B (85). These were not completed CP sequences. However, the same cleavage site was predicated by SignalP in the putative translation of CP from *Aa*CP and *A. variegatum* TC353. The alignments of *Dv*CP, *Aa*CP and *A. variegatum*, *R. appendiculatus*, *R. microplus* and *Ixodes scapularis* homologs suggest that this protein is

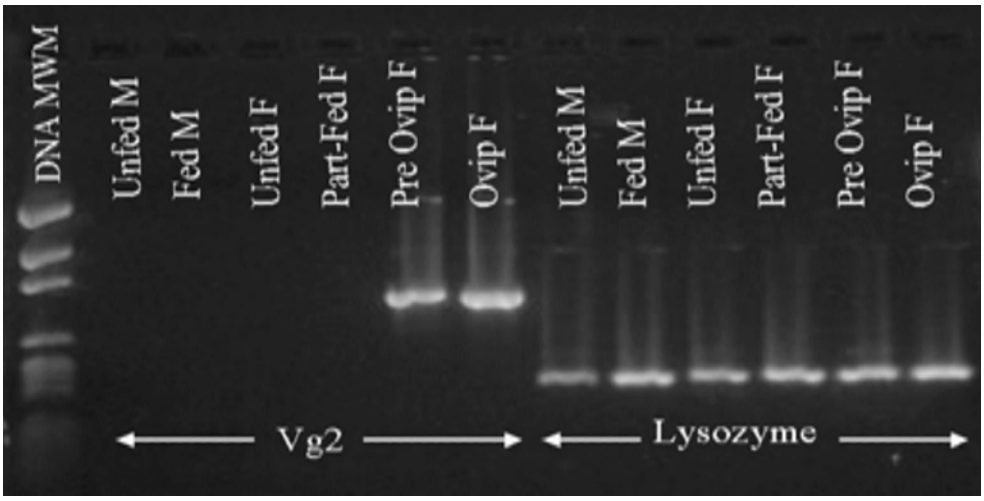

Figure 5. Agarose gel showing RT-PCR products from the American dog tick, *Dermacentor variabilis*, using Vg2 and lysozyme specific primers. Note that Vg2 mRNA is present only in replete mated females prior to ovipoistion (pre OvipF) and in ovipositing females (OvipF). No Vg2 message was found in unfed males (UnfedM), fed males (FedM), unfed virgin females (UnfedF) or partially fed, virgin females (Part-FedF). Lysozyme mRNA is present in all stages tested showing equal loading and a positive RT-PCR amplification with these primers.

highly conserved among ixodid ticks. Nene *et al.* (84) first demonstrated the similarity of AvTC353, RaTC1551 and RaTC1839 to the n-terminus data of apoHeLp-B (85). Nene *et al.* (84) also identified *R. appendiculatus* clones from the downstream portion of the CP gene. Donohue *et al.* (58) also located similar ESTs from *R. microplus* and *I. scapularis*. These alignments indicate that at least two forms of CP may exist in the same ixodid ticks.

Tissue-Specific Regulation of the *Dv*CP message is different from *Dv*Vg**.** To better understand the regulation of CP during adult development, Donohue *et al.* (58) used a 909 bp region of the CP message (bp 1279-2187) to examine its developmental regulation using Northern blots. From these studies it was apparent that the expression of CP in whole body of adult virgin females of *D. variabilis* was initiated after attachment to the host and with the initiation of blood feeding, and appeared to increase with host-attachment duration. After attachment for 6 d, expression of CP in female ticks was greatest in the fat body and salivary gland compared to the levels found in the ovary and midgut. These results appeared to be in agreement with the CP protein levels in the same tissues as reported previously (60). The appearance of both CP message and protein in the salivary gland was surprising and suggests that CP might be secreted in tick saliva. Although there is some evidence to support this view in the literature (reviewed in (58)), repeated attempts to find CP in the saliva and cement cone of *D. variabilis* were unsuccessful. Donohue and Roe (unpublished data) found that salivary glands held in organ culture, released CP protein into the incubation medium as determined by co-electrophoresis in separate lanes with hemolymph CP. However, it could not be determined whether the release was from the salivary ducts or directly from the cells into the medium. Because of the size of salivary gland tissue at the time of blood feeding and considering that CP gene expression is initiated at this time presumably to store digested products from the host blood, an argument can be made that the salivary glands based on shear bulk would be a reasonable source for hemolymph CP. Vg appears to be produced from multiple tissues but not the salivary gland since they degenerate during vitellogenesis. On the other hand, the secretion of the predominant hemolymph storage protein into saliva and its additional function in host complementation as well as serving as a storage protein would be surprising. Further work is needed to address these possible different roles for CP in ticks. Note that Gudderra *et al.* (60) reported that CP also might occur in tick coxal fluid. One of the problems with working with a highly abundant hemolymph protein like CP, is the potential of hemolymph contamination when collecting samples.

