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Isolation and Characterization of an 'Engorgement Factor' from the Testis/Vas Deferens of the Fed Male Tick, *Amblyomma hebraeum*

by

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Abstract

The lifecycle of ixodid ticks consists of four stages; egg, larva, nymph and adult. The feeding period of the adult stage is divided into preparatory, slow and rapid feeding phases. In *Amblyomma hebraeum* Koch, the transition from slow to rapid feeding occurs at ~ 10x the unfed weight and is marked by a developmental stage called the 'critical weight' (CW). The CW is characterized by numerous physiological and behavioral changes which signal the transition from a 'host seeking strategy' to a 'reproductive strategy'. Six parameters defining this transition (re-attachment to the host, hemolymph ecdysteroid titre, salivary gland (SG) degeneration, ovary weight, oocyte length and oocyte vitellin content) were evaluated. CW was found to vary somewhat depending on the parameter measured.

SG degeneration in engorged female *A. hebraeum* is triggered by the steroid hormone 20-hydroxyecdysone (20E). The release of 20E into the hemolymph occurs 4 days earlier in mated females than in virgins. This early release is caused by a 'male factor' (MF) produced in the testis/vas deferens (T/VD) of fed males and passed to the female during copulation. A previous study determined that another mating factor ('engorgement factor', EF; Pappas and Oliver, 1972) is the stimulus for rapid engorgement. MF and EF are probably the same substance. In an attempt to isolate this substance, a cDNA library was made from the testis/vas deferens of fed male *A.*

hebraeum, and feeding-induced genes were identified using a differentially cross-screening approach. Of the 35 feeding-induced genes isolated using this technique, only two shared some sequence homology with others catalogued in the GenBank database.

Twenty-eight of the 35 genes isolated were cloned into an insect-based plasmid expression vector and recombinant proteins ($_r$proteins) produced in an *Sf*21 cell line. These $_r$proteins were then tested in a newly developed EF bioassay. 'Recombinant *A. hebraeum* EF' ($_r$*Ah*EF) consists of two $_r$proteins: $_r$*Ah*EFα (16.1 kD) and $_r$*Ah*EFβ (11.6 kD). Injection of $_r$*Ah*EF stimulates virgin females to engorge, and also induces SG degeneration and a significant degree of ovary development. These results further the hypothesis that EF and MF are the same substance.

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Chapter 1. General Introduction

I) GENERAL TICK BIOLOGY

Ticks are among the most important vectors of human and animal pathogens including arboviruses, rickettsiae, spirochaetes, parasitic protozoa and possibly nematodes (Sonenshine, 1993). Some species of tick (e.g., *Dermacentor andersoni*, Schmitt *et al.*, 1969; *Ixodes holocyclus*, Stone *et al.,* 1989) secrete a paralytic toxin capable of disabling or killing their host. Furthermore, severe infestations can result in host anemia, loss of appetite, weakening of the immune system, disruption of liver metabolism and extensive hair loss (Nelson *et al.*, 1977). The salivary glands (SGs; a list of abbreviations is found in appendix 1) serve as the major pathway through which pathogens and toxins enter the host (Kaufman, 1989).

Ticks are divided into three families: Nuttalliellidae, Ixodidae and Argasidae (Fig. 1-1). The family Nuttalliellidae contains a single species (*Nuttalliella namaqua*) about which very little is known (Keirans *et al.*, 1976). Ixodid ticks are often referred to as 'hard ticks' because their dorsal body surface has a sclerotized plate called a scutum. While waiting for potential hosts, ixodid ticks 'quest' from the exposed, apical portion of vegetation. The prostriate ticks (genus *Ixodes*) can mate before or during feeding; the metastriate ticks (all other genera) mate only on the host (Sonenshine, 1991).

The ixodid life cycle comprises four stages: egg, larva, nymph and adult. Following hatching, molting to the subsequent instar requires a blood meal. Male ixodids must feed for a period ≥ 3 days to complete gonad maturation (Khalil, 1970). After this initial feeding period, males detach from the host and proceed to move around, copulating with females as they go. Because of this intermittent feeding behavior, determining when males engorge is not possible, and they may be correctly referred to as 'fed' at any given time proceeding the onset of mating (Snow, 1970).

The feeding period of adult female ixodid ticks lasts 7-14 days and comprises the following stages:

- The 24-36 hour preparatory phase: During this time the tick inserts its mouthparts into the host epidermis, prepares a feeding lesion and secretes the attachment cement.
- The 7-10 day slow feeding phase: During this phase the attached female gradually expands to approximately 10 times her unfed weight by imbibing blood and other tissue fluids.
- The 12-36 hour rapid feeding phase: Throughout the rapid feeding phase, the tick increases its weight a further 10-fold. Thus, females weigh approximately 100 times their unfed weight at repletion (Balashov, 1972).

Following engorgement, females drop off the host and begin oviposition approximately 10 days later. Larger species can lay up to 23,000 eggs during a single gonotrophic cycle, after which they die.

Unlike ixodid ticks, argasid ticks have a soft, leathery cuticle and lack a scutum. Argasid ticks mate off the host, and normally exhibit nidiculous host-seeking behavior (they inhabit the nests, caves and burrows of their hosts). The argasid tick life cycle comprises the same four stages as ixodid ticks, except that 2-8 nymphal molts may occur, usually depending on the species (and even varies within species; Hoogstraal, 1985). Adult argasid ticks feed rapidly, often engorging within 1 h. Females go through multiple gonotrophic cycles during each of which they lay a few hundred eggs (Sonenshine, 1991).

II) TICK SALIVARY GLANDS

Functions

Tick SGs serve numerous physiological functions:

• During periods of dehydration, ticks are capable of water vapor uptake from the atmosphere. They achieve this by secreting a hygroscopic liquid onto the mouthparts. Sorbed water is then imbibed (Rudolph and Knulle, 1974).

• After establishing a feeding lesion, ixodid ticks secrete a cement-like substance from the SG that hardens into a cone surrounding the hypostome, thus anchoring the mouthparts to the host's skin (Moorhouse and Tatchell, 1966).

• The SGs of some species secrete anticoagulants and vasoactive substances that facilitate the process of imbibition (Ribeiro, 1989; Waxman *et al.*, 1990).

• In females, the SGs are responsible for concentrating the nutrient portion of the blood meal by excreting excess fluid back into the host (Kaufman, 1983).

• Finally, males use saliva as a lubricant to aid transfer of the spermatophore into the female genital tract (Feldman-Muhsam and Borut, 1970).

Structure, development and degeneration

The SGs of female ixodid ticks consist of a pair of elongate, glandular masses of three alveolar types (I, II, III) extending from the anterior of the tick to the single pair of spiracles located posterior to the 4th pair of walking legs (Till, 1961; Barker *et al.*, 1984). The SGs of unfed female ticks have virtually no fluid-secretory ability (Kaufman, 1976). Upon initiation of feeding, significant ultrastructural, cytological and biochemical changes occur within the gland. These changes include the appearance of features characteristic of fluid transport epithelia (Fawcett *et al.*, 1981; 1982; Coons and Kaufman, 1988), increases in cAMP (Shelby *et al.*, 1987) and Na, K-ATPase activity (Kaufman *et al.*, 1976). Experiments by Coons and Kaufman (1988) on *A. hebraeum* suggest that a hemolymph-borne factor(s) stimulates these changes.

Within a few days of dropping off the host, the SGs of female *A. hebraeum* are resorbed (Harris and Kaufman, 1981). This process, which is triggered by a hemolymph-

borne substance ('tick salivary gland degeneration factor'; TSGDF), occurs only in ticks that have fed to above a 'critical weight' (CW) of approximately 10x the unfed weight (Harris and Kaufman, 1984; Kaufman and Lomas, 1996). Ticks forcibly removed from a host below the CW do not degenerate their SGs, but instead re-attach and resume feeding if a new host presents itself. TSGDF is the steroid hormone 20-hydroxyecdysone (20E; Harris and Kaufman, 1985).

The tissue(s) responsible for the production of ecdysteroids in ticks have remained an enigma until recently. Previous studies had suggested fat body and the lateral segmental organs of the central nervous system as possible sites of ecdysteroid production (Schreifer *et al.,* 1987). More recent work has demonstrated that the epidermis is capable of ecdysteroid production (Zhu *et al.*, 1991; Lomas *et al.*, 1997). The production of ecdysteroids by tick integumental tissue is not surprising because the prothoracic glands (source of ecdysteroids in most insects) and epidermal tissue are both ectodermal in origin. Also, in the few insects that lack prothoracic glands, epidermal tissues also synthesize ecdysteroids (Rees, 1985).

III) MALE REPRODUCTIVE BIOLOGY

Anatomy of the reproductive system

The genital system of adult male metastriate ticks consists of a pair of elongate, tubular testes. The testes connect anteriorly to a pair of convoluted vasa deferentia that fuse into a common seminal vesicle. Dorsal to the seminal vesicle is a large multi-lobed accessory gland. The seminal vesicle and accessory gland empty into the seminal atrium, a structure that is continuous with the ejaculatory duct that ends at the genital pore (Fig. 1-2; Oliver, 1982; Sonenshine, 1991).

Spermatogenesis, the spermatophore and mating

Sperm development in ticks occurs in two stages; spermatogenesis and spermiogenesis. Spermatogenesis, which is the sequence of mitotic and meiotic divisions leading to haploid spermatids, is initiated in the fed nymph, and completed in the fed adult. During nymphal ecdysis, germ cells undergo differentiation in 'waves' proceeding anteriorly through the testis. Following this differentiation event, the newly formed primary spermatocytes form agglomerations called spermatocysts and are arrested at the level of late prophase until ecdysis occurs. Spermatogenesis of the primary spermatocytes resumes following eclosion of the unfed male and the commencement of feeding. At this point the spermatids grow rapidly (enlarge 5-6x their previous size) and become elongated (Dumser and Oliver, 1981; Oliver, 1982).

Within five days of feeding, spermiogenesis has begun and the spermatids continue their differentiation into mature spermatozoa. Following numerous complex cytological changes (described by Sonenshine, 1991), the elongated, mature spermatids, now called prospermia, accumulate in the vasa deferentia and seminal vesicle. The final stage of spermiogenesis, capacitation, occurs after the spermatophore has been transferred to the female. Capacitation is triggered by a male-derived 'capacitation factor' which will be discussed in more detail below.

Before being transferred to the female, spermatids are packaged into a spermatophore. Formation of the spermatophore is in response to a chemical stimulus produced by a female after her genital aperture is probed by the mouthparts of a conspecific male (Sonenshine, 1985). The spermatophore is formed externally from materials such as mucopolysaccharides and proteins extruded by the male. These materials condense into a membranous, three-layered sac-like ectospermatophore into which flow the spermatids, seminal fluids and yeast-like sperm symbionts (Sonenshine, 1991; Feldman-Muhsam, 1991). A second droplet called the endospermatophore seals the

ectospermatophore. This entire process requires only ~30 seconds (Feldman-Muhsam, 1986).

Copulation commences following spermatophore formation. Observations of mating *D. andersoni* demonstrate that the male, which is already venter-to-venter with the female, contorts his body thus forcing the spermatophore anteriorly towards his mouthparts (which are still inserted in the female's genital pore). Once the spermatophore is in place, the male removes his mouthparts from the female, seizes the spermatophore with his chelicerae, and deposits it onto the female's genital pore. Finally, the spermatophore elongates and expels its contents into the female genital pore via pressure created from internally generated CO_2 (Sonenshine, 1991). Argasid males, on the other hand, use their mouthparts to transfer the spermatophore the entire distance from their genital aperture to the females. Males also salivate at this time to lubricate the spermatophore thus easing insemination (Feldman-Muhsam, 1986).

IV) PHYSIOLOGICAL CHANGES ASSOCIATED WITH MATING

Insect mating factors

Many male accessory sex gland proteins (Acps) alter female behavior and physiology subsequent to copulation (Gillott, 1988). Gillott (1988) divides these proteins into two groups, fecundity-enhancing substances (FESs) and receptivity-inhibiting substances (RISs). FESs result in a general increase in fecundity, mainly through induction of egg development, ovulation or oviposition. RISs render females unreceptive to males temporarily or for the rest of their lives.

Once transferred to the female, Acps may function by any of the following mechanisms:

1) They may pass through the wall of the reproductive tract without modification. For example, Lung and Wolfner (1999) demonstrated that two *Drosophila* Acps, Acp26Aa

and Acp62F, pass directly from the ventral posterior vagina into the female hemocoel within 10 min of mating.

2) In some female insects, the reproductive tract may become more permeable to Acps as a result of mechanical trauma sustained during mating. The male genitalia of several insect species induce such a condition (Lewis and Pollock, 1975).

3) They may stimulate the reproductive tract to produce another hormone that alters fecundity or receptivity. For example, an RIS from the grasshopper, *Gomphocerus rufus*, has effect only from within the spermathecae. Injections of accessory gland homogenates into the hemolymph do not render them refractory. A receptivity-inhibiting response is observed, however, when the same homogenate is micro-injected into the spermathecae (Hartmann and Loher, 1996; 1999; this system is discussed in more detail below).

4) Acps may be cleaved to release the active fraction once inside the female. *Drosophila* Acp26Aa, which stimulates ovulation (see below), has prohormone-like structural features. Once inside the female, Acp26Aa gets processed into its active form by a sequential series of proteolytic cleavages (Park and Wolfner, 1995). This processing requires activities donated by the male, one of which may be the enzyme seminal-fluid esterase 6 (Meikle *et al.*, 1990)

Fecundity-enhancing substances (FESs)

FESs are present in numerous insect species where they most commonly stimulate ovulation and oviposition. In no other insect model has this response been better documented than in *Drosophila melanogaster* (Wolfner, 2002). Heifetz *et al.* (2000) generated male mutants deficient in the gene coding for Acp26Aa. Mates of males lacking Acp26Aa laid fewer eggs than normal. Further experiments revealed that just one step in the egg-laying process is dependent on the transfer of Acp26Aa: the initial release of oocytes at ovulation. Because the above-mentioned females still exhibited some egg-

laying capacity, other Acps, such as Acp70A (Chen *et al.*, 1988), are presumed to function in conjunction with Acp26Aa as egg-laying modulators.

Neubaum and Wolfner (1999) investigated the role of Acp36DE in promoting sperm storage. Females mated to males lacking the Acp36DE gene stored 85% fewer sperm and produced 90% fewer adult progeny than normally mated controls. Furthermore, behaviors normally associated with stored sperm, such as increased rate of egg-laying and decreased receptivity, were suppressed in females mated with Acp36DE-deficient males. The authors showed that Acp36DE, in combination with a 'tissue barrier' in the oviduct, are essential for maximal sperm storage.

Similar substances are found in insects other than *Drosophila*. Some of the more well-studied, and exceptionally interesting, examples include:

• In the blood-sucking bug *Rhodnius prolixus*, an FES in the seminal fluid stimulates the spermatheca and adjacent region in mated females to produce a 'spermathecal factor' (SF) which promotes fecundity by increasing both egg production and laying (Kuster and Davey, 1986). Davey (1967) demonstrated that SF acts on medial neurosecretory cells in the brain to release a myotropin. This myotropin triggers ovulation and oviposition (Kriger and Davey, 1982; 1983) and is essential for the maintenance of sperm viability in the spermathecae (Kuster and Davey, 1986).

• In the cricket, *Acheta domesticus*, gravid, virgin females injected with PGE_2 or $PGF_{2\alpha}$ layed 60x more eggs than normal controls one day post-treatment (Destephano and Brady, 1977). Interestingly, however, the authors found that prostaglandin (PG)-synthetase (which is necessary to produce PGs from arachidonic acid) is not present in females, but is brought to them by males via the spermatophore. When normal mated females were injected with the PG-synthetase inhibitor *N*-acetyl-p-aminophenol, oviposition was inhibited by 90% (Destephano and Brady, 1977).

• Males of the Colorado potato beetle, *Leptinotarsa decemlineata,* produce an 8 kD protein called Led-MAGP that is deposited into the female's spermathecal duct before the transfer of sperm (Smid, 1998). As Led-MAGP-immunoreactivity disappears 4-6 min later, the author suggests it likely crosses the duct wall into the hemolymph. Once there, Led-MAGP may stimulate endocytosis of certain hemolymph proteins that bind to it. Smid *et al.* (1997) speculate that Led-MAGP may initiate yolk protein uptake by developing oocytes.

Receptivity-inhibiting substances (RISs)

RISs deter males from mating with females already inseminated with viable sperm. This may benefit a species because males are encouraged to seek out virgin females in the population (Gillott and Friedel, 1977). An interesting form of receptivity inhibition occurs in sexually mature, mated female grasshoppers (*G. rufus*). A 'secondary defensive' behavior, which involves strong directed kicks at the male to prevent further matings (something I've also experienced occasionally in the past!), lasts until oviposition concludes 3-4 days post-copulation. Hartmann and Loher (1996) found that females were unable to initiate this behavior if their spermathecae were denervated or their ventral nerve cord cut. Further experiments revealed that a 'white secretion' from a specific accessory gland tubule, in combination with the mechanical stimulus induced by ovulation, initiated secondary defense (Hartmann and Loher, 1999).

In the corn earworm moth, *Helicoverpa zea*, mating inhibits receptivity by decreasing the attractiveness of the female to other males. Kingan *et al.* (1995) isolated a 57 amino acid pheromonostatic protein from the accessory gland/ejaculatory duct complex of males. When injected into virgin females, this protein elicited a 15-fold decrease in sex pheromone production. When mated with males surgically altered to produce a spermatophore containing no spermatozoa or seminal fluids, pheromone

synthesis occurred normally. Injecting these females with purified protein reversed this effect. Finally, mated females with severed ventral nerve cords still produced pheromone, thus indicating the necessity of neural signals in pheromonostasis.

Several RISs have been characterized in *Drosophila*. A 377 amino acid secreted protein produced in the ejaculatory bulb, called PEB-me (Ludwig *et al.*, 1991), is transferred to the female within 3 min of the start of mating and 'solidifies' approximately 2 min later to form a major component of the posterior mating plug. Because the posterior mating plug forms before the transfer of spermatozoa, functions other than blocking further matings have been proposed for it. These include facilitating sperm movement (Bairati, 1968; cited in Lung and Wolfner, 2001) and preventing sperm loss (Lung and Wolfner, 2001).

Lung *et al.* (2002) ectopically expressed eight male *Drosophila* Acps and found that one, Acp62F, has toxic effects that resulted in decreased female lifespan. The authors found that while one exposure was toxic to immature male and females, multiple exposures were necessary to have toxic effects on mature females. Biochemical assays and sequence analysis revealed that 1) Acp62F has a 28 amino acid region sharing high sequence similarity with a neurotoxin from the Brazilian armed spider, *Phoneutria nigriventer* (Wolfner et al., 1997), and 2) that Acp62F encodes a trypsin inhibitor similar in structure to extracellular serine protease inhibitors from the nematode *Ascaris*. About 90% of the Acp62F transferred to the female remains in her reproductive system (where, coincidentally, it may act as an FES; Lung *et al.*, 2002) and ~10% passes into the hemocoel through a permeable region in the ventral posterior vagina (Lung and Wolfner, 1999). Once in the circulatory system, Acp62F may have a toxic effect by inhibiting essential proteolytic cascades. These cascades are necessary for the fly's immunity against microbial parasites (Imler and Hoffmann, 2000) and may alter pathways essential

for robust viability. Thus, the repeated introduction of Acp62F through multiple matings may result in a decrease in female fecundity (Wolfner, 2002).

Mating factors in ticks

Mating is also a trigger for behavioral and physiological changes in ticks. Normal engorgement, SG degeneration and genital tract maturation in both males and females are influenced by mating (Khalil, 1970; Oliver and Dotson, 1993). Studies done on *A. hebraeum* (unpublished observations) reveal that the vast majority of virgin females (≈ 90-95%) do not exceed a critical weight (CW) of approximately 10x the unfed weight, even after several weeks on the host. Experiments by Harris and Kaufman (1984) suggested that a factor contained within the male reproductive tract is part of the pathway stimulating SG degeneration. The results of their experiments were:

1) SG degeneration was prevented in engorged females which had their seminal receptacles surgically removed. When the excised seminal receptacles were replaced, degeneration proceeded normally.

2) SG degeneration could be restored in engorged females whose seminal receptacles had been removed by injecting male genital tract extracts into the hemocoel, but not by injecting male SG extracts. Thus, the effect on SG degeneration results from material contained specifically in male gonad extracts.

3) SG degeneration proceeded normally when nerves to the seminal receptacle were severed. Thus, SG degeneration is not caused by mechanical stimulation associated with copulation (Harris and Kaufman, 1984).

Harris and Kaufman (1984) named this substance from the male gonad 'male factor' (MF). At that time they thought MF to be an essential link in the pathway to SG degeneration. However, Lomas and Kaufman (1992a) found that, whereas mated females above the CW lost 95% of their fluid secretory ability within 4 days of removal from the

host (Kaufman, 1983), virgin females above the CW also lost 95% of their fluid secretory ability, but they required 8 days. These results indicated that MF plays a modulatory (rather than essential) role in SG degeneration.