The regulation of the *Dv*Vg message was discussed before in this review. The appearance of the *Dv*Vg message is regulated by mating in adult females and not by the initiation of partial blood feeding as is the case for expression and synthesis of CP. Mating also increases the levels of 20-hydroxyecdysone, and the injection of the same hormone into part-fed, virgin ticks attached to the host resulted in increased levels of the Vg message and the production of vitellogenic eggs similar to that produced by replete, mated females. Apparently, even though *Dv*CP and *Dv*Vg may share a common evolutionary origin and have similar functions in binding lipids, carbohydrates and heme, the whole body tissue sources and regulation of their messages are different. It is interesting that blood feeding initiates the synthesis of the CP message in part-fed, virgin females, but Gudderra *et al.* (59) found that mating and blood feeding to repletion reduced the levels of CP protein and Thompson *et al.* (54) found that mating and blood feeding to repletion increased the Vg message, Vg hemolymph protein and yolk deposition in the egg.

Apparently, in the adult stage before the initiation of vitellogenesis, CP is the major tick storage protein. Then this function shifts to Vg in replete ticks as a result of mating and with increased levels of ecdysteroids.

Role of CP and Vg in heme sequestration and evolution to blood feeding. Blood meal digestion in ticks is initiated in the midgut (86-87) with the lysis of host erythrocytes presumably to release serine proteases (88). Uptake of the blood meal components by the digestive cells in the midgut is facilitated by receptor mediated endocytosis via fluid-phase endocytosis and clathrin-coated pits (86). Intracellular digestion by heterophagy, the fusion of primary lysosomes and endosomes to form secondary lysosomes, results in the breakdown of host hemoglobin. The resulting heme becomes concentrated into dense residual bodies (87) or hemosomes (89) which serve as a hematin sequestration mechanism to prevent reduction-oxidation reactions from forming harmful free radicals. During intracellular digestion, the digest cells are found along the basal lamina where heme is transferred to the hemocoel. The exact transfer mechanism is not yet clear but preliminary data suggest that a membrane-protein transporter is involved (90). Apparently, heme is then sequestered in the hemolymph by CP in a 2:1 heme to CP molar ratio (85). During vitellogenesis heme is also transferred to vitellogenin directly and/or via CP (55) with Vg serving as the major route of heme trafficking into developing oocytes. Both CP and Vg appear to be critical components for the sequestration and utilization of host heme in the absence of *de novo* heme synthesis in the Ixodidae and for the extended periods of non-feeding that occurs in ticks between blood meals or during embryogenesis. As a result, these two proteins appear to be critical to the evolution of hematophagy.

Evolutionary Relationship between CP and Vg. A phylogenetic analysis of *Dv*CP, *Aa*CP and other vitellogenins and Vg-like proteins showed that CP grouped most closely with tick Vgs. Babin *et al.* (91) previously showed that apolipophorin II/I, apolipoprotein B, vitellogenin and microsomal triglyceride transfer proteins (MTP) are derived from a common ancestor and all belong to the large lipid transfer protein (LLTP) superfamily. Smolenaars *et al.* (92) compared vertebrate and invertebrate sequences from the LLTP superfamily that all contained a large lipid transfer module or motif located toward the n-terminal and reported three major groups-apoB-like LLTPs, MTPs and Vg-like LLTPs. Based on the current analysis, CP appears to be most closely related to the vitellogenins.

8. MOLECULAR BIOLOGY OF THE VG RECEPTOR AND ITS IMPORTANCE IN VITELLOGENESIS

Only a single Vg receptor (VgR) has been sequenced from ticks (Table 1) (93). The 5673 bp transcript includes an ORF that encodes for 1798 aa with a predicted MW of 198.7 kD and a 20 aa signal peptide (after cleavage, 196.7 kD). The cleavage site is found between nt 159-160. The protein has a number of expected domains. These include 13 LDL-receptor class A ($LDLR_A$) repeats at two sites. Each ligand-binding site is followed by an EGF-like domain containing a single calcium-binding element, YWXD and LDL-receptor class B ($LDLR_B$) repeats. The arrangement of the YWXD and $LDLR_B$ repeats leads to the formation of a characteristic beta-propeller in the final product. A putative O-linked sugar domain rich in serine and threonine residues is predicted at nt positions 5056-5106. A transmembrane domain encompassing positions 5107-7172, and a cytoplasmic domain (5173-5493) are also predicted. Potential internalization signals exist at positions 5320-5337 (following the consensus FXNPXF) and 5254-5259 and 5356-5361 (LL motif) (94).

Of the Vg receptors so far sequenced, the *Dv*VgR is most closely related to that from the American cockroach, 33% identical and 48% similar. Similar matches also occur with *Leucophaea maderae*, *B. germanica* and *A. aegypti*. Of special interest, is the high level of similarity with LDL receptors from vertebrates and invertebrates. The *Dv*VgR is distinct from that of most insects where there are 13 $LDLR_A$ repeats, five in the first and eight in the second ligand binding sites. Both are different from the classical LDLRs, vertebrate VgRs and VLDLRs, which have only a single ligand binding site (95). $LDLR_A$ repeats (approximately 40 amino acids long) consists of six disulfide-bond cysteines and a conserved negatively charged SDE region that form the binding site for Vg. The beta-propeller is important in ligand release and receptor recycling. The EGF-like domain (approximately 50 amino acids with three internal disulfide bridges) serves to bind calcium with high affinity to specific cell-surface receptors (96-97), presumably to induce a rigid conformation of this domain to reduce proteolytic degradation (98). The O-linked sugar domain appears to be important in the maintenance of a stable expression of the receptor on cell surfaces (99).

Northern analyses in the American dog tick show that the *Dv*VgR message only occurs in the ovary and is not found in the midgut or salivary glands of female adults as would be expected based on its function of transferring yolk protein from the hemolymph to the egg. The message is also only found in mated, replete vitellogenic females as would be expected. The VgR receptor is absolutely essential for egg development. When VgR message was silenced by RNAi, Vg accumulated in the hemolymph, no absorbance of vitellogenin by eggs occurred, and no eggs were oviposited (93). Therefore the timing for the appearance of the VgR message during vitellogenesis is critical to female tick reproduction.

It was discussed earlier in this review that the injection of 20-HE into part-fed American dog tick females still attached to the host initiated the expression of the Vg message, Vg protein synthesis and secretion into the hemolymph, and the absorption of Vg in developing eggs. Part-fed females do not have VgR message (93) and presumably do not have the Vg receptor protein. If the latter assumption is correct, it appears that 20-HE either directly or indirectly is responsible for the expression of the VgR message and the regulation of the uptake of Vg by the ovaries. Recall from RNAi experiments, VgR appears to

be essential for yolk uptake into the egg. Although further studies are needed to validate this hypothesis, there is some evidence in the mosquito, *Aedes aegypti*, that the upstream region of the VgR gene has binding sites for the ecdysone regulatory gene products E74 and BR-C, along with the necessary transcription factors (100) and in this insect, ecdysteroids initiate vitellogenesis as is also the case in ticks.

9. MOLECULAR BIOLOGY OF MALE PHEROMONES AND THEIR ROLE IN FEMALE REPRODUCTION

The earliest evidence that a factor from the male might be able to regulate blood feeding to repletion in ticks was that of Pappas and Oliver (101). Later, Lomas and Kaufman (102) found that this factor in male *A. hebraeum* was a protein originating from the testis/vas deferens/ejaculatory duct and not the male accessory gland. The protein activity was greatest from reproductive competent, fed males and nearly undetectable in unfed males, further suggesting its importance in female reproduction. The protein molecular weight was in the range of 20-100 kD (103). More recently, Weiss *et al.* (104) cloned the messages for 35 genes that were up regulated in the male testis/vas deferens of *A. hebraeum.* Two of these messages when expressed produced proteins that were later characterized by Weiss and Kaufman (105) as the engorgement factor (EF).