Lomas and Kaufman (1992a) found that MF activity occurred exclusively within the testis/vas deferens (T/VD) portion of the male gonad (and not in the accessory gland). MF activity was associated mostly with the middle fraction of a discontinuous sucrose gradient. The bottom fraction, which lacked MF-activity, contained the spermatozoa. Boiling reproductive tract extracts for 5 min destroyed male factor activity completely and treatment with proteinase K reduced male factor activity by 58%.

Lomas and Kaufman (1992b) considered two potential mechanisms whereby MF could act:

1) MF could act directly on the SG by sensitizing it to 20E, possibly up-regulating the ecdysteroid receptor. But since 20E is equipotent when applied to SGs of virgin and mated females (Lomas and Kaufman, 1992b), such a mechanism is unlikely.

2) MF could act indirectly on the SGs by hastening the appearance of 20E in the hemolymph. Injection of virgin females above the CW with hemolymph from mated (but not virgin) donors caused a significant reduction in secretory competence. This reduction in secretory rate was very similar to that of saline-injected, weight-matched, partially-fed mated controls. Injection of hemolymph from virgins above the CW did not cause a significant reduction in fluid secretory competence (Lomas and Kaufman 1992b). Furthermore, Lomas *et al.* (1997) demonstrated that a synganglial factor(s) appears to be a requirement for ecdysteroidogenesis in the integument. Therefore, MF may act by initiating the release of this neuropeptide.

As mentioned previously, most virgin female *A. hebraeum* will not exceed the CW even when attached to the host for several weeks. Using *D. variabilis,* Pappas and Oliver (1971; 1972) found that females did not feed to repletion when exposed to males

which had their genital apertures blocked. These experiments suggested the presence of an 'engorgement factor' (EF) contained in the spermatophore which promotes feeding to repletion. As females mated to males that were irradiated with cobalt-60 (which kills spermatozoa) were still able to engorge, EF was suggested not to be associated with the spermatozoa.

Capacitation, the final phase of tick sperm maturation, occurs following transfer of the spermatids to the female during mating. Extensive morphogenesis of the spermatids, including the rupture of an operculum at the anterior end of the cell and the emergence of mature spermatozoa now twice their original length, occurs at this time (Borut and Feldman-Muhsam, 1976; Oliver and Shephard, 1981). Using *O. moubata* and *D. variabilis,* Shephard *et al.* (1982) demonstrated this process to be induced by a 12.5 kD male Acp added to the spermatids during ejaculation. Although the two factors are the same size in both species, they appear to be distinct molecules, as each has no effect on spermatids from the other species. The authors speculated that capacitation factor might have a proteolytic effect that causes rupture of the operculum. However, treatment with trypsin and pronase caused no more capacitation than that which occurred in controls. Instead, they found that the factor acted more like a signaling molecule: a short exposure of spermatids to the factor is sufficient to trigger the entire maturation process.

Another FES, a vitellogenesis-stimulating factor (VSF) in argasid ticks, was described by Connat *et al.* (1986). Mature spermatids injected into the hemocoel of virgin female *O. moubata* induced oviposition while immature spermatids (prior to capacitation) did not. Following a 20 h incubation in a physiological medium, washed mature spermatids still induced egg-laying behavior when injected into virgins. This indicated that the chemical stimulus was within the spermatids rather than the seminal fluid. Furthermore, homogenates of testis and accessory gland did not stimulate a response. VSF-activity within the female was highest at 12 h post-copulation, by which time

capacitation was complete. These results suggested that VSF is bound within the spermatids while inside the male, and released into the female genital tract following capacitation (Sahli *et al.*, 1985).

Receptivity inhibition in ticks

The four aforementioned mating factors all relate to 'fecundity enhancement' in ticks. Evidence for the 'inhibition of receptivity' via a chemical cue in ticks is lacking. However, Khalil (1984) found a decrease in fecundity in the argasid tick, *Argas (Persicargas) arboreus*, when replete females were crowded together. These crowded individuals either oviposited fewer eggs or none at all. This reduction in fecundity was attributed to a fecundity-reducing pheromone that may be emitted from a specific gland(s) in a gaseous state subsequent to stimulation by feeding. This putative pheromone was presumed to be additive and its effect was increased when produced by many individuals.

Male ticks appear to inhibit females from mating with subsequent males by 'proximate mate guarding' rather than by transferring an RIS. Yuval *et al.* (1990) found that free males of the deer tick, *I. dammini* (now referred to more commonly as *I. scapularis*), would try to dislodge mating males when male: female ratios surpassed 3:1. However, mating males remained obstinately in position and no dislodgment was observed. Furthermore, almost half of males remained attached to their replete partners when they dropped from the host, thus further ensuring that a subsequent male does not inseminate the female.

V) THESIS ACHIEVEMENTS

During my thesis work I have achieved the following:

1) Establishment of a more precise estimate of critical weight for individual females (thesis chapter 2; Weiss, B.L., Kaufman, W.R. (2001). The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick, *Amblyomma hebraeum. J. Insect Physiol.* 47: 1261-1267).

While conducting experiments with large populations of *A. hebraeum*, Harris and Kaufman (1984) established the CW as 'approximately' 10x the unfed weight. Since then, the concept of CW has remained an enigma for tick biologists. Subsequent experiments in our lab have revealed this figure (10x) may be somewhat inaccurate. I developed a protocol for determining a precise value for the CW of individual ticks based on five distinct parameters, and determined that it varies depending on the parameter used to measure it.

2) Identification and characterization of feeding-induced genes (thesis chapter 3; Weiss, B.L., Stepczynski, J.M., Wong, P., Kaufman, W.R. (2002). Identification and characterization of genes differentially expressed in the testis/vas deferens of the fed male tick, *Amblyomma hebraeum. Insect Biochem. Mol. Biol.* 32: 785-793).

The work by Pappas and Oliver (1972) informed me that EF, which is produced by fed males and passed to their mates during copulation, was necessary for feeding to engorgement. Furthermore, the MF characterized by Lomas and Kaufman (1992a; 1992b) is 1) feeding-induced, 2) found only in the T/VD, and 3) likely to be the same substance as EF. With this in mind, I began the isolation of EF by identifying all genes up-regulated in the T/VD of fed males as the result of feeding. I constructed a cDNA library from the T/VD of fed males and, using a differential cross-screening protocol, isolated 35 clones that are up-regulated as a result of feeding. Up-regulated clones were confirmed by Northern blot analysis, sequenced, and their identities putatively determined by comparing their nucleotide and amino acid sequences with those catalogued in databases. Thirty-three of the 35 clones contain inserts of novel nucleotide sequence.

3. Purification of EF (thesis chapter 4; A combination of two feeding-induced proteins from the male reproductive system induce engorgement in the female tick, *Amblyomma hebraeum*. Weiss and Kaufman, manuscript in preparation).

I began this work by establishing an EF bioassay. I injected gonad homogenates into virgin females below the CW and returned them to fresh hosts to see if they would engorge. EF bio-activity was found exclusively in the T/VD portion of the male gonad. Once I had a functional bioassay, I produced 28 recombinant proteins ($_r$proteins; only 28/35 had full open reading frames) from the aforementioned feeding-induced transcripts. The bioassay of these $_r$proteins revealed that two of them are required together for EF bio-activity (see Table 4-1). Recombinant *A. hebraeum* EF ($_r$*Ah*EF) also stimulates in virgin females a degree of SG degeneration comparable to that of mated controls, thus supporting the hypothesis that EF and MF are the same substance. However, $_r$*Ah*EF on its own is unable to induce complete ovary development.

Fig. 1-1. Phylogenetic relationships among the ticks. Cladogram modified from Sonenshine (1991).

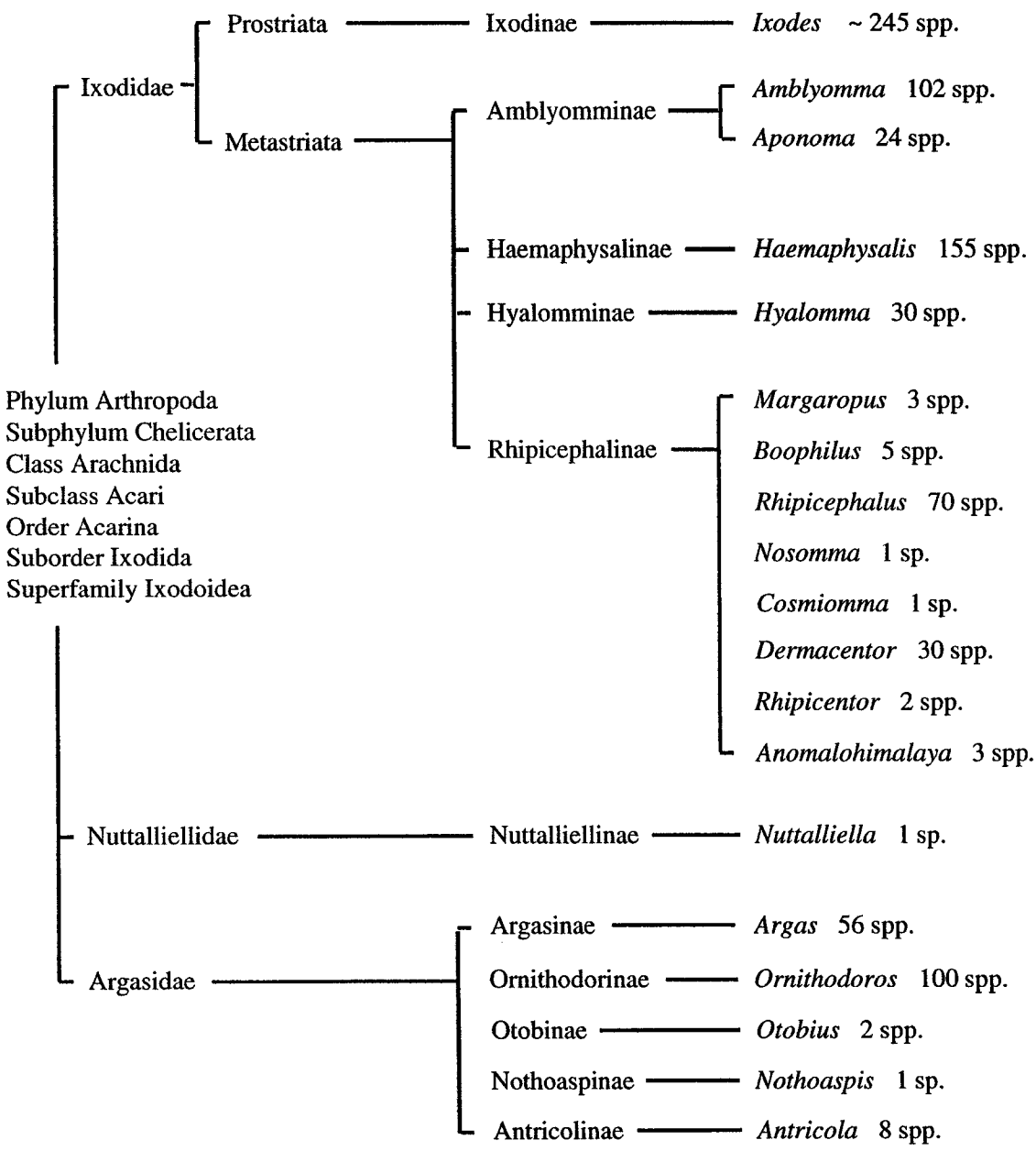
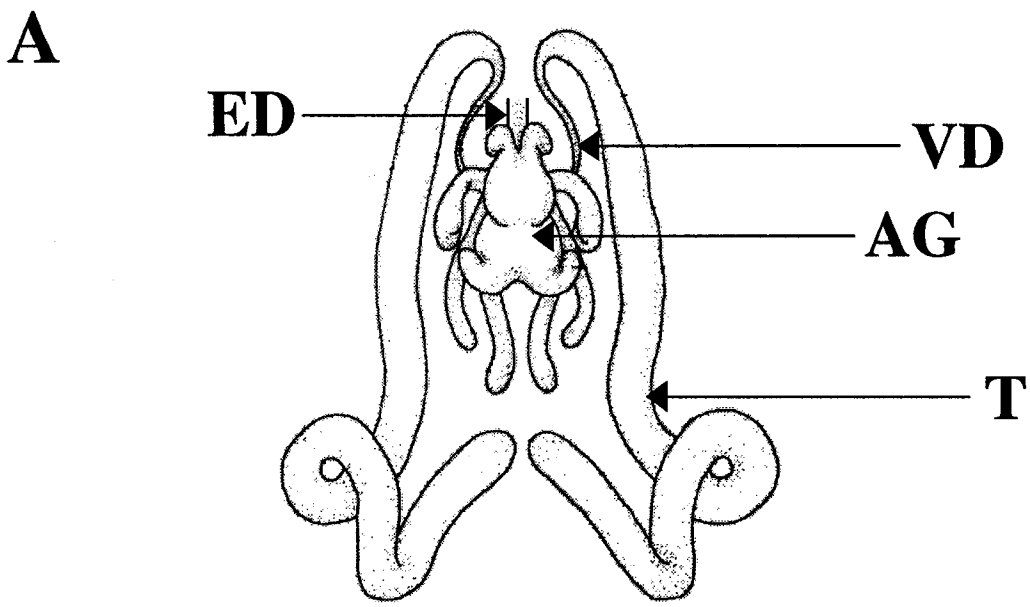
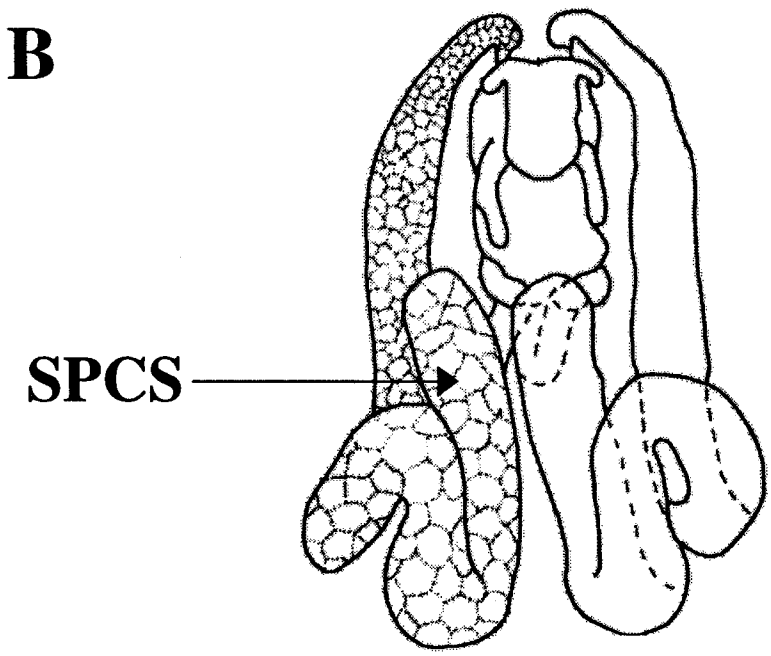


Fig. 1-2. Diagrammatic representation (dorsal aspect) of the reproductive system of male *Dermacentor andersoni*. From A) unfed, and B) fed individuals. ED, ejaculatory duct; VD, vas deferens; AG, accessory gland; T, testis; SPCS, spermatocysts. The seminal vesicle is hidden beneath the accessory gland. Modified from Sonenshine (1991).





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Chapter 2: The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick, *Amblyomma hebraeum*[1]

I) INTRODUCTION

Adult female *A. hebraeum* Koch require 10-14 days to engorge completely (approximately 100x the unfed weight). When ticks are held at 26°C, oviposition usually begins 10-12 days later and continues for 3-4 weeks. The feeding period comprises three distinct phases: 1) a 24-36 h *preparatory phase*, during which the female inserts its mouthparts, establishes a feeding lesion and secretes cement to secure attachment to the host epidermis, 2) a 7-10 day *slow feeding phase*, during which the female gradually expands to approximately 10x its unfed weight, and 3) a 12-36 h *rapid feeding phase*, during which the female increases its weight another 10-fold (Balashov, 1972).

Species of the genus *Ixodes* can copulate prior to finding a host, but in all other genera, copulation can occur only during the feeding cycle. Most feeding virgin females, approximately 85% (Kaufman and Lomas, 1996), remain attached to the host for several weeks at a small, partially-fed state (under 10x the unfed weight). Once fed males are introduced, copulation occurs and the females engorge rapidly (Snow, 1969). Approximately 15% of feeding virgins may achieve 10-20x the unfed weight, but full engorgement never occurs (Kaufman and Lomas, 1996).

If females are removed from the host prematurely, their subsequent behavior is influenced mostly by two factors: 1) how much blood they have imbibed to that point, and 2) whether or not they have mated. If either virgin or mated females are removed from the host before they have achieved approximately 10x the unfed weight, they will

[1] A version of this chapter has been published as: Weiss, B.L., Kaufman, W.R. (2001). *Journal of Insect Physiology* 47: 1261-1267 (see Appendix 2)

readily re-attach and resume feeding if presented to a host once again. If mated females are removed from the host during the rapid phase of feeding, however, they will generally not re-attach if presented to a host. Instead, their salivary glands (SG) degenerate within about 4 days, and an egg mass is laid subsequently, the size being approximately proportional to the amount of blood consumed. The hormone directly responsible for SG degeneration is 20-hydroxyecdysone (20E; Kaufman, 1991). Vitellogenesis in *D. variabilis* is also triggered by 20E (Sankhon *et al.*, 1999). The source of hormonal ecdysteroid (ES) is the epidermis, and synthesis of ES by this tissue is controlled by a neuropeptide (Lomas *et al.*, 1997). If virgin females are removed at a weight exceeding 10x the unfed weight, SG degeneration occurs within about 8 days, and any eggs laid are infertile (Kaufman and Lomas, 1996). Because this transition from the slow to rapid phase of feeding, at about 10x the unfed weight, results in such major changes in physiology and behavior, it has been termed a 'critical weight' (CW) in the feeding cycle (Harris and Kaufman, 1984; Lomas and Kaufman, 1992a, b).

When compared to mated females, virgins take longer to degenerate their SG (8 days versus 4 days) because of an equivalent delay in the secretion of ES (Lomas and Kaufman, 1992b). A protein in the spermatophore transferred to the female during copulation is responsible for hastening the secretion of ES and thus hastening the process of SG degeneration (Lomas and Kaufman, 1992a, b). The site and mechanism of action of this 'male factor' (MF) are not yet known.

Although the concept of CW has been recognized for over 15 years, its value has never been measured precisely. In previous studies we have not known the individual unfed weights of the partially-fed ticks removed from the host, and the range of unfed weights in our colony of *A. hebraeum* can vary considerably (~15-45 mg, with an approximate average of 20-30 mg). Furthermore, we have assumed, but never demonstrated, that the CW is a unique value for all of the physiological and behavioral

changes that become evident at this point in the feeding cycle. The purpose of this study was to establish a precise weight range for CW in individual females based on four measures: hemolymph ES-titre, SG degeneration, ovary maturation and re-attachment to the host. Our results indicate that, contrary to earlier assumptions, the CW varies somewhat according to the parameter measured.

II) MATERIALS AND METHODS

Tick rearing and feeding

Adult ticks (*A. hebraeum* Koch) were obtained from our laboratory colony maintained in darkness at 27°C and > 95% relative humidity. Adult ticks were confined to the backs of rabbits within a foam and cloth 'backpack' as described by Kaufman and Phillips (1973). For rearing of mated females, equal numbers of males and females were confined together. Ticks were removed at various stages of feeding or left to engorge spontaneously. Those ticks weighing < 350 mg are referred to here as "small partially fed", ticks between 350 mg and engorgement are referred to as "large partially fed" and ticks which have been allowed to feed to repletion are referred to as "engorged". Experimental ticks were kept, as necessary, in the colony incubator. Fig. 2-1 shows the relative sizes of an unfed female, a female at approximately the CW, and an engorged female.

The complete protocol for all critical weight experiments is shown in Fig. 2-2. For each set of experiments, 24 unfed adult females were weighed and individually coded using a colored thread tied to a distinct leg. An equal number of males and females were confined together on the rabbits. Ticks were reared as described above and removed for experimentation within a weight range known to overlap the CW considerably (~5.0 - 20.0x unfed weight). When at this point, females were removed, re-weighed, held in the colony incubator for 12 h and then placed back on a host for 12 h to test for re-attachment. All ticks were then placed in the colony incubator for eight days.

Radioimmunoassay (RIA) procedure

The RIA procedure used is that of Kaufman (1990). Ticks were glued ventral surface down to a Petri dish and chilled at 4°C for 25 min. Cooling reduces gut volume, thereby reducing risk of cutting the gut and contaminating the hemolymph sample with

gut content. Hemolymph was collected from small incisions made through the integument along the dorsolateral margins. Hemolymph exuding from these wounds was collected in 5 or 10 µl volumetric capillary tubes, transferred directly into glass distilled methanol and frozen at -70°C until needed.