Weiss and Kaufman (105) demonstrated that recombinant protein *recAh*EF derived from the male testes/vas deferens when injected into the hemolymph of virgin female adults of *A. hebraeum* elicited normal engorgement and an increase in ovary weights greater than that of controls, as well as salivary gland degeneration. *recAh*EF consists of two polypeptides, *recAh*EFalpha and *recAh*EFbeta (16.1 and 11.6 kD, respectively). No known homologs for the engorgement factor could be found in non-tick species. Donohue, Sonenshine and Roe (unpublished) recently completed the sequence of a *D. variabilis* male transcript (*Dv*EFalpha; EF203418) that is homologous to *recAh*EFalpha. The message is 69.5 and 78.9% identical at the nucleotide and amino acid level, respectively. In preliminary rt-PCR analysis, *Dv*EFalpha message was found only in fed males (not in unfed males) of *D. variabilis* as would be expected. *Dv*EFalpha has a predicted molecular weight of 16.9 kD and is 40 residues longer than *recAh*EFalpha at the carboxy terminus. The first 30 aa at the n-terminus are 80.0.% identical; however the 5' UTR of *Dv*EFalpha is 335 bp in comparison to 128 bp for that of *recAh*EFalpha. *Dv*EFalpha also contains an $AU_{(3)}A$ element in the 3' UTR of the message which is not present in the *recAh*EFalpha sequence. These elements have been shown to decrease mRNA stability (106), but its significance in *Dv*EFalpha is not yet clear. Attempts to obtain a homolog to *recAh*EFbeta in *D. variabilis* have so far been unsuccessful. A blast search with the *recAh*EFbeta nucleotide sequence against the Genbank databases, omitting the first 24 nucleotides of the published sequence (which corresponds to a 12-mer Stratagene *Eco*RI adapter in the forward and reverse orientations), does not return results with a significant expect value (≤1e-10). Although Donohue and Roe (unpublished) were successful in finding alpha in the *I. scapularis* genome, beta was not found. It remains to be seen whether a homolog to *recAh*EFbeta exists in other ixodid ticks or if EF activity can be elicited by *Dv*EFalpha alone in the American dog tick.

Since the molecular weight of the *A. hebraeum* mating factor was within the range of a 20-100 kD polypeptide, the combined molecular weight of *recAh*EFalpha and *recAh*EFbeta (27.7 kD) was in this same range, and EF expression is initiated by blood feeding in male ticks, it has been hypothesized that the mating factor and EF are the same protein (105, 107). Further evidence for this is that the combined molecular weight is different from tick sperm-capacitation factor (12.5 kD) and vitellogenesis-stimulating factor (>100 kD). Moreover, the injection of the two *rec* proteins resulted in salivary gland degeneration which has been shown to be one of the effects of mating factor activity (102). The fact that EF can initiate female blood feeding and ovary development by injection into the hemolymph, suggests that the male pheromones act independently of the female genital tract and either on the synganglion or epidermis to initiate vitellogenesis. There has been essentially no research conducted on the direct role of male pheromones on the female reproductive system and synganglion function or on the specific mechanism by which they regulate vitellogenesis. In respect to the latter, the working hypothesis has been that the male initiates vitellogenesis via the synganglion by a PTTH-like hormone acting on the epidermis to produce ecdysteroids. Donohue and Roe with the assistance of Dr. Larry Gilbert at the University of North Carolina (Chapel Hill, NC) (unpublished data) found that synganglia from replete females of *D. variabilis* co-incubated in organ culture with epidermis from part-fed females, increased the level of ecdysteroids. This supports the hypothesis that a hormone originating from the synganglion at the time of vitellogenesis triggers ecdysteroid synthesis and it has been discussed before that ecdysteroids have been shown to initiate egg development. Whether male pheromone acts on the synganglion and/or also on the epidermis is unknown. Since the most complete understanding of EF and it role in the regulation of female reproduction is in *A. hebraeum*, research is needed to better understand the molecular biology of this pheromone and its function in other tick models.