When needed, samples stored in methanol were centrifuged at 8000 g for 5 min in a microcentrifuge. The resulting supernatant was removed, placed in another eppendorf tube and vacuum centrifuged until all methanol was removed and only dried ecdysteroid remained. Meanwhile, bovine serum albumin (BSA; Sigma) was dissolved to 5 mg/ml in 0.2 M borate buffer (pH 8.5; BSA-borate buffer). Ecdysone antibody (E-22- succinyl-thyroglobulin; supplied by Dr. L.I. Gilbert, University of North Carolina) and [23,24-^{3}H(N)]-ecdysone (New England Nuclear) was diluted in BSA-borate buffer prior to use. Hemolymph samples and 20-hydroxyecdysone standards (20E) were mixed with ecdysone antibody-BSA borate buffer (which also contains [23,24-^{3}H(N)]- ecdysone) vortexed well and allowed to incubate 4 h to overnight at 4°C. Antibody-bound ligand was then separated using protein A and centrifuged at 8000 g for 5 min. The supernatant was aspirated away and the remaining pellet resuspended in scintillation fluid (Scintiverse E; Fisher Scientific) and double-distilled water. Finally, radioactivity was monitored on a LS 6500 Multi-Purpose Scintillation Counter (Beckman Instruments). The limit of detection using this protocol is about 10 pg 20E. Because the ecdysone antibody cross-reacts with several ES analogues, all data is expressed as 20E equivalents.

Assay for salivary fluid secretory competence

Ticks were glued ventral surface down to a Petri dish and bathed in ice-cold TC medium-199 (Gibco, Long Island, N.Y.), supplemented with 2.09 g 3-N-morpholinopropanesulfonic acid (MOPS; Sigma Chemical Co., St. Louis, MO.) and 2.1 g NaCl per litre, pH 7.3. The SGs were excised and the main duct of each was ligated with

one strand peeled from 8-0 surgical silk thread (Davis and Geck, Pearl River, NY). Glands were gently blotted, weighed to the nearest 0.01 mg, and allowed to incubate for 12 minutes in fresh TCM-199 containing 10 μM dopamine (Sigma Chemical Co.). Glands were then re-weighed after gentle blotting. Net weight gain of the tissue was used as a measure of fluid secretory competence, which is inversely proportional to the degree of SG degeneration (Harris and Kaufman, 1984). A marked reduction in fluid secretory competence was used as another criterion for determining CW.

Ovary maturation assays

Ovaries were removed, gently blotted, weighed to the nearest 0.01 mg and the length of the 10 largest oocytes measured using a compound microscope fitted with an ocular micrometer. Ovaries were then homogenized in 3 ml distilled water, centrifuged at 20,000g for 10 min and the supernatant frozen for later spectrophotometric analysis. The absorbance at 400 nm (specific for the haem moiety of vitellin) and 500 nm (non-specific to vitellin) was recorded and the difference was taken as a measure of total ovary vitellin content (Kaufman *et al.*, 1986). Marked increases in ovary wet weight, oocyte length and ovary vitellin content were used as further criteria for the determination of CW.

Re-attachment assay

Partially fed females, approaching and exceeding the CW, were removed from the host, weighed and left in the colony incubator for 5, 7 or 10 days. After the predetermined time had elapsed, females were returned to a fresh host and allowed up to 24 h to re-attach (re-attachment was monitored every hour for the first 12 h). Those that re-attached were considered to be below the CW based on the criterion of re-attachment.

Effect of 20E on re-attachment to the host

From the results presented in Fig. 2-4 we hypothesized that an increase in hemolymph 20E-titre may be the factor that ultimately inhibits re-attachment in ticks exceeding the CW. To test this, we injected seven groups of partially fed ticks weighing 194 ± 16 (SEM) mg with 0.2 (n=14), 0.6 (n=10), 1.3 (n=10), 2.1 (n=14), 6.4 (n=11), 10.4 (n=19) or 31.2 (n=7) µmoles 20E/kg body weight (20E was purchased from Sigma Chemical Co.), all delivered in 10 µl of sterile medium TC199 (Gibco-BRL) per tick. An eighth group of ticks (n=15) were injected with 10 µl 1.2% NaCl containing 1.7% ethanol to serve as vehicle injected controls. Following injection, all ticks were returned to fresh hosts and monitored hourly (for the first 12 of 24 h) until re-attachment. All ticks that had re-attached, or were clearly in the process of re-attaching, were removed immediately and a hemolymph sample taken for ES-RIA.

Statistics

All data are expressed as mean ± SEM (*n*). Statistical significance was determined by Student's t test using Microsoft Excel software on a Macintosh computer.

III) RESULTS

Fig. 2-3 shows CW as defined by hemolymph ES-titre (2-3A), SG degeneration (2-3B), ovary weight (2-3C), oocyte length (2-3D) and absorbance (A_{400} - A_{500}) of ovary homogenates (2-3E). The CW was identified as a marked discontinuity in the values of the dependent variables, and occurred at 10x, 10x, 12x, 12x and 13x the unfed weight respectively.

Fig. 2-4 shows the CW as defined by re-attachment to the host, as a function of the number of days spent off the host. In all cases there was some overlap in the weight range defining the CW, but the overlap diminished appreciably as a function of duration off the host. The overlap range for 5, 7 and 10 days was 7.3-12.0 x unfed weight, 8.0-10.5x unfed weight and 7.8-8.8x unfed weight respectively (more limited data for 2 days off the host suggested an overlap range similar to that of 5 days). If one accepts the lowest value on day 10 above which there was no overlap, the CW based on the criterion of re-attachment was 9x the unfed weight.

Regarding the criterion of re-attachment to the host as a measure of CW: Fig. 2-4 demonstrates a clear increase in precision as a function of time off the host. This observation suggested that the increase in hemolymph ES-titre that occurs above the CW, and which triggers SG degeneration and vitellogenesis, might also be the factor that inhibits re-attachment to the host. To test this hypothesis we injected partially fed ticks below the CW with increasing amounts of 20E, returned them to the host, and checked hourly for re-attachment (see Materials and Methods). Fig. 2-5 demonstrates clearly that 20E inhibited re-attachment. Vehicle injected controls re-attached within 2.8 ± 0.6 h (n=15), significantly sooner than ticks receiving 1.3 µmoles 20E/kg body weight (7.3 ± 0.6 h, n=10; $p<0.0001$). The latency for re-attachment did not change significantly thereafter up to 10.4 µmoles 20E/kg body weight (7.1 ± 1.21 h, n=19; $p=0.67$).

Fig. 2-5 also demonstrates the equilibrated hemolymph ES-titre in the same ticks at the time of re-attachment. Comparable concentration units are used for both axes (μmoles/kg body weight for the abscissa and μmoles/litre for the ordinate). Because hemolymph volume accounts for approximately 25% of body weight (Kaufman *et al.*, 1980), the initial concentrations in the hemolymph following injection would be approximately 4-fold higher than those indicated on the abscissa. Table 2-1 indicates that most of the RIA-detectable ES disappeared rapidly from the hemolymph during the 4-7 h required for re-attachment; the rate of disappearance increased almost 32-fold (from 0.18 to 5.8 μmoles/h) over the total range of hemolymph ES-titre at time of injection (0.8 to 41.6 μmoles/kg body weight; Table 2-1).

IV) DISCUSSION

While conducting experiments with large populations of *A. hebraeum*, Harris and Kaufman (1984) established the CW as 'approximately' 10x the unfed weight. This study reveals that the value for CW differs significantly according to the parameter measured. SG degeneration in *A. hebraeum* (Harris and Kaufman, 1985) and yolk synthesis by the fat body in *D. variabilis* (Sankhon *et al.*, 1999) can be triggered *in vitro* by similarly low doses of 20E (~30-40 ng/ml). Our results, however, indicate that the CW for hemolymph ES-titre and SG degeneration is 10x the unfed weight whereas that for ovary maturation is higher (12x-13x the unfed weight). The full expression of oocyte development depends on vitellogenesis followed by yolk uptake. Although vitellogenesis is triggered by 20E (Sankhon *et al.*, 1999), this hormone is not sufficient to stimulate yolk uptake *in vivo* whether injected alone or in combination with juvenile hormone (Lunke and Kaufman, 1993; Kaufman, 1997). So if vitellogenesis was triggered in our ticks at 10x the unfed weight, a higher weight is required for sufficient yolk uptake to be detected in our spectrophotometric assay.

Re-attachment to the host is a less well-defined measure of CW because its value changed with duration off the host. This finding suggested that rising hemolymph ES-titre between 5-10 days post-removal may inhibit re-attachment, and the data presented in Fig. 2-5 support this hypothesis. Although the specific mechanism for this effect is unknown, ES inhibit feeding in some insects. The 5th larval stage of *Manduca sexta* experiences a brief rise and fall in hemolymph ES-titre during the 4th to 5th days of the instar. This commitment peak causes the larva to stop feeding, void its gut contents and wander in search of a suitable location for pupation (Nijhout, 1994). A similar brief pulse of ES also occurs during the rapid phase of engorgement in *A. hebraeum* (Mao and Kaufman, 1999), although its physiological significance has not been clearly established yet.

In our experiments, the inhibitory effect of 20E was recorded as merely a delay in re-attachment (Fig. 2-5). We assume this was because of the rapid clearance of exogenous 20E that occurred following a bolus injection. Among numerous insects, the half-life of injected ES ranges from 4 min to 35 h (Koolman and Karlson, 1985). *In vivo*, however, hemolymph titres probably remain elevated in ticks above the CW due to ongoing ES-synthesis (Kaufman, 1991). Initially we were surprised that the latency to re-attachment did not continue to rise at doses over 1.3 μmoles 20E/kg body weight (Fig. 2-5). The reason appears to be the marked increase in clearance rate that occurs at higher injected doses (Table 2-1). Several processes may contribute to the clearance of ES from hemolymph, but the relative importance of these routes is unknown. ES may be transported actively or passively to various tissues, or excreted by the Malphigian tubules. ES may be inactivated by a number of catabolic pathways. Finally, long chain fatty acids form apolar conjugates with ES in ticks (Diehl *et al.*, 1985). Such conjugates may have greatly reduced hormonal activity or immuno-reactivity in our RIA. Conjugation, even if not followed by excretion or storage (in the ovary, for example), would represent a component of the clearance recorded in Table 2-1. Using the numbers generated in Table 2-1, we attempted to calculate an 'apparent K_m' and an 'apparent V_{max}' for the clearance process, but the experimental design clearly did not satisfy the criteria for Michaelis-Menten kinetics (initial rates of reaction with no significant reduction in substrate concentration). Nevertheless, the 32-fold rise in clearance rate occurred over the sub-micromolar to low-micromolar range (Table 2-1). Perhaps more than coincidentally, the K_m values for a number of enzymes that metabolize E and 20E fall over a similar range in a variety of insects (Webb *et al.*, 1996; Williams *et al.*, 2000; Young, 1976; Smith *et al.*, 1979). For a better understanding of the physiological regulation of hemolymph ES-titres in ticks, we shall have to evaluate the relative importance of the factors responsible for clearance from the hemolymph.

Fig. 2-1. Female *A. hebraeum* ticks at three defined stages of the feeding cycle (unfed, CW and fully engorged).

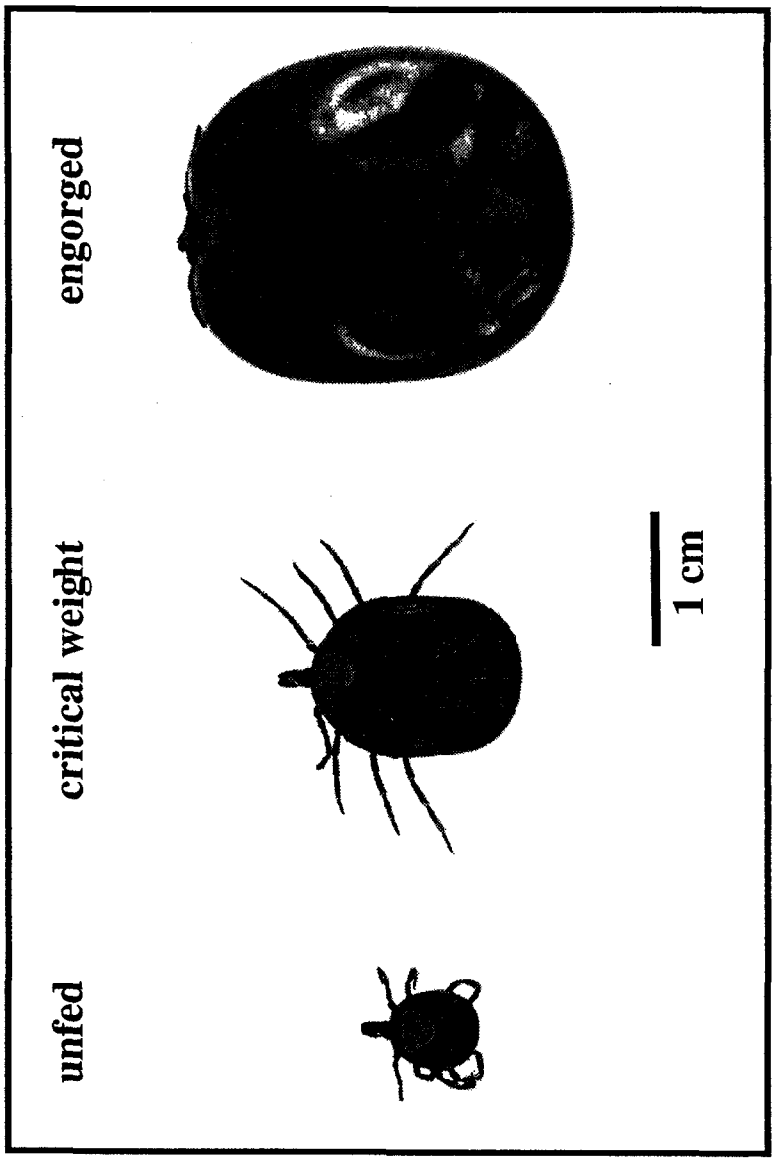


Fig. 2-2. Flow chart of the complete experimental protocol to determine CW.

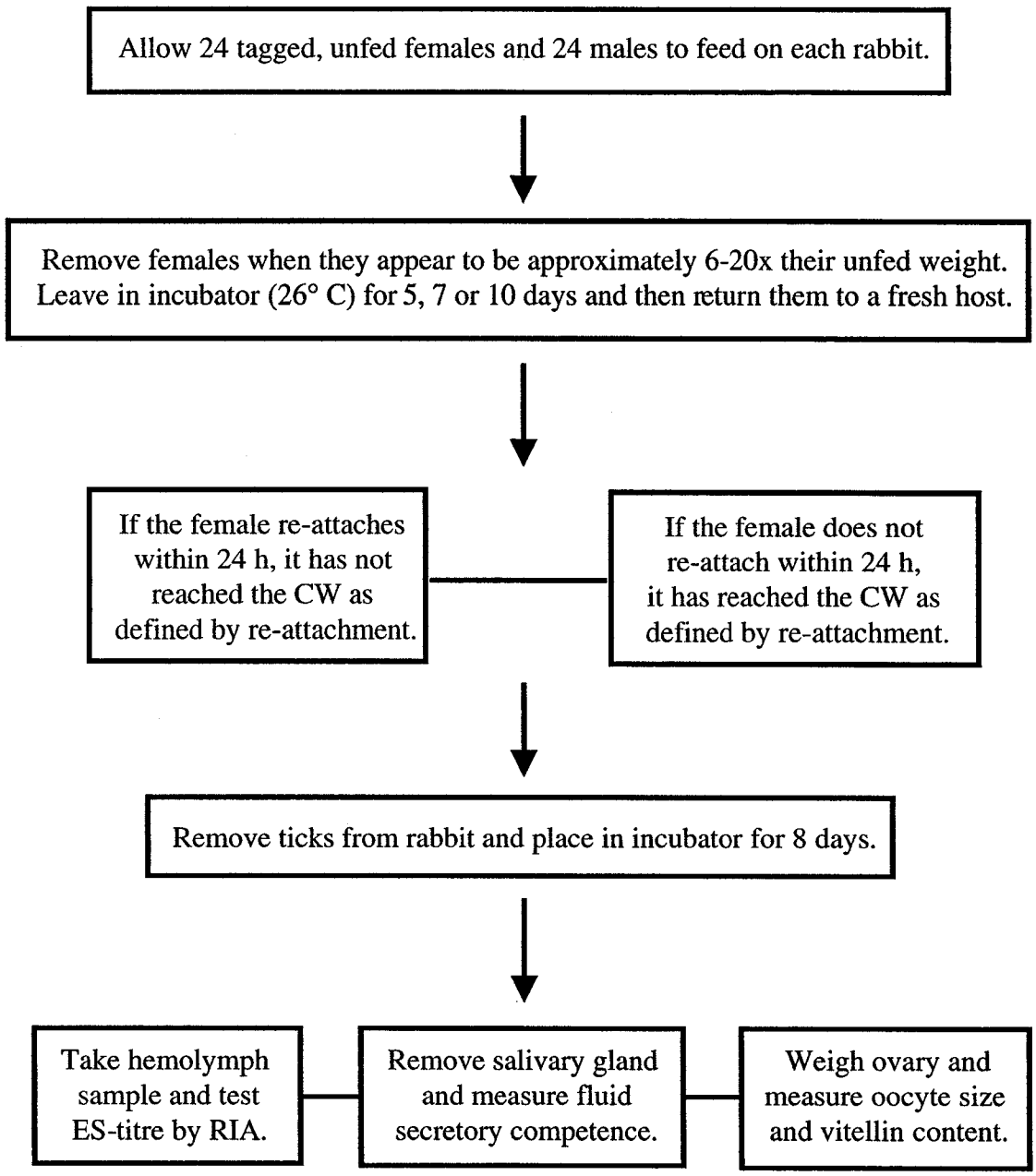
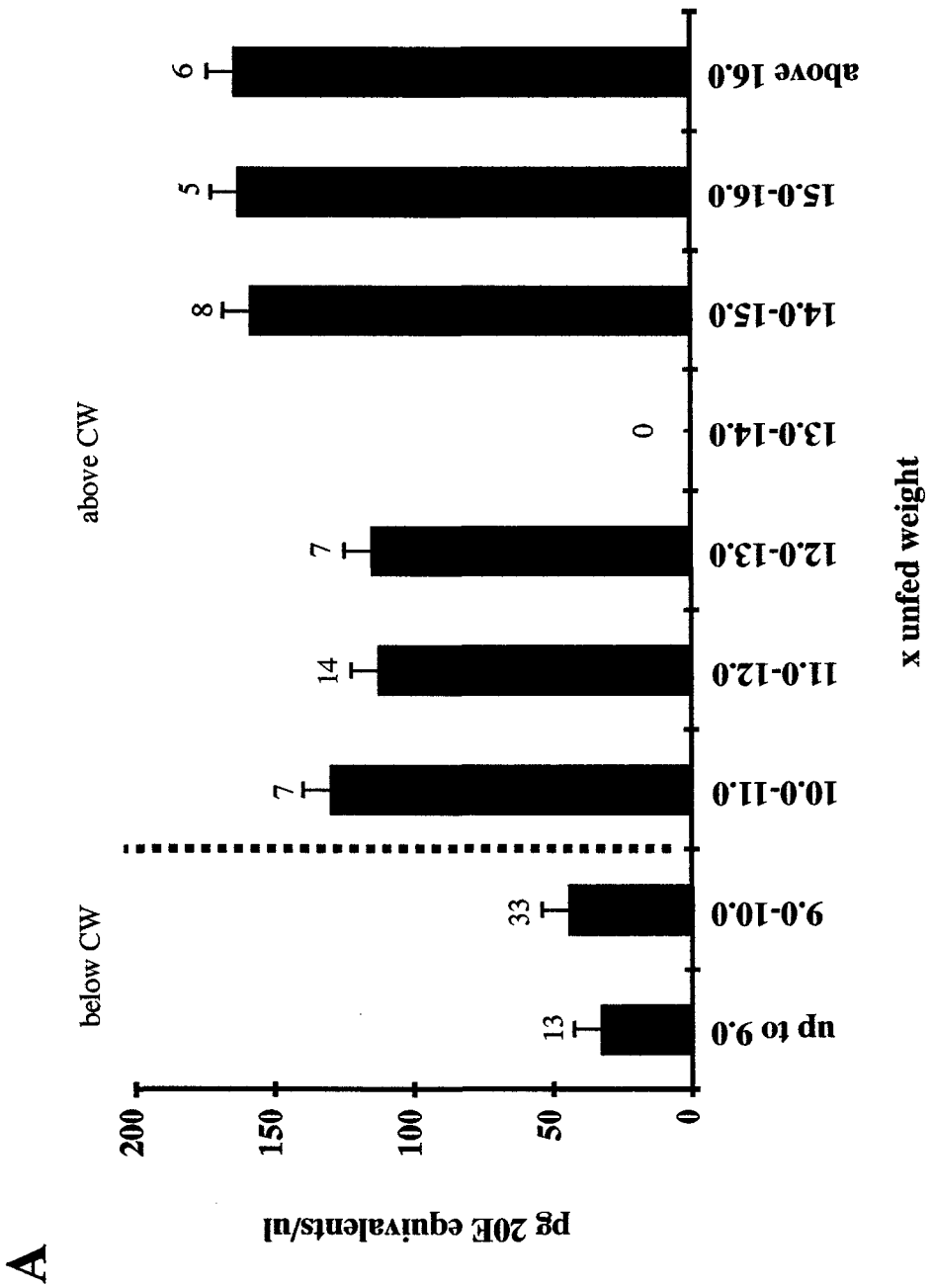
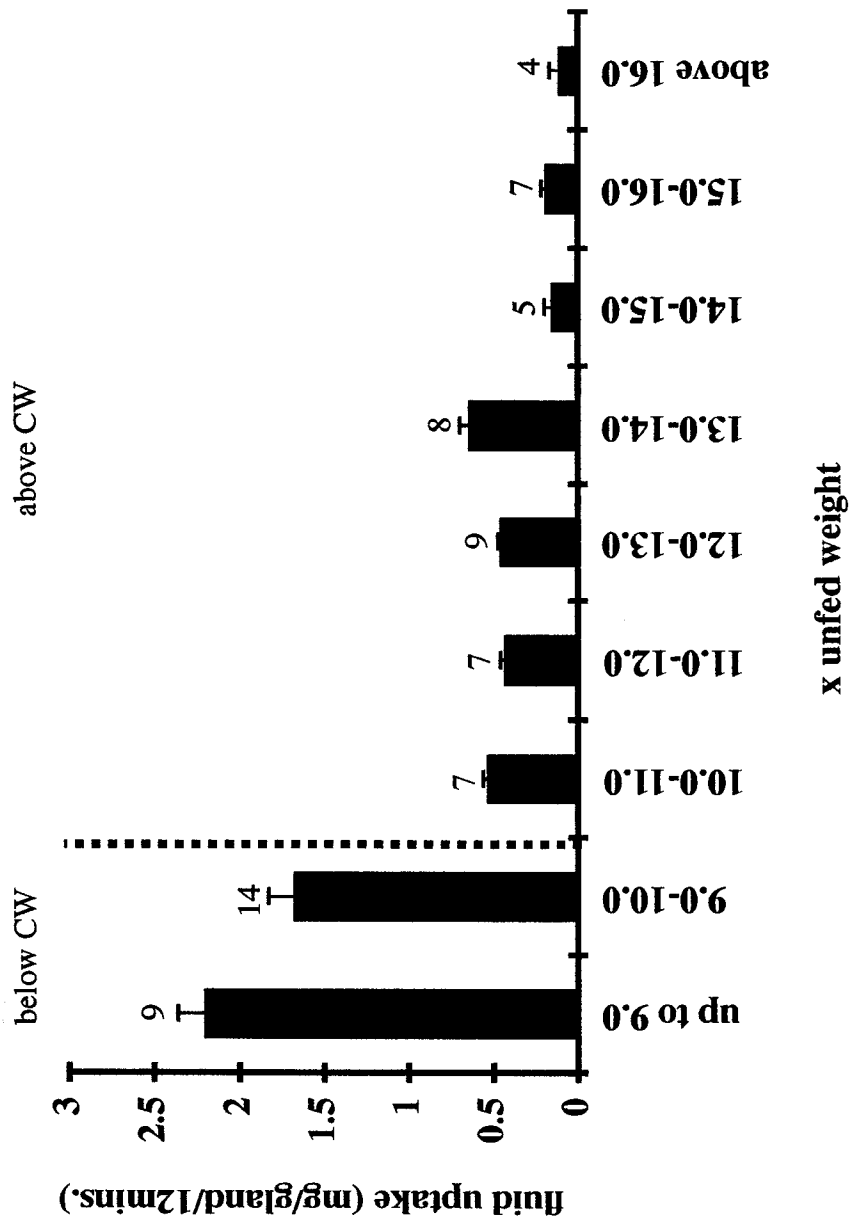
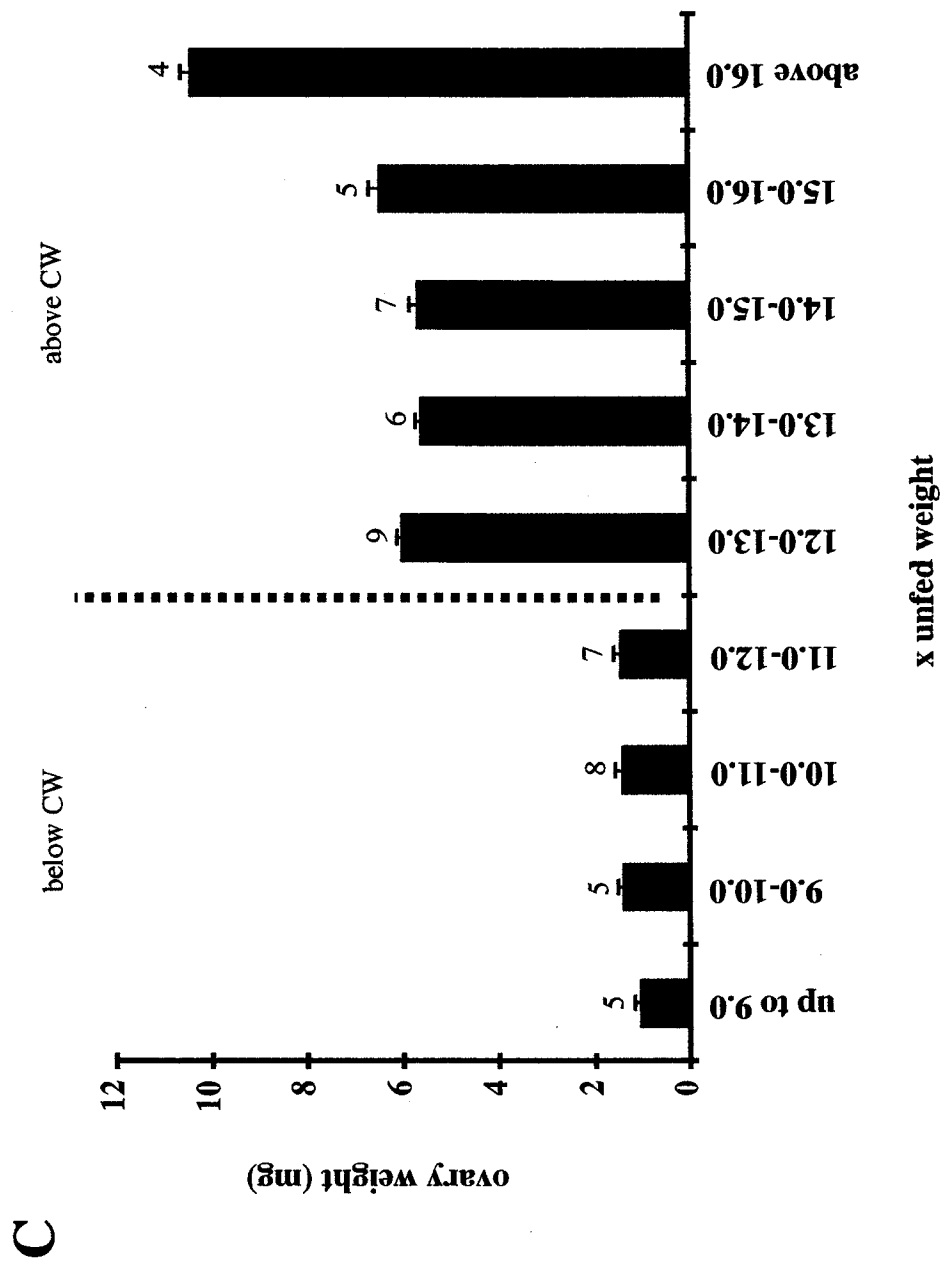


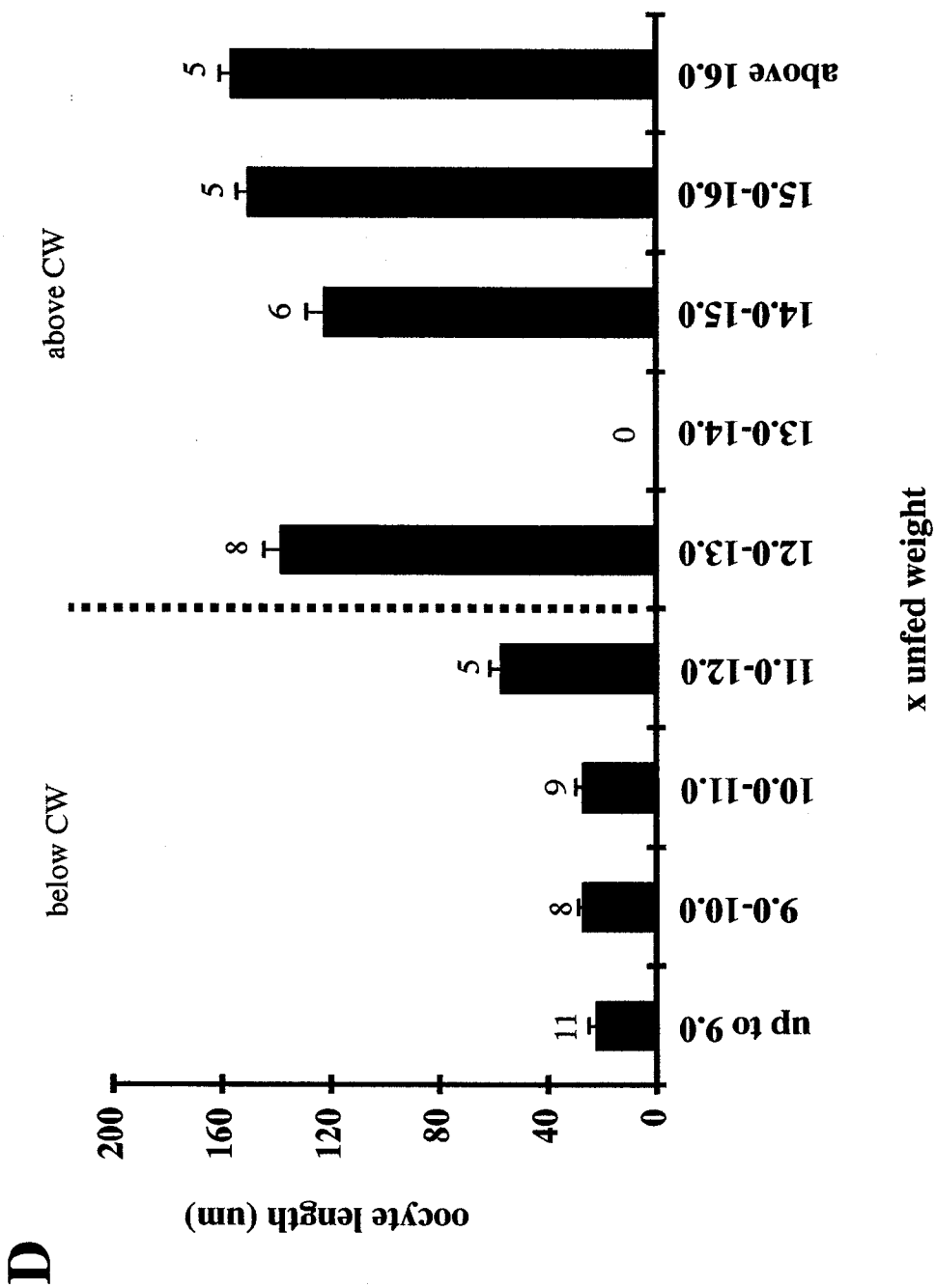
Fig. 2-3. Estimating CW by (A) hemolymph ES-titre, (B) rate of salivary fluid transport, (C) wet weight of ovary, (D) length of largest oocytes, and (E) oocyte vitellin content. See Materials and Methods for the respective techniques. In all panels, the vertical dotted line indicates the threshold of CW according to the parameter measured.



B







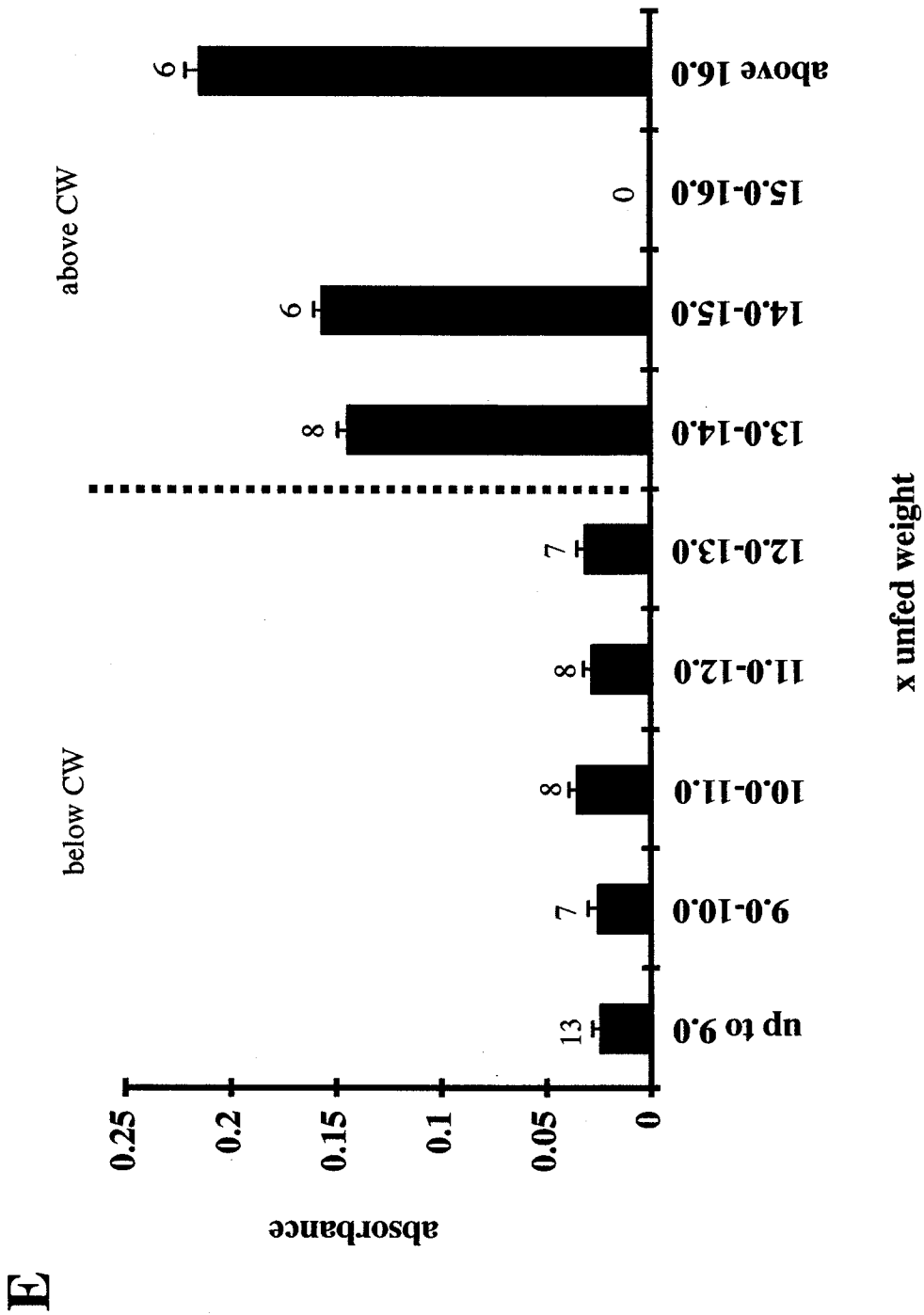
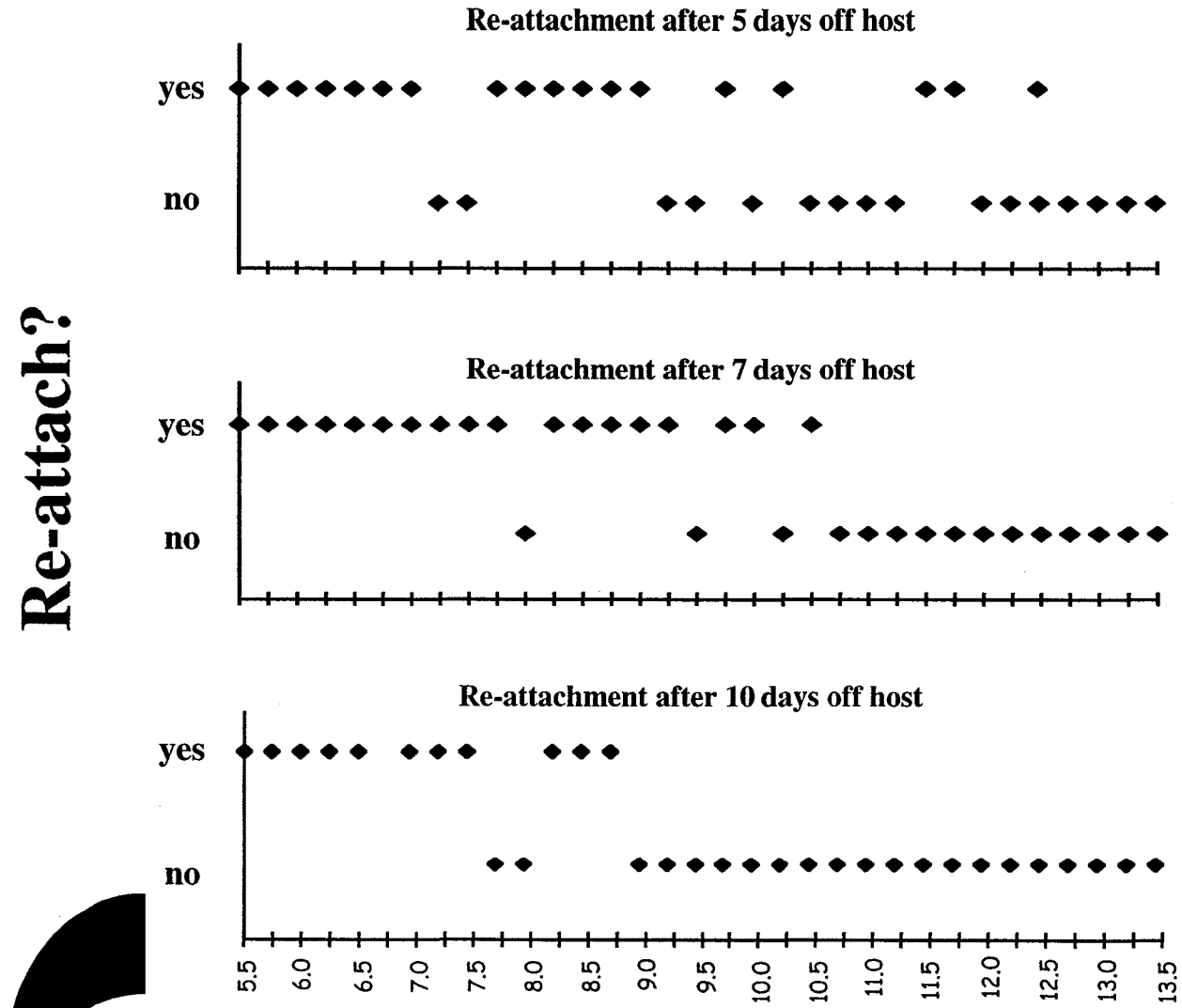


Fig. 2-4. CW as estimated by re-attachment to the host. Partially-fed ticks were removed between 5.0 and 20.0x the unfed weight and left off the host at 26°C for 5, 7 or 10 days before being presented to a new host. The range of overlap between those individuals which re-attached and those which did not re-attach narrowed as a function of time off the host. CW was determined as the lowest value above which no overlap was evident. By day 10 the CW was 8.8x the unfed weight.



Days off host	CW as defined by re-attachment
5	12.5 x unfed weight
7	10.5 x unfed weight
10	8.8 x unfed weight

Fig. 2-5. The effect of exogenous 20E on delaying re-attachment. Small, partially-fed ticks (194 ± 16 mg) were injected with increasing concentrations of 20E, returned to fresh hosts, and monitored hourly until re-attachment. Hours until re-attachment increased in a dose-dependent manner up to 1.3 μmoles 20E/kg body weight. In all cases, most of the exogenous ES (>85%) was cleared from the hemolymph before the ticks re-attached. Seven ticks injected with 31.2 μmoles 20E/kg body weight died within 10 h, without re-attaching (data not shown). SEM are shown wherever they exceed the dimension of the symbol, and n=10-19.