10. CONSENSUS MODEL FOR THE REGULATION OF FEMALE REPRODUCTION IN TICKS

To summarize what is currently understood about the molecular biology of tick reproduction just discussed, a model was developed to describe the mechanism for the regulation of egg development in ticks (Figure 6). This model is limited in that it represents a consensus of information on tick reproduction developed from different tick species. As a consequence, it may not be representative of any one species. The model is also limited in that it represents a relative small number of studies and a great deal of the work is conducted on hard ticks.

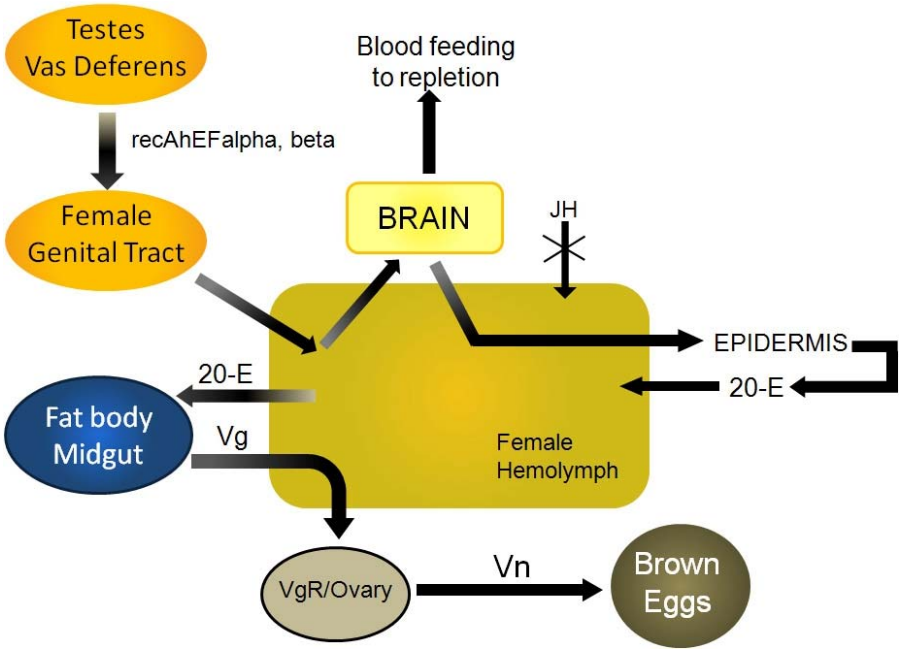

Figure 6. Model for the endocrine regulation of vitellogenesis in ticks by ecdysteroids. JH, juvenile hormone; *recAh*EFalpha,beta, male protein sex pheromones; Vg, vitellogenin; VgR, Vg receptor; Vn, vitellin; 20-E, 20-hydroxyecdysone.

Substantial evidence now exists that ticks regulate reproduction by increases in the level of circulating ecdysteroids and not that of juvenile hormone as once thought. Studies currently suggest that ecdysteroids regulate the transcription of Vg mRNA, Vg protein synthesis, and Vg secretion into hemolymph. The lack of physical and bioassay evidence for JH in ticks; the developmental expression of the Vg message correlated with the appearance of increased hemolymph ecdysteroid titers and Vg protein levels; and the induction of Vg mRNA, Vg protein synthesis and egg development in partially fed, virgin females by 20-HE support this new perspective. Although ecdysteroid injections into part-fed, virgin adult females can initiate vitellogenesis and the growth of ovaries to weights similar to mated females, 20-HE has no effect on the promotion of blood feeding to repletion for the American dog tick that is partially fed and still attached to the host. This suggests that other factors are regulating blood feeding to repletion. The ecdysteroids that regulate vitellogenesis are synthesized by the tick epidermis, which is apparently regulated by a peptidic hormone from the tick synganglion. The specific site of synthesis of the ecdysiotrophic hormone and information on its sequence is not known.