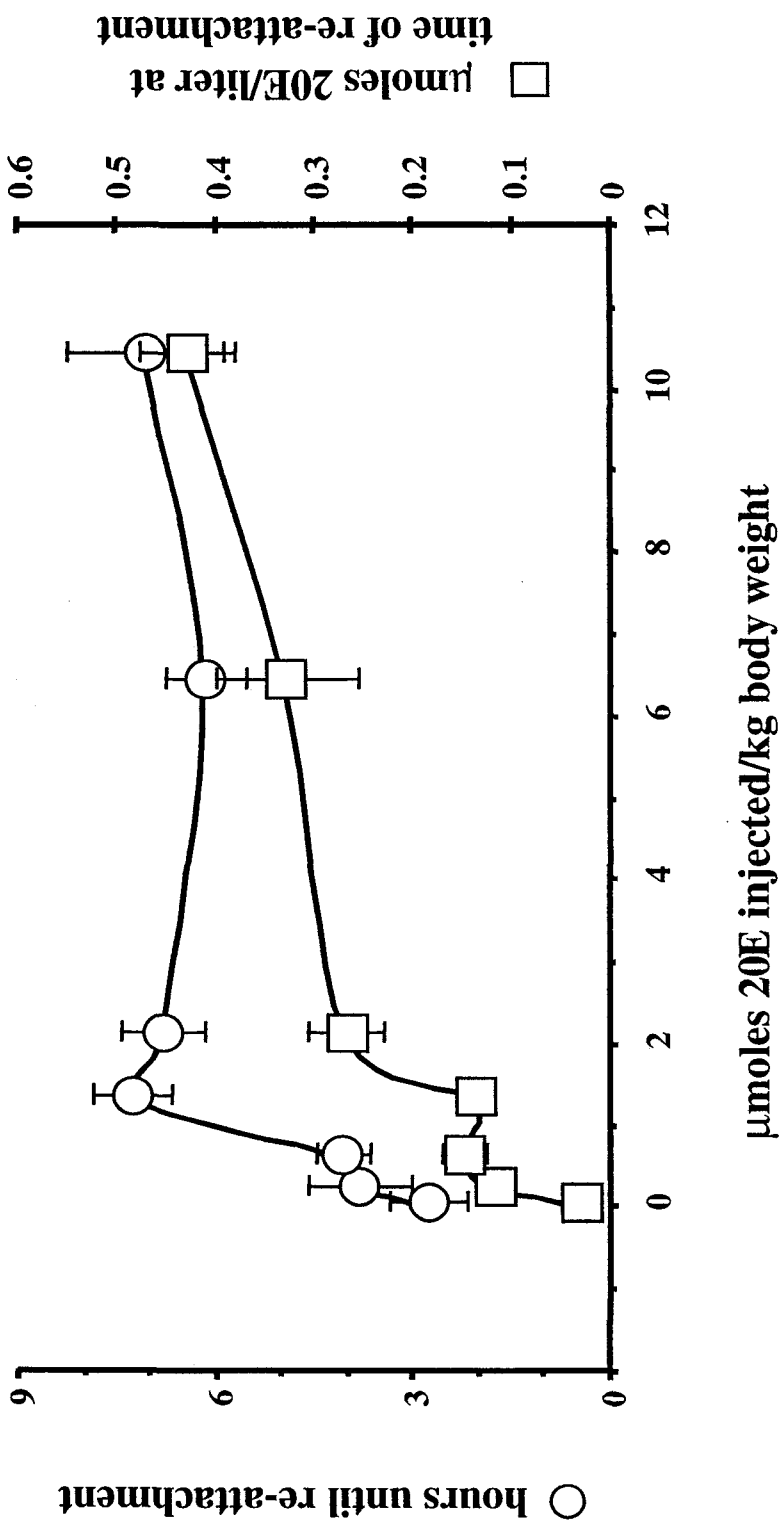


TABLE 2-1

Clearance of 20E equivalents from hemolymph in ticks injected with exogenous 20E.

Amount 20E injected (µmoles/kg body weight)	(A) Approximate hemolymph ES-titre at time of injection (µmoles/l)[a]	(B) Time until re-attachment (hours)	(C) Hemolymph ES-titre at time of re-attachment (µmoles/l)	% ES metabolized or cleared[b]	Approximate rate of ES disappearance (µmoles/hour)[c]
0.2	0.8	3.8	0.12	85	0.18
0.6	2.4	4.1	0.15	94	0.55
1.3	5.2	7.3	0.14	97	0.69
2.1	8.4	6.8	0.27	97	1.20
6.4	25.6	6.2	0.33	99	4.08
10.4	41.6	7.1	0.43	99	5.80

[a] Calculated as 4x the amount injected.

[b] Calculated as [(A - C)/A] x 100

[c] Calculated as (A - C)/B

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Chapter 3. Isolation and characterization of genes differentially expressed in the testis vas deferens of male *Amblyomma hebraeum*[2]

I) INTRODUCTION

Female ticks of the family Ixodidae usually require 7-10 days to engorge fully. During this period, numerous developmental changes occur, the most dramatic and best studied of which occur in the female salivary gland (SG; Sauer *et al.*, 1995). Within 4 days following engorgement, however, the SG degenerate, a process triggered by the hormone 20-hydroxyecdysone (20E; Kaufman, 1991).

Although gonadal development begins during metamorphosis between the nymphal and adult stages, in most ixodid ticks it remains arrested at the primary oocyte stage (female) and late prophase primary spermatocyte stage (male) until the adult begins feeding (Kiszewski *et al.*, 2001). The onset of feeding in females is characterized by the 'great cytoplasmic growth' phase during which the oocyte cytoplasm increases substantially in volume. Following engorgement, oocytes begin to migrate out of the ovary wall, thus increasing the surface area in contact with hemolymph where vitellogenin accumulates. At this stage oocytes begin to enlarge considerably and fill with brown yolk granules (Brinton and Oliver, 1971; Diehl *et al.*, 1982).

Substances produced by the male gonad [testis (T), vas deferens (VD) and accessory gland (AG)] are transferred to the female via the spermatophore and cause physiological and behavioral changes in the female. Pappas and Oliver (1972) reported that the male gonad of *D. variabilis* produces an 'engorgement factor' (EF) that triggers full engorgement in the female (virgin females rarely exceed 10-20% of the normal

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[2] This chapter is modified slightly from: Weiss, B.L., Stepczynski, J., Wong, P., Kaufman, W.R. (2002). *Insect Biochem. Mol. Biol.* 32: 785-794. (see Appendix 3)

engorged weight even after a lengthy sojourn on the host). Homogenates of gonad from unfed males do not stimulate the engorgement response, suggesting that EF is synthesized during feeding.

Previous work in our laboratory on the control of SG degeneration established that large partially fed virgin females require 8 days to degenerate their SG, whereas weight-matched mated females require only 4 days (Lomas and Kaufman, 1992a). The factor responsible for this is a protein from the male gonad (which was named 'male factor'; MF; Harris and Kaufman 1984) that acts somewhere in the female to trigger an early release of 20E (Lomas and Kaufman, 1992b). Little MF bio-activity is present in crude gonad homogenates from unfed males, suggesting that feeding induces synthesis of MF. MF is probably a gonad-specific product, as it cannot be detected in SG homogenates from fed males (Harris and Kaufman 1984; Lomas and Kaufman, 1992a).

Feeding-induced male gonad development is necessary for successful reproduction. Although specific aspects of the physiology involved are known (Kaufman, 1997), the gene expression underlying these changes has not yet been explored. Using a differential approach to screen a fed tick T/VD cDNA library, we have characterized the molecular phenotype of the gonad in fed male *A. hebraeum* and identified changes in gene expression in this tissue that are induced by feeding. Thirty-five genes were confirmed to be differentially expressed in the T/VD of fed compared to unfed males. Single-pass sequencing and comparison with the Genbank database revealed that the majority of clones are novel. Two significant homologies with confirmed expressed sequence tags were however present. We further characterized these two genes using Northern and Southern blot analysis, and discuss their potential biological relevance within the male gonad.

II) MATERIALS AND METHODS[3]

Ticks

Male *A. hebraeum* were taken from a laboratory colony maintained in the dark at 26°C and at a relative humidity of >95%. To allow for sufficient tissue maturation, 30 male ticks were fed per rabbit for > 4 days in a foam and cloth backpack as described by Kaufman and Phillips (1973). A total of 2500 male ticks were used in this study.

Tissue / RNA isolation

Males were stuck ventral surface down to a petri dish using a cyanoacrylate glue (Loctite, Rocky Hill, NJ), flooded with DEPC treated water and the T/VD, AG, SG, Malphigian tubules (Mt), synganglion (SYN) and gut were dissected out. Tissues were frozen immediately on dry ice. Total cellular RNA was extracted by grinding tissues with a mortar and pestle and then further homogenizing in a glass tissue homogenizer in the presence of TRIzol reagent (Gibco-BRL, Rockville, MD). Poly (A)+ RNA was extracted using an Oligotex mRNA mini kit (Qiagen, Carlsbad, CA.) according to the manufacturer's protocol.

cDNA library construction

A cDNA library was constructed from 4 μg fed tick T/VD poly (A)+ RNA's using a Uni-ZAP XR cDNA library synthesis kit and the Gigapack II Gold Packaging Extract (Strategene, La Jolla, Ca.) according to the manufacturer's protocol. The fed-T/VD library contained between 1 x 10^6 to 2 x 10^6 independent cDNA clones. Twenty randomly chosen clones were amplified by PCR, and then subjected to electrophoresis on

[3] Some of the techniques used in this materials and methods section are presented in more detail in Appendix 4.

a 1% agarose gel for 2 h at 80 volts. The gel was stained with ethidium bromide and viewed over UV light to determine average insert size.

Preparation of DNA probes

Poly (A)+ RNA was prepared from fed and unfed testis as described above. One microgram of mRNA was reverse transcribed using a Timesaver cDNA synthesis kit (Amersham Pharmacia, Piscataway, NJ) to produce a mixed population of double-stranded cDNA probe representative of the mRNA population in each of the tissues. Insert DNA from selected clones were prepared by PCR amplification as described below in the section 'PCR and secondary screening'.

Probes for all experiments were labeled using random primers and a mixture of dNTP's and Klenow fragment (Random Primers DNA Labeling System; Gibco-BRL, Rockville, MD). Probes made for the primary and secondary differential screens were triple-labeled ([^{32}P]αdATP, [^{32}P] αdCTP and [^{32}P] αdGTP) while those made for Northern and Southern blots were single labeled ([^{32}P] αdCTP). Unincorporated nucleotides from each reaction were removed by Sephadex G-50 chromatography.

Differential cross-screening of fed T/VD cDNA library

The library was screened unamplified. Differential screening was performed as described by Benton and Davis (1977). Clones from the fed-T/VD library, using XL1-Blue *E. coli* cells as a host, were plated at a density of 1500 pfu/150 mm plate. Nylon colony plaque screen hybridization transfer membranes were marked for later re-orientation with plates and screened as defined by the manufacturer (NEN-Dupont, Boston, MA.). The first of each duplicate set of plaque lifts was screened with [^{32}P]-labelled fed-T/VD mixed cDNA probe and the second with [^{32}P]-labelled unfed-T/VD mixed cDNA probe. Lifts were hybridized with the respective T/VD cDNA probe and

processed under stringent conditions (final wash with 0.1x SSC/0.1% SDS for 10 min at 65°C) in Hybrisol II (Intergen Co., Purchase, NY.). Screened blots were exposed for 1-3 days at -70°C to Kodak X-O Mat film. Unless otherwise noted these conditions were used for all hybridization experiments performed for the current study. In the case of the library screening, plaques with different intensities of hybridization signal between the two probes were identified and isolated (Sambrook *et al.*, 1989).

PCR and secondary screening

PCR was performed on all putative feeding-induced clones isolated after primary screening. A 5 µl sample of each plaque was added to a 95 µl reaction mixture containing ddH_20, dNTP's (200 µM), PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, 50 mM $MgCl_2$), T3 primer (0.5 µM; 5'-ATT AAC CCT CAC TAA AGG GA-3'), T7 primer (0.5 µM; 5'-TAA TAC GAC TCA CTA TAG GG-3'; BioServe, USA) and 10 units of Taq DNA polymerase. PCR was conducted using an Eppendorf (Westbury, NY) thermal cycler. The amplification program consisted of a 3 min hotstart at 94°C, followed by 30 cycles at 94°C for 1 min (DNA denaturation), 50°C for 1 min (annealing of primers), 72°C for 3.5 min (DNA elongation) and a final elongation/extension at 72°C for 7 min. Amplified products were verified by agarose gel electrophoresis.

For secondary screening, 0.2 µl of PCR product from each putative feeding-induced clone isolated after primary screening was arrayed onto three gridded nylon membranes (secondary blot). Each membrane was then allowed to hybridize with either [^{32}P]-labeled fed-T/VD mixed cDNA probe or [^{32}P]-labeled unfed-T/VD mixed cDNA probe. Prehybridization, hybridization, wash conditions and the final processing of the blots for the secondary screen were the same as those used for the primary screen.

Sequencing and sequence analysis

cDNA clones, which passed the secondary screening process, were purified using either the QIAquick Gel extraction kit or the QIAquick PCR purification kit (Qiagen, Mississauga, Ontario). Clones isolated from the secondary screen were submitted to single pass sequencing using a DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia, Piscataway, NJ) in order to generate an expressed sequence tag (EST) for each gene in question. Sequenced inserts were run on a PE Applied Biosystems 377 automated sequencer. Sequence data were analyzed using Genetool™ (Biotools Inc., Edmonton, Canada) and comparisons with the Genbank database performed by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Northern blots

Three micrograms of total RNA was subjected to electrophoresis on an agarose gel and transferred overnight to Genescreen Plus nylon membranes (NEN-Dupont, Boston, MA.) following the protocol of Sambrook *et al.* (1989). Blots were screened with the relevant radio-labeled probe under stringent conditions (as described for the library screens) and then exposed to Kodak X-O Mat film between two intensifying screens.

The intensity of bands on autoradiographs was quantified using the Kodak Digital Science ID image analysis system (Eastman Kodak Co., Rochester, NY). In order to normalize the band intensities to possible variations in RNA loading, we also quantified the relative level of 18S RNA in each lane of the gel used to generate the Northern blot analyzed. The normalized value of any transcript is the intensity of the corresponding band on the autoradiograph divided by the intensity of the 18S RNA band in the photograph of the corresponding sample in the original agarose gel photograph (Correa-Rotter *et al.*, 1992). Statistical analysis was performed using Microsoft Excel software (Microsoft, WA.).

Southern blots

Genomic DNA was extracted from seven species of arthropod (Acarina: *A. hebraeum, Dermacentor andersoni;* Diptera: *Glossina morsitans morsitans, Drosophila melanogaster;* Orthoptera: *Acheta domestica;* Blattodea: *Blaberus gigantia;* Hemiptera: *Gerris argentatus*) using the procedure of Sambrook *et al.* (1989). Separate *Cla*I and *Sau*3AI restriction endonuclease digests were set up for each genomic DNA sample according to the manufacturers of the enzyme (Gibco-BRL, Rockville, MD). Southern blot analysis using 3 μg of digested genomic DNA from each species considered was performed under reduced stringency (final wash in 0.1x SSC/0.1x SDS at 57°C for 10 min) for *Ah*T/VD16 and *Ah*T/VD146 as described by Sambrook *et al.* (1989). Autoradiography was done using Kodak X-O Mat film between two intensifying screens.

III) RESULTS

cDNA library and screening

Fig. 3-1A and 3-1B displays the considerable development occurring within the T/VD and AG following 5 days of feeding. On average, the gonad weight of fed males exceeded that of unfed males by 6-7 fold (Fig. 3-1C). In order to gain some insight into the genes that underlie this feeding-induced change, we made and screened a T/VD cDNA library from tissues taken from fed male ticks.

A cDNA library of 10^6 clones has a >99% probability of including a copy of all rare transcripts (<10 copies per cell) at a detectable frequency (Sambrook *et al.*, 1989). Thus, our library, which contains 1 x 10^6 to 2 x 10^6 independent cDNA clones, is likely to contain representatives of abundant, medium and low abundance transcripts from the fed T/VD.

Analysis of the primary differential screen of 15,000 clones on duplicate plaque lifts, using [^{32}P]-labeled fed-T/VD cDNA as probe on the first lift and [^{32}P]-labeled unfed-T/VD cDNA as probe on the duplicate plaque lift, allowed us to isolate 247 clones which apparently displayed higher levels of hybridization with the fed testis compared to unfed testis probe (results not shown). Analysis of the secondary screen confirmed 35 putative differentially expressed sequences (Fig. 3-2A). PCR analysis was used to amplify out the inserts from the 35 above-mentioned clones. Insert sizes range from 300 to 2150 bp (average insert size was 650 bp) and 31 (89%) contained only one insert (Fig. 3-2B).

Sequence analysis

Sequence comparison of up-regulated genes with the Genbank database revealed that two are significantly similar to known entries and the remaining 33 represent unique, previously unidentified transcripts. Clone *Ah*T/VD16 contains a 460 nucleotide insert with a 288 nucleotide open reading frame (ORF) followed by a polyadenylation (polyA+)

signal at position 413. The ORF, with a start codon (atg) at position 28 and a stop codon (tga) at position 316, encodes a 96 amino acid protein (10.4 kD; Genbank/EMBL accession number AF31662; Fig. 3-3A). The putative polypeptide has sites for phosphorylation by protein kinase C, cAMP-dependent protein kinase A and heavy chain myosin I. This amino acid sequence is 53% similar to an acylphosphatase muscle type isozyme found in a number of vertebrate species (Stephani *et al.*, 1997; Fig. 3-4A). Further bioinformatic analysis of clone *Ah*T/VD16 is presented in Table 3-1. This information indicates our putative protein is hydrophilic, as are the acylphosphatases of the other species examined. The molecular weight and isoelectric point of acylphosphatase in all species examined fall within a narrow range (10.5-11.3 kD and 8.6-9.7 pI, respectively). Furthermore, the tertiary structure of these amino acid sequences is highly conserved (based on predictions of estimated radius of folded proteins and % alpha helix, beta sheet and coil).

Clone *Ah*T/VD146 contains a 370 nucleotide insert with a 255 nucleotide ORF. This ORF, which encodes an 85 amino acid protein (9.8 kD; AF316621), has a start codon (atg) at position 33 and a stop codon (tga) at position 288. The poly (A+) signal in this nucleotide sequence is at position 333 (Fig. 3-3B). This putative protein has sites for phosphorylation by protein kinase C, caesin kinase II and proline-dependent kinase. The putative protein is 44% similar to a *Drosophila melanogaster* 9 kD basic protein (Ghabrial *et al.*, 1998; Fig. 3-4B).

Northern blot and Southern blot analysis

Northern blot analyses of clones *Ah*T/VD16 and *Ah*T/VD146 are shown in Fig. 3-5. Clone *Ah*T/VD16 (Fig. 3-5A) hybridized to three fed-T/VD transcripts and one unfed-T/VD transcript. A 1.5 kb transcript is up-regulated in the fed-T/VD as the result of feeding (Fig. 3-5A). This 1.5 kb transcript was also detected in a number of other organs,

although expression was highest in the fed-T/VD. Additional 3.6 kb and 6.0 kb transcripts were observed only in the fed-T/VD. Clone *Ah*T/VD146 hybridized with two transcripts (2.7 kb and 0.6 kb) from the fed-T/VD (Fig. 3-5B). The 0.6 kb transcript is expressed in all tissues examined. Expression of this transcript is highest in the Mt, fed-T/VD and gut. The 2.7 kb transcript was expressed only in the fed-T/VD. In the course of our study we confirmed the differential expression pattern of a total of 35 of the clones identified (data not shown). Thus, 0.2% of the 15,000 fed-T/VD clones originally screened represent differentially up-regulated genes.

Southern blot analysis was performed using genomic DNA from seven species of arthropod to determine whether their genomes contained sequences similar to clones *Ah*T/VD16 and *Ah*T/VD146. The restriction endonucleases used to cut the genomic DNA were chosen based on their cut sites within each clone (*Ah*T/VD16, *Cla*I, 5'-AT↓CGAT-3'; *Ah*T/VD146, *Sau*3AI, 5'-↓GATC-3'). Clone *Ah*T/VD16 hybridized to two *ClaI* genomic DNA fragments (6.0 kb and 2.8 kb) from *A. hebraeum.* This clone further hybridized to four genomic DNA fragments from *G. argentatus*, each 12.2 kb, 6.6 kb, 6.1 kb and 2.2 kb in size (Fig. 3-6). Clone *Ah*T/VD146 hybridized to two *Sau3AI* genomic DNA fragments from *A. hebraeum.* These fragments were 9.4 kb and 8.5 kb in size (Fig. 3-6). No hybridization was observed between clone *Ah*T/VD146 and genomic DNA from the other species examined (data not shown).

IV) DISCUSSION

We used a differential screening approach to begin characterizing the molecular phenotype of the T/VD from fed males. I chose this approach for the following reasons. 1) This technique was the most feasible considering the small amount of available starting material (mRNA from fed and unfed T/VD). 2) The library was screened unamplified, so no PCR-based bias occurred in favor of short transcripts. 3) Differential cross-screening detects those mRNA species present in quantities $\geq 0.05\%$ of the total cellular mRNA population (Liang and Pardee, 1995). Because a 'typical' cell contains $5x10^5$ mRNAs (Bishop *et al.*, 1974), as few as 250 copies of a given transcript need be present in a cell to be identified using this technique. 4). Analysis of results acquired using this technique allowed me to obtain fundamental information about feeding-induced gene expression in the T/VD of male ixodid ticks, something which had not been determined previously.

This study represents the first mass screening for clones that are differentially expressed in the gonad of ticks. In this study we identified 35 genes that are induced by feeding. The fact that 33 out of 35 clones contain novel sequences suggests that the tick T/VD cDNA library is a valuable resource for defining new genes.

Two of these clones, *Ah*T/VD16 and *Ah*T/VD146, have amino acid sequences that are similar to previously described sequences. The Genbank database currently (as of 5 January, 2001) contains 242 protein sequences and 2983 gene sequences from the family Ixodidae. Of these catalogued protein sequences, none come from *A. hebraeum.* Furthermore, all four of the catalogued *A. hebraeum* nucleotide sequences correspond to ribosomal RNA sequences. Therefore, the contribution of the sequence data contained in this paper, as well as likely future submissions, adds significantly to the collection of known *A. hebraeum* ESTs.

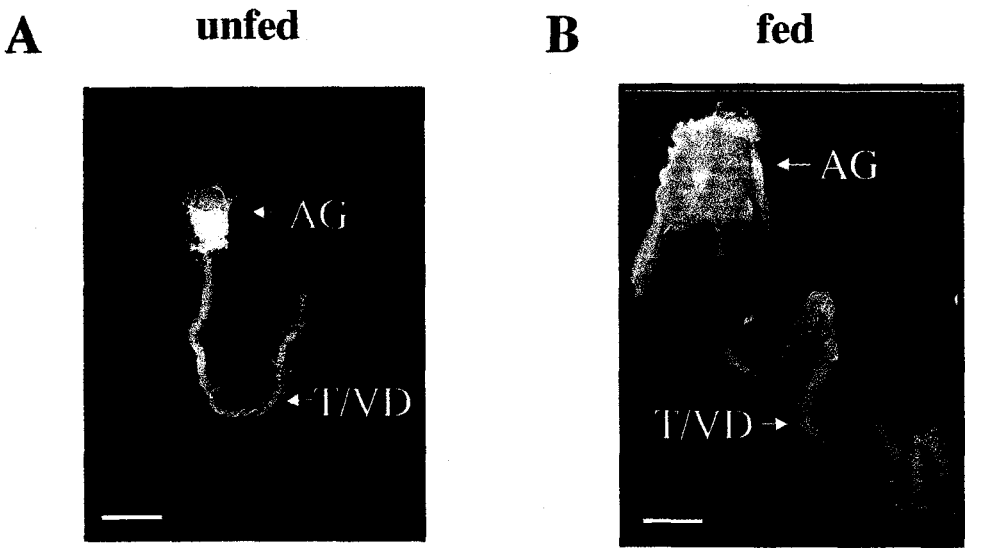
Possible orthologues of *Ah*T/VD16 and *Ah*T/VD146 are produced in the reproductive tissues of other species and play roles in cell differentiation, cell membrane permeability and ribonucleotide production. The amino acid sequence corresponding to clone *Ah*T/VD16 from our cDNA library is significantly similar (53%) to acylphosphatase, a small cytosolic enzyme widely distributed in the tissues of numerous animals (Thunnissen *et al.*, 1997; Pieri *et al.*, 1998). Southern blot analysis revealed that clone *Ah*T/VD16 hybridized to genomic DNA fragments from *A. hebraeum* and *G. argentatus*, supporting the concept that *Ah*T/VD16 represents an evolutionarily conserved DNA sequence. This enzyme is reported to serve multiple functions. Acylphosphatase is capable of hydrolyzing the phospho-aspartyl intermediate formed during the action of membrane pumps such as Na^+/K^+-ATPase and Ca^{2+} -ATPase. This action could influence the ion concentrations across cell membranes by increasing the turnover rate of these enzymes (Nassi *et al.*, 1991, 1993; Thunnissen *et al.*, 1997). Kaufman *et al.* (1976) demonstrated that Na^+/K^+-ATPase activity in the salivary glands of female *A. hebraeum* increased with feeding duration. Although undocumented in the reproductive system of ticks, we speculate that ATPase activity in the male gonad may also increase as the result of feeding and in conjunction with spermatozoa maturation. This putative feeding-induced increase in reproductive system ATPase activity may correlate with the up-regulation of acylphosphatase.

Recent studies demonstrate that increases in acylphosphatase expression are accompanied by ribonucleotide production (Paoli *et al.*, 2000) and cell differentiation in many cell lines including germ cells (Chiarugi *et al.*, 1997). In ticks, germ cell differentiation is complete after spermatogenesis and spermiogenesis (Oliver and Brinton, 1972; 1973; Feldman-Muhsam and Filskie, 1976). In ticks *Ah*T/VD16 hybridized to a 1.5 kb transcript that was observed in all tissues examined, and a pair of transcripts (6.0 kb and 3.6 kb) that represent fed T/VD specific messages (Fig. 3-5A). The two fed T/VD

specific transcripts may underlie a specific function pertaining to T/VD and germ cell development.

The amino acid sequence of clone *Ah*T/VD146 is similar (44%) to a predicted 9.0 kD basic protein from *D. melanogaster*. In *D. melanogaster* the 550 bp gene encoding this protein is located in region c550 at position 88B on the polytene chromosome (Ghabrial *et al.* 1998). Analysis of the putative amino acid sequence revealed that both proteins are hydrophilic. Furthermore, the predicted tertiary structure of these two proteins is virtually identical (Table 3-1). The function of these proteins is unknown. Our cDNA library is the first one reported from any tissue of *A. hebraeum* and it's screening represents the initial steps in the characterization of the molecular phenotype of the fed T/VD. As mentioned earlier, MF bio-activity has been found only in the fed T/VD (Harris and Kaufman, 1984; Lomas and Kaufman, 1992a). Thus, transcripts hybridizing to clones *Ah*T/VD16 and *Ah*T/VD146 in tissues other than the T/VD are not likely to be candidates for EF and MF. The three transcripts exclusive to the fed T/VD (6.0 kb and 3.6 kb transcripts detected by clone *Ah*T/VD16 and the 2.7 kb transcript detected by clone *Ah*T/VD146), and the 33 other differentially expressed transcripts, will be investigated to determine their possible relevance to factors secreted by the tick T/VD and their subsequent effects on female engorgement, reproduction and SG degeneration.

Fig. 3-1. Feeding-induced growth of male gonad in *A. hebraeum*. A) unfed tick, B) tick fed for 5 days. In A and B, only one of the testes is shown. Scale bar for A and B = 150 μm. C) Weight change of gonad after 5 days of feeding. Mean ± SEM and n are shown for each group. Gonads were dissected out under 1.2% NaCl (isosmotic with tick hemolymph).



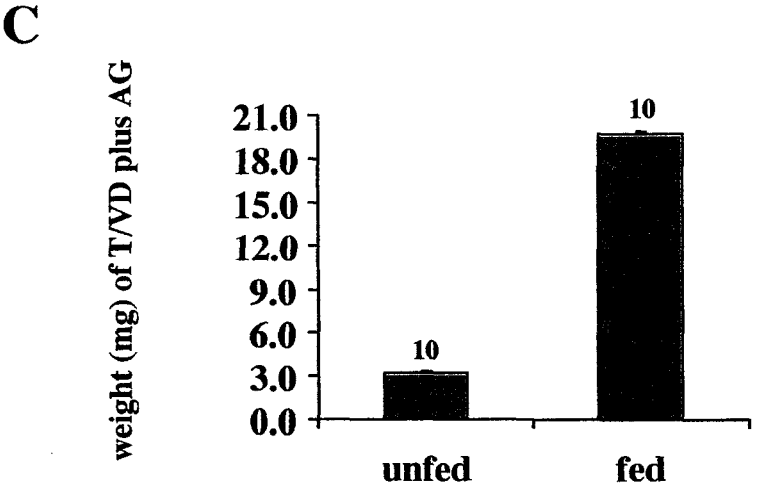
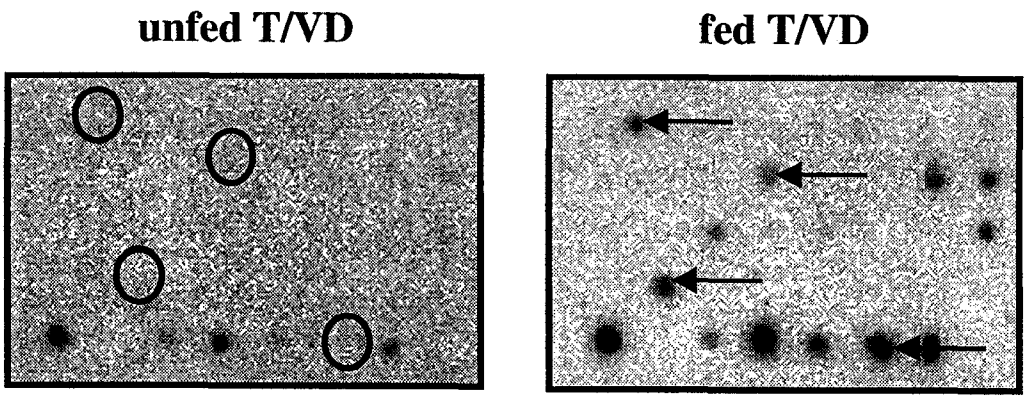


Fig. 3-2. Differential screening and PCR analysis of fed T/VD cDNA library clones. A) Secondary screening of putative fed testis cDNA clones. Each PCR-amplified cDNA clone isolated from the primary screen (not shown) was spotted onto 2 nylon membranes. The first membrane was screened with a mixed cDNA unfed T/VD probe and the second with a mixed fed T/VD cDNA probe. Clones up-regulated by feeding were kept for bioinformatic, Northern and Southern blot analysis. Four such examples are indicated by arrows. B) PCR-amplification of the 35 putative feeding induced clone inserts following the secondary differential screen. Amplified products were electrophoresed on a 1.2% agarose gel at 80 volts for 2 h. Amplification of clone inserts revealed 89% were pure (contained only 1 insert). Those with more then 1 insert were purified as stated in the Materials and Methods section (*Sequencing and sequence analysis*).

A



B

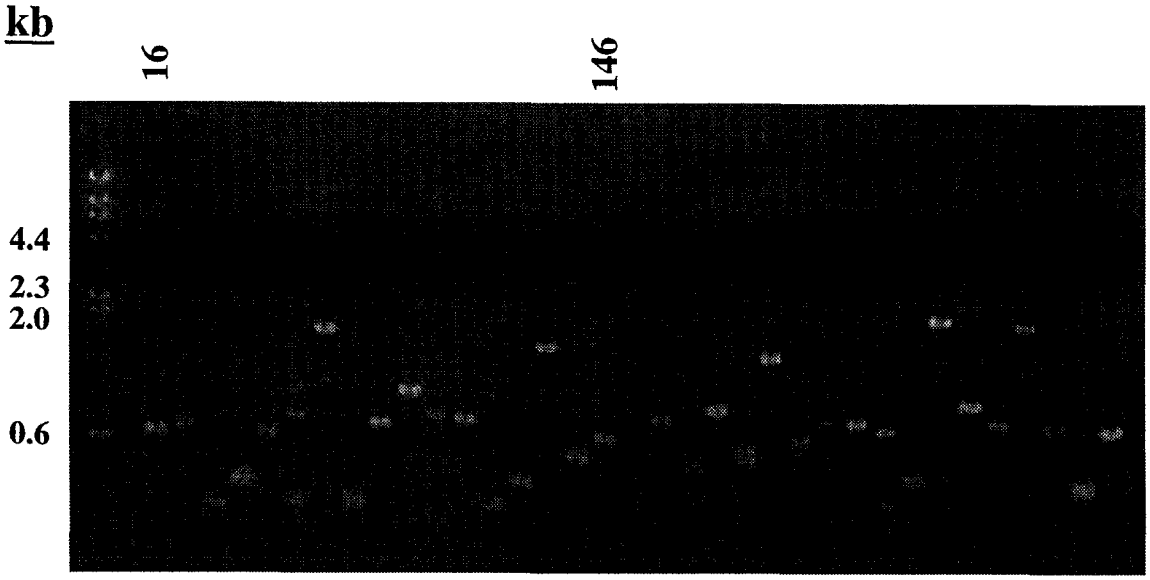


Fig. 3-3. Nucleotide and putative amino acid sequence of (A) *Ah*T/VD16 (460 kb) and (B) *Ah*T/VD146 (370 kb). The start codon (atg), stop codon (tga) and polyadenylation signals (aataa) are bolded and Kozak consensus sequences are bolded and underlined (Kozak, 1990). *Cla*I (A) and *Sau*3AI (B) restriction sites are underlined. Potential amino acid sequence post-translational modifications are as follows: protein kinase C phosphorylation sites are boxed, cAMP-dependent protein kinase A phosphorylation site, *myosin I heavy chain phosphorylation site*, *proline-dependent phosphorylation site* and caesin kinase II phosphorylation site. Upper numbers adjacent to each sequence indicate nucleotide position and bolded numbers indicate amino acid position.

A

```
agctcgccantggnaggttcgtcgacaatggtgcgcagcgc   41
                          M  V  R  S  A     5

aaattttgaagtcttcggcaaagtgcaaggtgttttcttcc   82
  N  F  E  V  F  G  K  V  Q  G  V  F  F    18

gaaagtgcaccaaggacaaggccgacagtttggggatctac   123
R  K  C  T  K  D  K  A  D  S  L  G  I  Y    32

ggatgggtccgcaacacgcgaaaaggcaccgtcgagggaat   164
 G  W  V  R  N  T  R  K  G  T  V  E  G  I   46

tatacaaggcagcacggataagatggacaccatgaagcact   205
  I  Q  G  S  T  D  K  M  D  T  M  K  H     59

ggcttagagaagttggcagcccacagtcaaccatcgataag   246
W  L  R  E  V  G  S  P  Q  S  T  I  D  K    73

tgcacctttaccaatgagaaggacattgaccaagctgaatt   287
 C  T  F  T  N  E  K  D  I  D  Q  A  E  F   87

tgaagacttcagcattaaaaagactgcctgatccacacatg   328
  E  D  F  S  I  K  K  T  A                 96

cacgactatctcgtgctccgacaggtccgcattagcatagt   369
ctgcacaagtgtcgtggagccatgcacagaaatccttatat   410
gtaaataaatatcttatgttgcacaaaaaacaaacacaaaa   451
aaaaaacc                                    460
```

B

```
tgaattaatcgactcatttcaaagacgaaaatatggtcgaa   41
                              M  V  E       3

ttagcccctccagtttccgtatcacctttcatcagggcttg   82
 L  A  P  P  V  S  V  S  P  F  I  R  A  C   17

ccggtggggctttctcgccgctggaatttttacggagcct   123
  R  W  G  F  L  A  A  G  I  F  Y  G  A     30

ttaactataggcggctatcccgcaaggaagcaaaggtgcgc   164
F  N  Y  R  R  L  S  R  K  E  A  K  V  R    44

gagtatgaagccaagcaaatggagattctcaaagtcaagag   205
 E  Y  E  A  K  Q  M  E  I  L  K  V  K  R   58

agaagcagaaaagcagaggcagacaagagaggaaatgatca   246
  E  A  E  K  Q  R  Q  T  R  E  E  M  I     71

ctctggcaaaagatgtgggcgttccgattcctccaaacttc   287
T  L  A  K  D  V  G  V  P  I  P  P  N  F    85

tgaaaagcacgtgcagcctctattcttgggatttcctagtt   328
acataataatgaagcattaaagcttgttttcttacttaaaa   369
a                                           370
```

Fig. 3-4. Putative amino acid sequence of (A) *Ah*T/VD16 compared to that of a number of other species (Genbank/EMBL accession numbers: *R. norvegicus*, P35745; *M. musculus*, P56375; *S. scrofa*, P24540; *M. gallopavo*, P00821, *H. sapiens*, NP001099), and (B) *Ah*T/VD146 compared to *D. melanogaster* 9.0 KD basic protein (AAC42662). The residues involved in the formation of the active site are boxed. Bolded residues are identical in all sequences and underlined residues indicate the positions at which conservative substitutions are found. Sequence alignment and bioinformatic analysis was obtained by using the Peptool™ software.

A *Ah*T/VD16

```
R. norvegicus  -------AEPLKSVDYEVF GTVQGVC F R MYTEGEAKKRGLVGWVK N TSKGTVTGQVQGPEEK
M. musculus        MTELLKSVDYEVF GTVQGVC F R MYTEGEAKKRGLVGWVK N TSKGTVTGQVQGPEEK
S. scrofa      -----STARPLKSVDYEVF GRVQGVC F R MYTEDEARKIGVVGWVK N TSKGTVTGQVQGPEEK
M. gallopavo   -SALTKASGALKSVDYEVF GRVQGVC F R MYTEEEARKLGVVGWVK N TRQGTVTGQVQGPEDK
O. cuniculus   -----STAGPLKSVDYEVF GRVQGVC F R MYTEGEAKKIGVVGWVK N TSKGTVTGQVQGPEDK
H. sapiens     ----------LKSVDYEVF GRVQGVC F R MYTEDEARKIGVVGWVK N TSKGTVTGQVQGPEDK
A. hebraeum          MVRSANFEVF GKVQGVF F R KCTKDKADSLGIYGWVR N TRKGTVEGIIQGSTDK

R. norvegicus  VNSMKSWLSKVGSPSSRIDRADFSNEKTISKLEYSNFSIRY
M. musculus    VDAMKSWLSKVGSPSSRIDRADFSNEKTISKLEYSDFSIRY
S. scrofa      VNSMKSWLSKIGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
M. gallopavo   VNAMKSWLSKVGSPSSRIDRTNFSNEKEISKLDFSGFSTRY
O. cuniculus   VNSMKSWLSKVGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
H. sapiens     VNSMKSWLSKVGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
A. hebraeum    MDTMKHWLREVGSPQSTIDKCTFTNEKDIDQAEFEDFSIKKTA
```

B *Ah*T/VD146

```
D. melanogaster    MSQAPVRVSPLIKFGRWSLLLVGIAYGAAHQSRLSKKEEKLREIEAQQKAVRDAKLAEEK
A. hebraeum        MVELAPPVSVSPFIRACRWGFLAAGIFYGAFNYRRLSRKEAKVREYEAKQMEILKVKREAEK

D. melanogaster    KRSAEAEARALAELSKPTPKH
A. hebraeum        QRQTREEMITLAKDVGVPIPPNF
```

Fig. 3-5. Northern blot analysis of differentially expressed clones. A) Radio-labeled clone *Ah*T/VD16 insert DNA was used to probe 3 μg/lane of total RNA from the following tissues: unfed testis/vas deferens (U), fed testis/vas deferens (FT), fed synganglion (SYN), fed Malphigian tubule (Mt), fed accessory gland (AG), fed salivary gland (SG) and fed gut. RNA was electrophoresed on 1.0% agarose-formaldehyde gels and subsequently transferred to nylon membranes. B) The same procedure using PCR product of clone *Ah*T/VD146 as a probe. 18S ribosomal RNA was used as a loading standard. Three independent densitometry readings from two autoradiographs of all hybridization signals from clones *Ah*T/VD16 and *Ah*T/VD146 were taken. Ordinate units for each densitometry graph are arbitrary. The standard deviation for each set of densitometry determinations is provided above each column.