Multiple putative tissue sources for the synthesis of Vg found in tick hemolymph have been identified by detection of the Vg message in fat body, midgut and ovary. The relative importance of each in contributing to hemolymph and egg Vg levels is uncertain. There also appears to be multiple Vgs per tick, and the expression of both appears to be controlled by elevated levels of 20-HE. The sequence of Vg2 from *D. variabilis* is not yet complete, and therefore the comparative molecular biology of tick Vgs is not possible at this time. Vg1 in *Dv* apparently exists as a dimer of the full-length message after removal of the secretion signal and is also post-translationally modified to several smaller peptides as predicted and not-predicted by known cleavage sites in the conceptual aa sequence.

Vg is the major egg storage protein that appears also to be important in the transport of heme to developing eggs. The later is especially significant since ticks have lost the ability to synthesize heme. The synthesis of CP which is the major larval, nymph and adult storage protein prior to commitment to egg laying in females ticks also appears to be important in heme sequestration. A reduction in CP expression was noted at the time in adult female development of the American dog tick when an increase in Vg expression occurs. Both Vg and CP which share a common ancestry appear to be critical to the evolution of hematophagy by serving as a source of heme in the tick and egg, respectively, during the long periods between blood feeding but have different tissue sources and appear to be regulated by different mechanisms. The uptake of Vg from the hemolymph into developing oocytes requires the appearance of the message for a Vg receptor in the tick ovary. Evidence suggests that VgR is regulated by ecdysteroids but more studies are needed to confirm this. When the VgR message was suppressed by RNAi, yolk deposition into the egg and oocyte development was stopped.

A protein pheromone, *recAh*EF, cloned from the male testes/vas deferens is transferred to the female during copulation in *A. hebraeum*. This protein in the hemolymph of virgin female adults elicits engorgement and an increase in ovary weight. *recAh*EF consists of two peptides (*recAh*EFalpha and *recAh*EFbeta) which separately had no

effect on engorgement or egg development. Although a similar protein to *recAh*EFalpha was sequenced from the American dog tick, studies to find *recAh*EFbeta have so far been unsuccessful. Further studies are needed to understand whether these male pheromones are present in other ticks species, are they involved directly in any regulation of gene expression and function in the female reproductive system, and do they move from the female system into the hemolymph and if so by what mechanism, and how do they regulate feeding and vitellogenesis. It is also possible that the male engorgement factor might stimulate the female reproductive system to produce a hormone that regulates vitellogenesis.

11. CONCLUSIONS AND PERSPECTIVES

Significant advances have been made in understanding the molecular biology of tick storage proteins, the vitellogenin receptor and male pheromones responsible for the initiation of female blood feeding and reproduction or vitellogenesis. However, our understanding of tick hormones is still in its infancy compared to insects and Crustacea; and significant advances are needed to determine whether ticks have peptidic hormones that regulate ecdysteroid biosynthesis, lipid and carbohydrate biochemistry, digestion, molting, scleritization, metamorphosis and other physiological processes. Our understanding of the interaction of the ovary and synganglion relative to female reproduction has not been studied. Also one of the great challenges will be to understand how ticks regulate immature development and metamorphosis. The latter is essentially a black box at this time, although most likely ecdysteroids will play some role. Comparative metabolomic and genomic studies are needed to study the evolution of the JH synthetic pathway in arthropods including ticks. Whether ticks have a "status quo" hormone like in insects is uncertain. Worthwhile goals in the future should also include improving our understanding of the molecular endocrinology of ticks relative to host complementation and vector competence, which are of critical importance to the transmission of animal and human diseases.

One of the greatest impediments for advancements in better understanding tick physiology is the difficulty of raising ticks in the laboratory and the necessity in using animals as hosts. A significant effort is needed to develop methods to feed and breed ticks on artificial membranes and if possible artificial media. Also more research is needed on multiple tick species, to better qualify developing ideas about tick endocrinology. On the other hand, the possibility of future advances in tick molecular endocrinology is exciting due to recent advances in tick genomics and the availability of new, ultra-high throughput sequencing technologies like 454 sequencing. It is likely that advancement will be more rapid than ever and great changes are expected in the near future. It is also important that the knowledge developed for ticks should also be extended to the even less studied mites, which have significant economic importance in agriculture as well as animal health. It is also the hope that researchers will not only advance our basic understanding of tick biology but extend this knowledge to new applied technologies both relative to the acarines and applications in other areas.

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