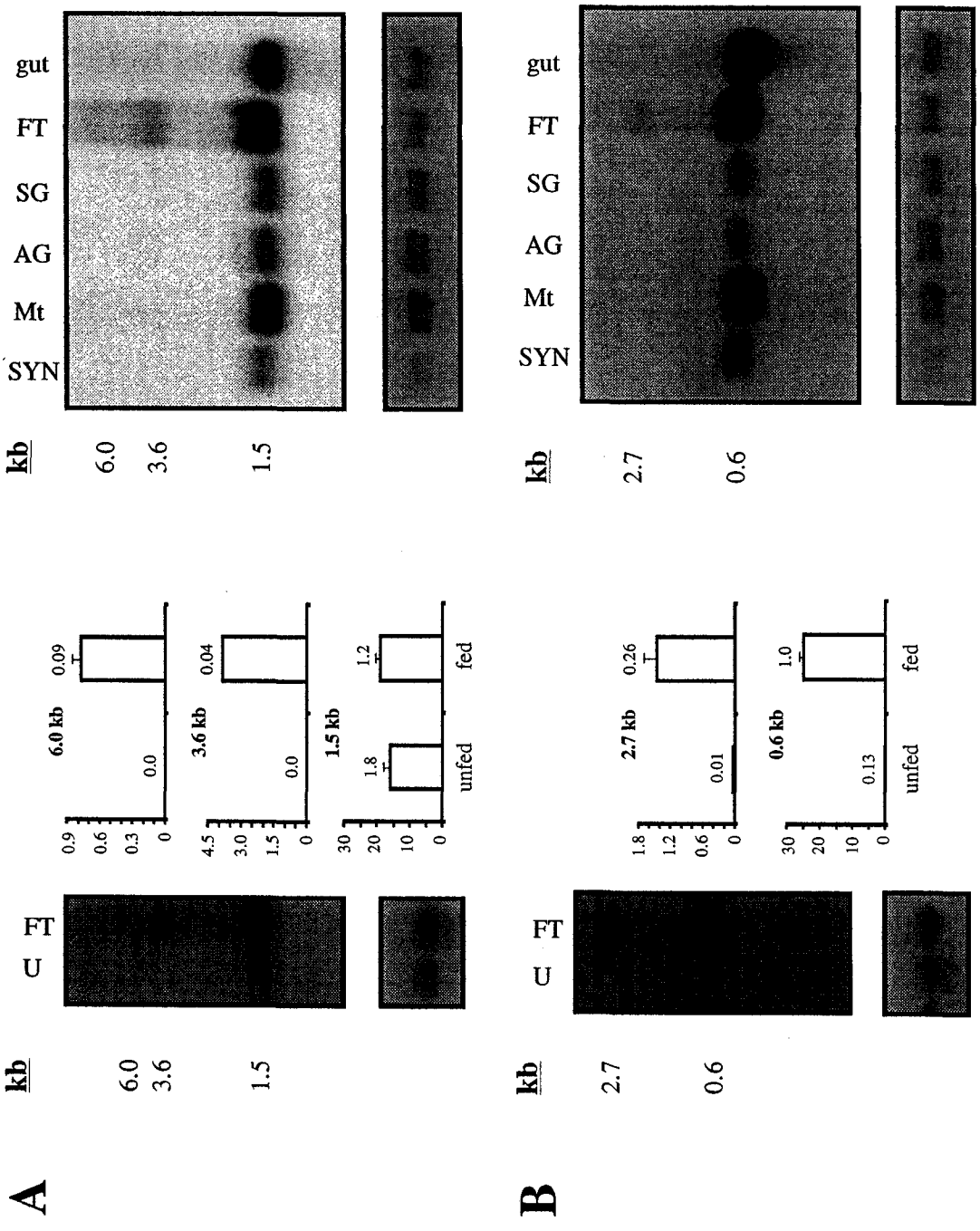
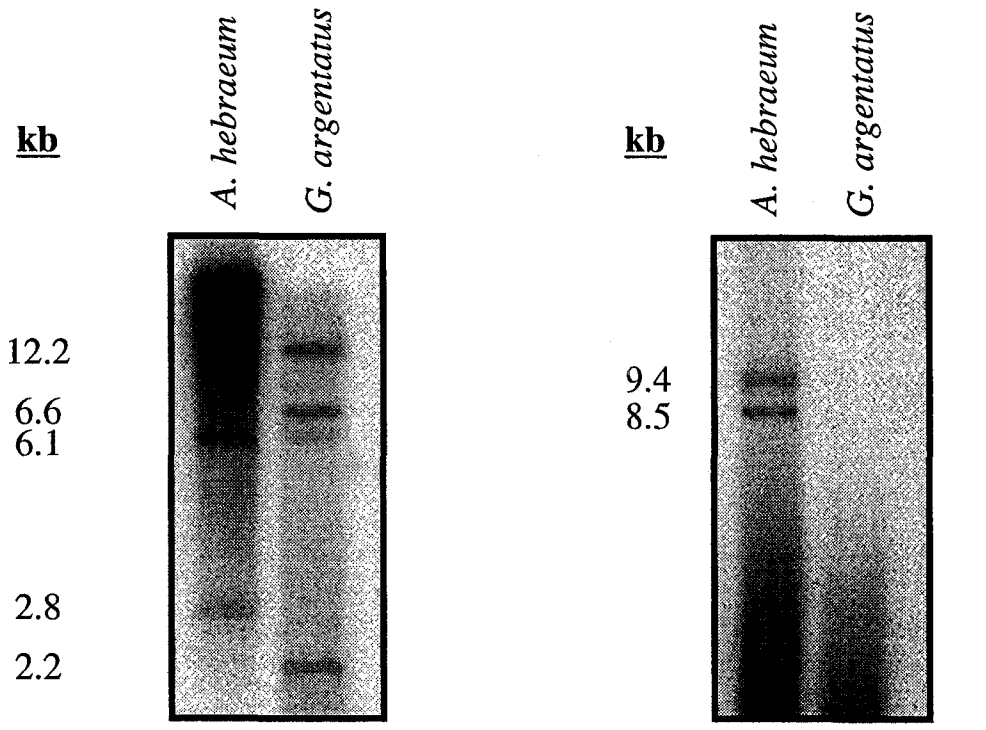


Fig. 3-6. Two preparations of genomic DNA, from seven species of arthropod, were digested to completion with either *Cla*I and *Sau*3AI. Samples were subjected to electrophoresis on 0.8% agarose gels and subsequently transferred to nylon membranes. Preparations were probed with insert DNA from clones *Ah*T/VD16 and *Ah*T/VD146, respectively, to determine the extent of sequence similarity within closely related animals. Clone *Ah*T/VD16 hybridized with two bands in *A. hebraeum* and 4 bands in *G. argentatus* (water strider), while clone *Ah*T/VD146 hybridized with two bands from *A. hebraeum*. Although some smearing was evident in the samples from the other species examined (*D. andersoni, G. morsitans morsitans, Drosophila melanogaster, A. domestica, B. gigantia*), no significant hybridization was detected (data not shown).



clone *Ah*T/VD16

clone *Ah*T/VD146

Table 3-1
Protein statistics for the putative amino acid sequences of clones AhT/VD16 and AhT/VD146 from *A. hebraeum* in comparison to similar sequences found in other species*.

Species	Molecular Weight (kD)	Hydro-phobicity (kcal/mol)	Estimated isoelectric point (pI)	Estimated radius of folded protein (Å)	Expected % alpha helix content	Expected % beta sheet content	Expected % coil content
(1) R. norvegicus	10.7	-6.3	9.4	17.7	26.0	30.2	43.8
(2) M. musculus	10.9	-5.4	9.0	17.8	25.8	32.0	42.2
(3) S. scrofa	11.3	-6.1	8.6	18.0	14.0	35.0	51.0
(4) M. gallopavo	11.3	-5.6	9.7	18.1	14.7	30.4	54.9
(5) O. cuniculus	10.9	-5.7	9.7	17.9	11.2	39.8	49
(6) H. sapiens	10.5	-6.2	9.6	17.6	11.8	41.9	46.2
(7) A. hebraeum	10.9	-6.2	9.0	17.7	24.0	28.1	47.9
(8) D. melanogaster	9.0	-6.1	10.1	16.8	75.3	0.0	24.7
(9) A. hebraeum	9.8	-3.6	10.0	17.0	75.3	0.0	24.7

* Species 1-7, acylphosphotase protein statistics (AhT/VD16); species 8-9, predicted 9.8 kD basic protein (AhT/VD146) statistics.

Acyphosphatase and *D. melanogaster* predicted 9.0 kD basic protein amino acid sequences for the above-mentioned species were obtained for protein statistic analysis from the Genbank database.

Protein statistic analysis was done using Peptool software (Biotools Inc., Edmonton, Canada).

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Chapter 4. A combination of two feeding-induced proteins from the male reproductive system trigger engorgement in the female tick, *Amblyomma hebraeum*

I) INTRODUCTION

Adult female *Amblyomma hebraeum* (an African cattle tick) require 10-14 days to feed to repletion. The feeding cycle consists of three phases: 1) a preparatory feeding phase (1-2 days), during which the female inserts her mouthparts into the host epidermis, establishes a feeding lesion and secretes a cement-like cone to securely attach herself to the skin, 2) a slow feeding phase (7-10 days), during which the female feeds to approximately 10x her original unfed weight, and 3) a 24-36 h rapid feeding phase, during which the female increases her weight a further 10-fold, so that at engorgement she weighs approximately 100x her original unfed weight (Balashov, 1972).

In *A. hebraeum,* the transition weight (10x the unfed weight) between the slow and rapid phases of feeding was named the 'critical weight' (CW) by Harris and Kaufman (1984). The CW, which varies somewhat depending on which parameter is used to measure it (Weiss and Kaufman, 2001), is characterized by some marked behavioral and physiological changes (Kaufman and Lomas, 1996). If a virgin or mated female is removed from a host while still below the CW, she: 1) will re-attach to a new host if given the opportunity, 2) will not resorb her salivary glands (SGs), and 3) will not lay a batch of eggs. A mated female, on the other hand, if removed from the host having exceeded the CW: 1) will not resume feeding even if given the opportunity, 2) will resorb her SGs within 4 days, and 3) will lay a batch of eggs, the size of which depends on the amount of blood she consumed before removal, and die. Recent observations from our lab indicate that ~ 90-95% of virgin females do not exceed the CW even if left on the host for a few weeks. But if a virgin is forcibly removed from the host when above the

CW, she 1) will not re-attach to another host if given the opportunity, 2) will resorb her SGs within 8 days (Lomas and Kaufman, 1992a), and 3) will oviposit a batch of infertile eggs, and die. Harris and Kaufman (1984) demonstrated that the difference between mated and virgin females is due to a substance (male factor; MF) passed to the female in the spermatophore. MF is a protein produced in the testis/vas deferens (T/VD) portion of the male gonad, but is not associated with the spermatozoa (Lomas and Kaufman, 1992b).

Pappas and Oliver (1971; 1972) demonstrated that the stimulus for rapid engorgement in *Dermacentor variabilis* is an 'engorgement factor' (EF), also produced by the male gonad and passed to the female during copulation. EF was not associated with the spermatozoa, because females engorged normally when mated with males in which the spermatozoa were killed by cobalt-60 irradiation.

For some time we have suspected that MF and EF are the same protein. As the synthesis of MF is markedly stimulated in fed males, Weiss *et al.* (2002) made a cDNA library from the T/VD portion of the fed male gonad, and used a differential cross-screening approach to identify the feeding-induced genes in this tissue. We identified 35 such genes, only two of which have sequences similar to those catalogued in the GenBank database.

For this study we successfully expressed 28 of the 35 up-regulated genes in an insect-based expression system (InsectSelect kit; Invitrogen Co.). This system was chosen to produce the recombinant proteins ($_r$proteins) because: 1) The expression vectors used (pIB/V5-His and pIB/His A, B and C) are plasmids genetically engineered to contain the highly active *OpIE2* promoter. *OpIE2* is located upstream from the vector's multiple cloning site and utilizes the host cell's transcriptional machinery to activate $_r$protein expression (Theilmann and Stewart, 1992). The promoter, which is from a

baculovirus whose natural host is the Douglas fir tussock moth, induces an exceptionally high level of $_{r}$protein expression. 2) The *Sf*21 cell line used to make the $_{r}$proteins is hardy and easily maintained in culture. The *OpIE2* promoter is often used to induce high levels of constitutive protein expression in this cell line (Hegedus *et al.,* 1998). 3) Recombinant tick proteins produced in insect cells (as opposed to mammalian cells) might have a higher probability of undergoing any post-translational modifications necessary to confer bio-activity.

Following their synthesis, the 28 $_{r}$proteins were tested in an EF bioassay; two of them were necessary for the expression of EF bio-activity. Further results presented here also support our hypothesis that MF and EF are the same substance.

II) MATERIALS AND METHODS

Ticks

Adult *A. hebraeum* Koch were obtained from our laboratory colony, which is maintained in darkness, at 26°C and >95% relative humidity. Ticks were fed partially on rabbits as described by Kaufman and Philips (1973), and removed for experimentation while still below the CW.

Isolation of feeding-induced genes

All inserts used in this study were identified previously by differentially cross-screening a cDNA library made from the T/VD of fed male *A. hebraeum*. Their differential status was confirmed by Northern blot analysis as described by Weiss *et al.* (2002).

Construct design and preparation

Prior to experimentation, all constructs used in this study were drafted using the computer program Gene Construction Kit 2 (SciQuest Inc., Research Park, NC). All PCR primers, designed used Genetool software (Biotools Inc., Edmonton, Canada), were engineered with 5'-*Eco*RI and 3'-*Xho*I restriction endonuclease cut sites (Invitrogen Co., Carlsbad, CA). Appendix 5 contains the sequences of all primers used in these experiments.

PCR of cDNA inserts

PCR was performed on all clones containing inserts having complete open reading frames (28 of the 35 clones up-regulated by feeding). A 5-µl sample of each plaque was added to a 95-µl reaction mixture containing ddH_20, dNTP's (200 µM), PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, 50 mM $MgCl_2$), the appropriate above-

mentioned PCR primers (0.5 μM) and 10 units of a combination of Taq and Pfu (10:1) enzymes. PCR was conducted using an Eppendorf (Westbury, NY) thermal cycler. The amplification program consisted of a 3-min hotstart at 94°C, followed by 30 cycles at 94°C for 1 min (DNA denaturation), 50°C for 1 min (annealing of primers), 72°C for 2.5 min (DNA elongation) and a final elongation/extension at 72°C for 7 min. Amplified products were verified by agarose gel electrophoresis, and appropriately sized bands extracted using a Qiagen gel extraction kit according to the manufacturers protocol.

Cloning

Basic cloning protocols are modified from Ausubel *et al.* (1994). Five microlitres (~1 μg) of purified insert and vector DNA (pIB/V5-His or pIB/His; Invitrogen Co.) were added to separate 40-μl restriction reactions containing 5 μl of 10x restriction buffer, 1 μl (10 U) of *Eco*RI and *Xho*I restriction endonuclease (Gibco-BRL, Rockville, MD) and 33 μl of ddH_20. Following a 2 h incubation at 37°C, samples were electrophoresed on a 1% agarose gel and bands extracted as mentioned above. Ligation reactions (10 μl) were set up containing the following reagents: 3 μl digested insert DNA, 1 μl digested vector DNA, 5 μl 2x ligation buffer and 1 μl T4 DNA ligase (3 Weiss U; Gibco-BRL). Reactions were incubated for 1 h at room temperature (or overnight at 4°C).

Constructs were propagated in DH5α competent cells (Gibco-BRL). Between 1-3 μl of each ligation reaction were added to a 50-μl aliquot of DH5α competent cells. Reactions were incubated on ice for 30 min, heat-shocked for 20 sec at 37°C and returned to ice for 2 min. S.O.C. medium (Gibco-BRL; 950 μl) was added to each reaction mixture. Reactions were placed in a shaking incubator at 37°C for 1 h at 225 rpm.

Subcloned plasmid constructs were isolated using a Qiagen plasmid mini-prep kit according to the manufacturer's protocol. All purified plasmids were subjected to *Eco*RI

and *Xho*I restriction endonuclease digestion followed by electrophoresis on 1% agarose gels to verify the presence of insert and vector DNA (Fig. 4-1).

Sequencing and sequence analysis

All subcloned plasmids were sequenced using a DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia, Piscataway, NJ). Sequencing reaction products were run on a PE Applied Biosystems 377 automated sequencer. Sequence data were analyzed using Genetool and Chromatool™ software (Biotools Inc.) to confirm that all inserts were ligated into the vector in the proper open reading frame (ORF).

Production and detection of $_r$proteins from feeding-induced T/VD genes

Transfections

*Sf*21 cells were maintained in culture prior to transfections. The technique used to culture these cells is presented in more detail in Appendix 6. At time of transfection, cells were plated at 60-80% confluency in 60 mm cell culture dishes and left undisturbed for 30 min to allow adhesion to the dish.

Liposome/DNA complexes were all formed in serum-free medium according to the manufacturer's protocol (Invitrogen Co.). Briefly, 1 μg (~ 10 μl) of purified plasmid DNA (construct containing the gene of interest), and 7.5 μl of Cellfectin reagent, were each diluted into separate 100-μl aliquots of serum-free medium (Sf-900 II serum-free medium (SFM); Gibco-BRL) and allowed to stand for ~10 min at room temperature. The contents of both tubes were then mixed together and incubated at room temperature for ~20 minutes. Positive (pIB/V5-His CAT) and negative (no liposome) control transfections were also performed. Sf-900 II SFM (800 μl) was added to each tube containing newly formed liposome/DNA complexes. Each dish of cells was washed with

2 ml of Sf-900 II serum-free medium and gently overlayed with liposome/DNA complex. Dishes were incubated for 7-10 h at 27°C. Following the incubation, the transfection solution was removed and replaced with 2 ml of serum-containing cell culture medium. All dishes containing transfected cells were placed in an airtight plastic bag containing moist paper towel to inhibit evaporation.

Detection of $_r$proteins

Expression products were harvested 48 h post-transfection. Medium from each transfection dish was stored at -80°C prior to assay for secreted proteins by Western blot analysis. Cell lysis buffer (100 µl; 50mM Tris pH 7.8, 150mM NaCl, 1% (v/v) Igepal CA-630) was repeatedly streamed over cells until all were sloughed from bottom of the dish. Complete lysis was assured by vortexing rapidly for 15 sec, and cellular debris was pelleted at 10,000x g for 15 min at 4°C.

Protein concentration of culture medium and cell lysis supernatant was determined by a Bradford assay (Bradford, 1976) using bovine serum albumin as standard. Lysate containing 30 µg of protein was combined with 4x SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 50% glycerol, 0.02% bromophenol blue, Sigma) and heated at 95°C for 5 min. Samples were electrophoresed in 1x SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) for approximately 90 min through 3% stacked, 12% continuous seperating polyacrylamide gels. Protein bands were visualized by staining the gels for 2-24 h with coomassie brilliant blue (Sigma, St. Louis, MO) dissolved in 40% methanol/10% acetic acid.

Recombinant protein production was confirmed by Western blot analysis (Fig. 4-2A; only data from the two $_r$proteins subsequently found to have EF bio-activity are shown). Proteins were electrophoresed as described above. Polyacrylamide gels and 0.2

μm nitrocellulose membranes (BioRad, Hercules, CA) were equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (w/v) methanol, pH 8.3) for 5 min. Proteins were blotted onto the membranes at 100V for 1 h, and protein transfer was confirmed by reversible staining with Ponceau S (Sigma). Following protein visualization, Ponceau S stain was removed by washing blots with milli-Q water. Nitrocellulose membranes were incubated in blocking buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 3% (w/v) ovalbumin, 0.1% (v/v) Triton X-100, 0.1% (w/v) NaN_3) for 30 min at room temperature. Old blocking buffer was removed and the membrane was covered with anti-6x histidine antibody (diluted at 1:3000 in fresh blocking buffer). Nitrocellulose membranes were incubated on a rocking platform for 2 h at room temperature, or overnight at 4°C.

Protein bands were visualized using a goat anti-mouse secondary antibody conjugated to an IRDye 800 (a near-infrared fluorophore). Following the removal of anti-6x histidine primary antibody solution by washing 4x 15 min in Tween-20/Tris-buffered saline (TTBS: 0.1% Tween-20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5), nitrocellulose membranes were again blocked in 10 ml blocking buffer for 20 min. Fluorescently-labeled secondary antibody was then diluted 1:2500 in blocking buffer and added to the nitrocellulose membrane. Following a 1-h incubation at room temperature on a rocking platform, non-bound secondary antibody was removed by washing 4x with TTBS (incubation with secondary antibody and all subsequent wash steps were performed in the dark). Protein bands were visualized using a LI-COR Odyssey infrared imaging system.

EF bioassay

Unfed virgin females were placed on rabbits along with a number of fed males which had their gonopores blocked with a small drop of cyanoacrylate glue. The presence of fed males strongly induces females to attach. Females were allowed to feed for 7 days,

at which point they were all below the CW (~ 250 mg in *A. hebraeum*). Individuals were divided into the treatment groups shown in Table 4-1 and identified by colored thread tied to a leg segment. All injections were made into the hemocoel via a coxal leg segment, using a 30-gauge needle attached to a Hamilton microlitre syringe. Following injection, ticks were allowed up to 14 days to feed on fresh rabbits (except in the initial experiment (Fig. 4-4) in which only 7 days were allowed). During this time any engorged females were weighed and stored in the colony incubator. All ticks still attached at 14 days were removed, weighed and stored in the colony incubator.

Following removal, some ticks were dissected at 4 days to measure SG degeneration and others at 10 days to measure ovary development. SG degeneration was determined by measuring rate of fluid secretion *in vitro* as described by Harris and Kaufman (1984). Ovary development was assayed by ovary weight, and compared to data reported for normally engorged females by Friesen and Kaufman (2002).

Bioassay of crude T/VD homogenates

A partially purified tissue extract of EF was prepared as follows. T/VD of fed males were dissected, homogenized (using glass tissue homogenizers) in chilled saline (1.2% NaCl; 7.5 µl per T/VD) and centrifuged at 8,000 g for 5 min at 4°C. The pellet was discarded and the supernatant stored frozen at -80°C until required for injection. Partially fed females (all below the CW) were injected with several doses of the partially purified T/VD extract. Control groups were injected with nothing, or 1.2% NaCl, or with one accessory gland equivalent from a fed male, or with one T/VD equivalent from an unfed male. Injected females were applied to a fresh rabbit and checked regularly over the next 7 days.

Bioassay of the 28 rproteins

The 28 rproteins (shown in Table 4-1) were initially divided arbitrarily into two groups, each containing 14 rproteins. Ticks were injected with one or the other group, but EF bio-activity was not detected in either. This negative result suggested that at least two proteins were necessary for EF bio-activity, one of them being among rproteins 1-14 and the other being among rproteins 15-28. Subsequent groupings of rproteins were tested in order to eliminate those without EF bio-activity. The following control injections were also performed: 1) non-transfected cell lysates, and 2) 5 μg of vector DNA (both pIB/V5-His and pIB/His C). The groupings used, and the bioassay results (which show the mean weight (± SEM) as a function of the indicated treatment), are shown in Table 4-1. All injections contained 10 μg of total protein (but were of differing quantities) from *Sf*21 cell lysates from each separate transfection reaction.

*Bioassay of purified r*Ah*EF*

The two rproteins necessary for EF bio-activity were purified from cell lysates using a HisTrap 6x-histidine binding column according to the manufacturer's protocol (Amersham-Pharmacia Biotech, Quebec, Canada) and purity confirmed by SDS-PAGE (Fig. 4-2B). Purified protein was stored at -20°C in elution buffer containing 500 mM imidazole.

A dose response curve of the two rproteins was performed (0.0-1.0 μg of each rprotein) using the EF bioassay. Two controls were 1) normally-mated females and 2) normally-mated females receiving 7.5 μl of 500 mM imidazole.

/*Statistics

All data are expressed as mean ± SEM. Statistical significance was determined by Student's t-test using Microsoft Excel software, or one-way analysis of variance (ANOVA) using Statview software, on a Macintosh computer.

III) RESULTS

Differential expression of EF RNA transcripts

Differential expression of RNA encoding the two feeding-induced proteins ultimately found to contain EF bio-activity was confirmed by Northern blot analysis. In both cases RNA encoding these proteins was significantly up-regulated as the result of feeding. RNA from the SGs of fed males was also subjected to Northern blot analysis as a control to confirm tissue specificity (Fig. 4-3).

Bioassay of T/VD homogenates for EF bio-activity

T/VD from fed males induced virgins to feed beyond the CW (250 mg) for this population of *A. hebraeum* (Fig. 4-4). The difference in fed weight among the three doses (431 ± 129, 510 ± 43 and 528 ± 61 mg) was not statistically significant ($P > 0.05$; ANOVA). The difference in weight among the four controls (231 ± 11, 284 ± 21, 181 ± 6 and 192 ± 9 mg) also was not statistically significant ($P > 0.05$; ANOVA). However, all of the ticks receiving 0.5-1.5 T/VD per female fed significantly more (495.6 ± 42 mg; n=52) than any of the control groups (231.8 ± 10.9 mg; n=32; $P < 0.0001$; Student's t-test).

Bioassay of the 28 $_r$proteins

Table 4-1 presents the sequence of bioassays performed to identify the two $_r$proteins that together are necessary for EF bio-activity. Data in this table shows that the average weight of virgins at time of injection was always below the CW (~ 250 mg). EF bio-activity is indicated in instances where the average tick weight at time of detachment exceeds about 1000 mg. This occurred only when virgins were injected with group 6 ($_r$proteins 8-14 plus 21-28), group 10 ($_r$proteins 8-14 plus 21-24), group 13 ($_r$proteins 8-

10 plus 21-22) and finally group 20 ($_r$proteins 9 and 22). Results from group 1 and group 2 demonstrate that neither $_r$protein 9 nor $_r$protein 22, when isolated from each other, had significant EF bio-activity. Thus, both $_r$proteins are necessary and sufficient for EF bio-activity. Henceforth, $_r$9 is designated as '$_r$*Ah*EFα' and $_r$22 as '$_r$*Ah*EFβ', and the two together as '$_r$*Ah*EF'.

Table 4-1 also demonstrates that $_r$*Ah*EF possesses MF activity because it triggers SG degeneration in 4 days and stimulates a significant degree of ovary development within 10 days. Fluid secretory competence of SGs from virgins injected with $_r$*Ah*EF (groups 6, 10 and 13) averaged 0.36 ± 0.06 mg/gland/15 min (n=31), a value expected for glands that have undergone complete degeneration (Harris and Kaufman, 1981). Fluid secretory competence of SGs from ticks injected with all other groups averaged 4.2 ± 0.19 mg/gland/15min (n=58). Furthermore, virgins injected with $_r$*Ah*EF showed a significant degree of ovarian development; 16.1 ± 1.0 mg (n=12) 10 days post-engorgement as compared to 1.9 ± 0.1 mg (n=32) in virgins not injected with $_r$*Ah*EF. Finally, latency to oviposition in $_r$*Ah*EF-injected virgins averaged 15.3 ± 1.4 days (n=7) and total egg mass size averaged 25% of initial engorged weight (n=7; Fig. 4-5). These values were significantly different from those reported for normally mated females by Friesen and Kaufman (2002).

Recombinant *Ah*EFα (16.1 kD) is a protein encoded by a 333 nucleotide ORF and contains 111 amino acids. The ORF, with a start codon (atg) at position 125 and a stop codon (tag) beginning at position 458, is followed by a polyadenylation (polyA+) signal at position 560. The ORF constitutes 57% of a 580 nucleotide insert from clone *Ah*T/VD

9 (Fig. 4-6A). Recombinant *Ah*EFβ (11.6 kD) is a protein encoded by a 213 nucleotide ORF and contains 71 amino acids. The ORF, with a start codon (atg) at position 104 and a stop codon (tga) at position 317, is followed by a polyA+ signal at position 400. The ORF constitutes 42% of a 509 nucleotide insert from clone *Ah*T/VD 22 (Fig. 4-6B). A database search using the basic alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) and the cDNA and putative amino acid sequences of $_{r}$*Ah*EFα and $_{r}$*Ah*EFβ revealed no significant homology with any catalogued genes or proteins.

Purification and bioassay of $_{r}$AhEF

Recombinant *Ah*EFα and $_{r}$*Ah*EFβ were purified from their respective cell lysates by elution from a 6x histidine-binding column (see Materials and Methods). Analysis using NIH image software (http://rsb.info.nih.gov/nih-image/) of the bands from lane E3 on both gels (Fig. 4-2B) indicates that $_{r}$*Ah*EFα and $_{r}$*Ah*EFβ constitutes ~ 89% and ~ 96% respectively of the total protein after passing through the column.

Purified $_{r}$*Ah*EF also stimulated engorgement, SG degeneration and a limited degree of ovarian development in virgin females below the CW. Data in Fig. 4-7A shows that virgins injected with 1.0, 0.3, 0.1 and 0.03 μg of pure $_{r}$*Ah*EF fed significantly more (1534.7 ± 100.9 mg; n=35) than those injected with 0.01 and 0.003 μg of pure $_{r}$*Ah*EF (498.4 ± 19.9 mg; n=12; $P < 0.0001$; Student's t-test), thus suggesting that the latter two doses are not potent enough to stimulate engorgement. Fluid secretory competence of SGs from virgins injected with 1.0, 0.3, 0.1 and 0.03 μg of pure $_{r}$*Ah*EF averaged 0.61 ± 0.07 mg/gland/15 min (n=21; Fig. 4-7B), a value significantly lower (thus indicating

degeneration) than SGs from virgins injected with 0.01 and 0.003 μg of pure $_r$*Ah*EF (3.5 ± 0.22 mg/gland/15 min; n=12; $P < 0.0001$; Student's t-test). Furthermore, ovary weights from the former group averaged significantly more (18.6 ± 1.33 mg; n=14) than those from the latter group (2.4 ± 0.44 mg; n=8; $P < 0.0001$; Student's t-test).

IV) DISCUSSION

Male *D. variabilis* ticks stimulate engorgement in co-feeding females by transferring a factor (EF) to them during copulation (Pappas and Oliver, 1971; 1972). In this study we demonstrate that extracts of T/VD from fed *A. hebraeum* males also contain EF bio-activity (Fig. 4-4). Moreover, we have identified a 'recombinant *A. hebraeum* EF' ($_r$*Ah*EF) from a group of 28 $_r$proteins produced *in vitro*. Recombinant *Ah*EF is a combination of two distinct proteins, $_r$*Ah*EFα and $_r$*Ah*EFβ. Whether these proteins are the products of two separate genes, or splice products of a single gene, is not known.

Our data in Table 4-1 and Fig. 4-7A indicates that $_r$*Ah*EF is able to induce SG degeneration, however on its own cannot stimulate a full degree of ovary development (Table 4-1, Fig. 4-5 and Fig. 4-7B). Thus, whereas mean ovary weight of virgins injected with $_r$*Ah*EF was 12.5-18 mg 10 days post-engorgement, mean ovary weights of normal mated females of this species is about 104 mg 10 days post-engorgement (Friesen and Kaufman, 2002). Moreover, the latency to oviposition was longer in the engorged virgins displayed in Table 4-1 (14-16 days) compared to normal, mated engorged females (~ 10 days; Friesen and Kaufman, 2002) and the total egg mass was significantly less than that laid by normal engorged females (25% of initial engorged weight vs. 40% respectively). Neither $_r$*Ah*EFα nor $_r$*Ah*EFβ on its own, nor any of the other 26 $_r$proteins, display EF or MF bio-activity.

Harris and Kaufman (1984) and Lomas and Kaufman (1992a) demonstrated that extracts of unfed T/VD had little, if any, MF activity. The results from our bioassay of crude T/VD homogenates (Fig. 4-4) also indicate that EF bio-activity is markedly enhanced as the result of feeding, and this is confirmed by Northern blot analysis (Fig. 4-3).

The MW of native MF, as determined by gel filtration, was reported to be in the range of 20-100 kD (Kaufman and Lomas, 1996). Western blots (Fig. 4-2A) and computer analysis using Peptool software (Biotools Inc., Edmonton, Canada) both indicate that the combined MWs of $_{r}$*Ah*EFα and $_{r}$*Ah*EFβ fall within this weight range (~27.7 kD). This MW is different from tick sperm-capacitation factor (12.5 kD; Shepherd *et al.*, 1982) and vitellogenesis-stimulating factor (100-200 kD; Connat *et al.*, 1986), the only two other known mating factors from male ticks. Native EF is likely a dimer (possibly larger than 27.7 kD) which, like other male insect sex peptides of similar size (~ 200-400 amino acids; Monsma and Wolfner, 1988; Yi and Gillott, 1999), may be cleaved into smaller subunits thus making it better able to pass into the female's hemocoel where it presumably has bio-activity.

The site and mode of action of native EF and MF have not been determined. Both factors are introduced to the female via her genital tract and both factors are effective when injected into the hemocoel. Moreover, the hemolymph of mated (but not virgin) females contains MF bio-activity (Harris and Kaufman, 1984; Lomas and Kaufman, 1992b). This suggests the following. 1) EF and MF may be transported to the hemolymph from the female genital tract, as occurs with some insect sex peptides (Yamaoka and Hirao, 1977; Smid, 1998; Lung and Wolfner, 1999). 2) EF and MF may stimulate the release of a substance from the wall of the seminal receptacle to the hemolymph (as occurs in the bloodsucking bug, *Rhodnius prolixus*; Davey, 1967) that conveys bio-activity. We may be better able to determine if one of the above mentioned mechanisms applies to EF once an ELISA is developed.

MF is characterized by its ability to hasten the onset of SG degeneration by 4 days, a process initiated by an early release of 20E (Lomas and Kaufman, 1992b). Lomas *et al.* (1997) demonstrated that a peptide produced by the synganglion stimulates integumental ecdysteroidogenesis via a cAMP-dependent second messenger system. That

the aforementioned synganglial peptide is necessary for this ecdysteroid production suggests the synganglia is likely MF's target tissue. Because we assume that EF and MF are the same substance, then EF must pass into the hemocoel to stimulate engorgement. Whether EF passes unaltered from the female's seminal receptacle into the hemocoel is unknown.

Fig. 4-1. Restriction endonuclease analysis of all constructs was performed to confirm the presence of PCR-amplified feeding-induced clone inserts. All purified constructs were digested to completion using *Eco*RI and *Xho*I restriction enzymes and then subjected to electrophoresis on 1.0% agarose gels. The first 15 inserts were cloned into the pIB/V5-His expression vector and the remaining 13 into the pIB/His expression vector (which incorporates the 6x histidine detection tag on the opposite end of the protein). The continuous line of bands across the gel at ~ 3540 kb represent vector DNA and the variably-sized bands (ranging from 211-540 kB) at the bottom of the gel represent construct inserts. The two constructs containing inserts coding for protein eventually found to have EF bio-activity are underlined.

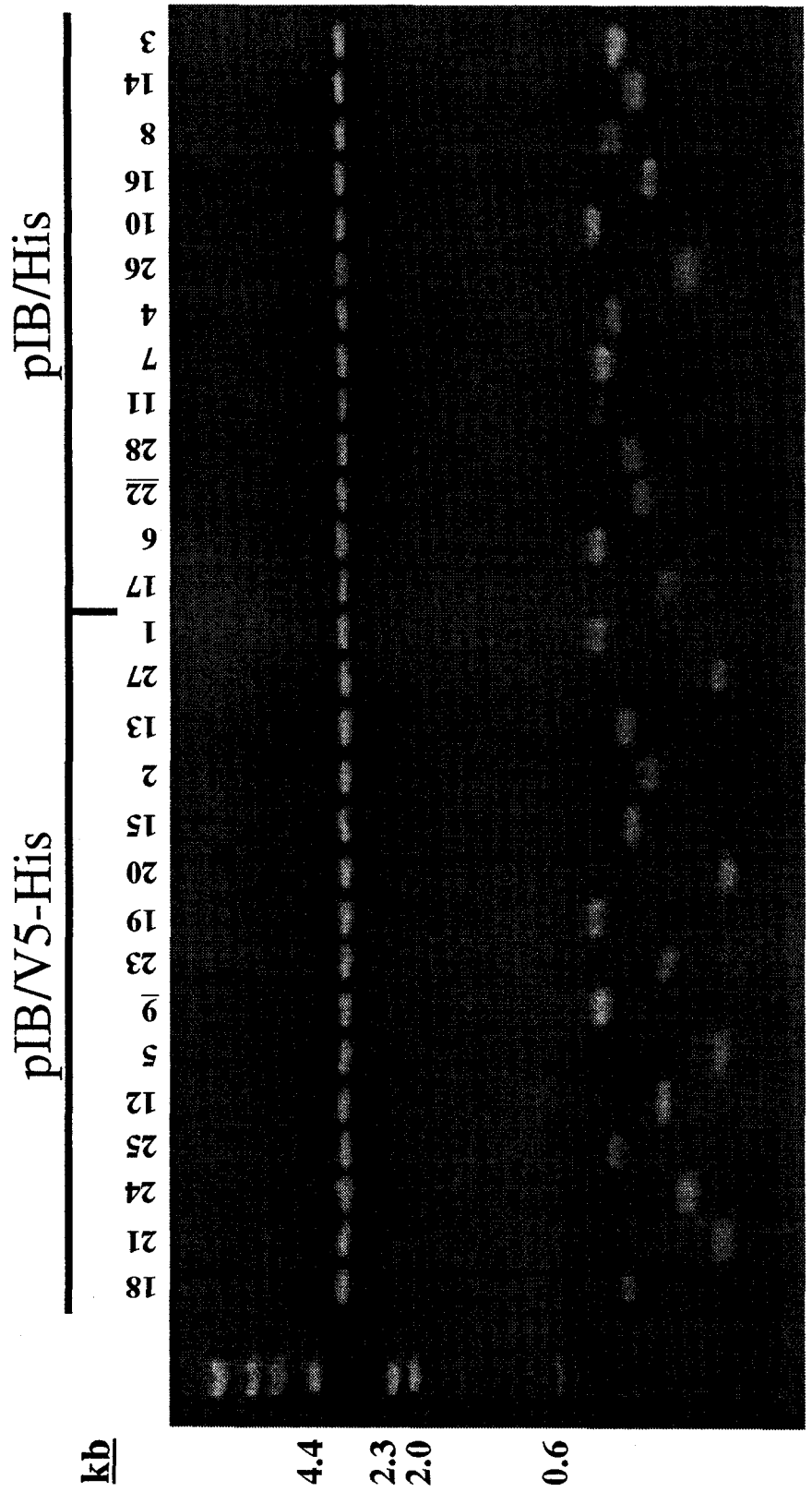

Fig. 4-2. Western blot analysis and SDS-PAGE of $_r$proteins required for EF bio-activity. A) Western blots of crude cell lysates containing $_r$*Ah*EFα and $_r$*Ah*EFβ (the expression products of constructs 9 and 22 respectively shown in Fig. 4-1). *Sf*21 cells used for transfection were lysed, centrifuged and the resulting supernatants subjected to electrophoresis on 10% polyacrylamide gels (see Materials and Methods). Proteins were transferred to nylon membranes and blots probed with an anti-6x histidine antibody. Following confirmation of $_r$protein production (by western blot analysis), and the identification of those $_r$proteins having EF bio-activity, *Sf*21 cell lysates containing the two $_r$proteins were passed through 6x histidine-binding columns. Bound $_r$proteins were eluted in five successive 1-ml fractions. B) SDS-PAGE of crude lysate (L) and the five 1-ml elutions (E1-E5), stained with Coomassie brilliant blue. In both cases E3 contained the most purified $_r$protein. Molecular weight standards on all gels are as follows (from top down): 220, 148, 98, 64, 50, 36, 16 and 6 kD.

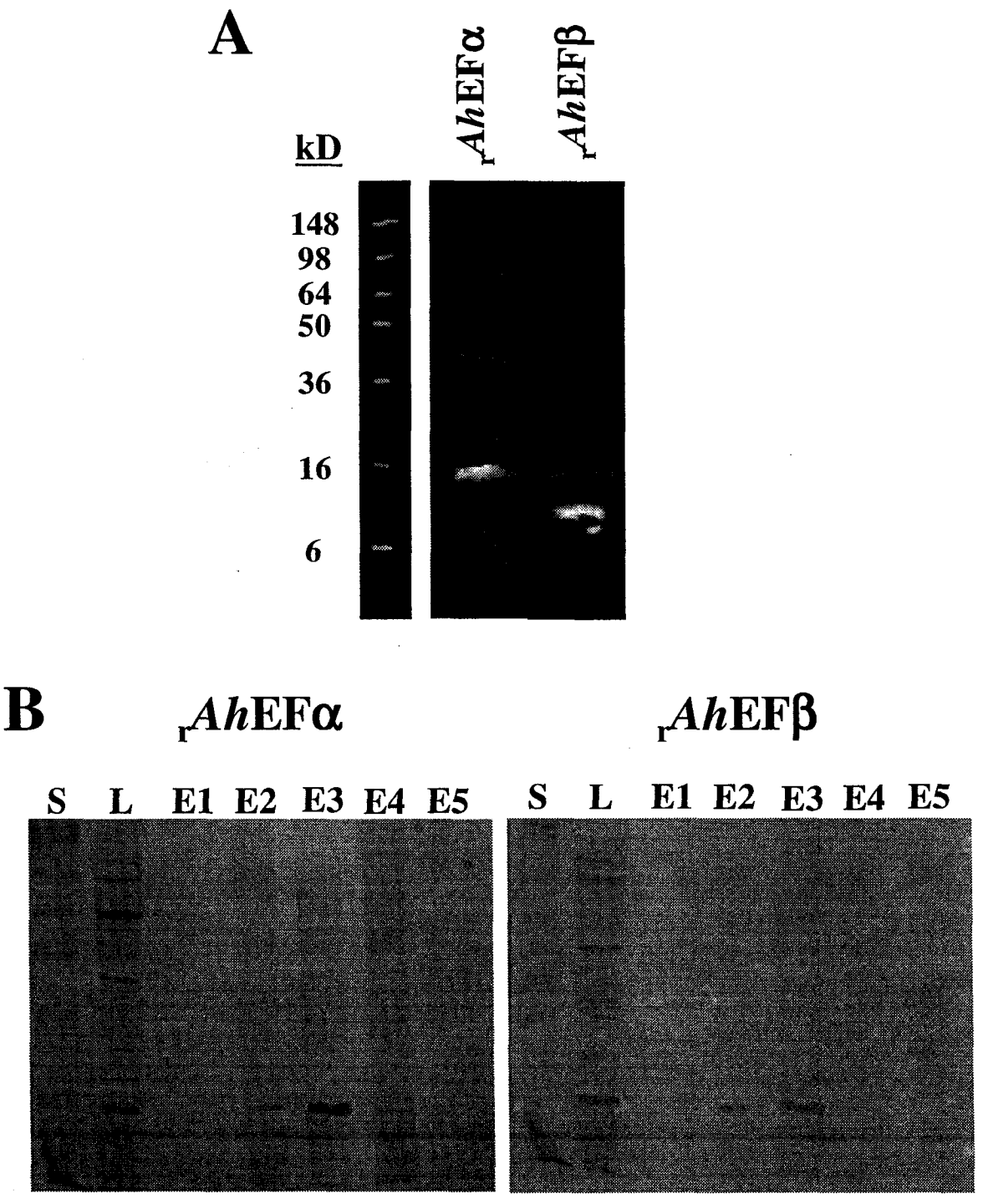

Fig. 4-3. Northern blot analysis of differentially expressed clones. A) Radio-labeled clone *Ah*T/VD 9 PCR product was used to probe 3 μg/lane of total RNA from the following tissues: fed salivary gland (SG), fed T/VD (F) and unfed T/VD (U). B) The same procedure using PCR product of clone *Ah*T/VD 22 as a probe. RNA was electrophoresed on 1.0% agarose-formaldehyde gels and subsequently transferred to nylon membranes. 18S ribosomal RNA (bottom panels) was used as a loading standard.

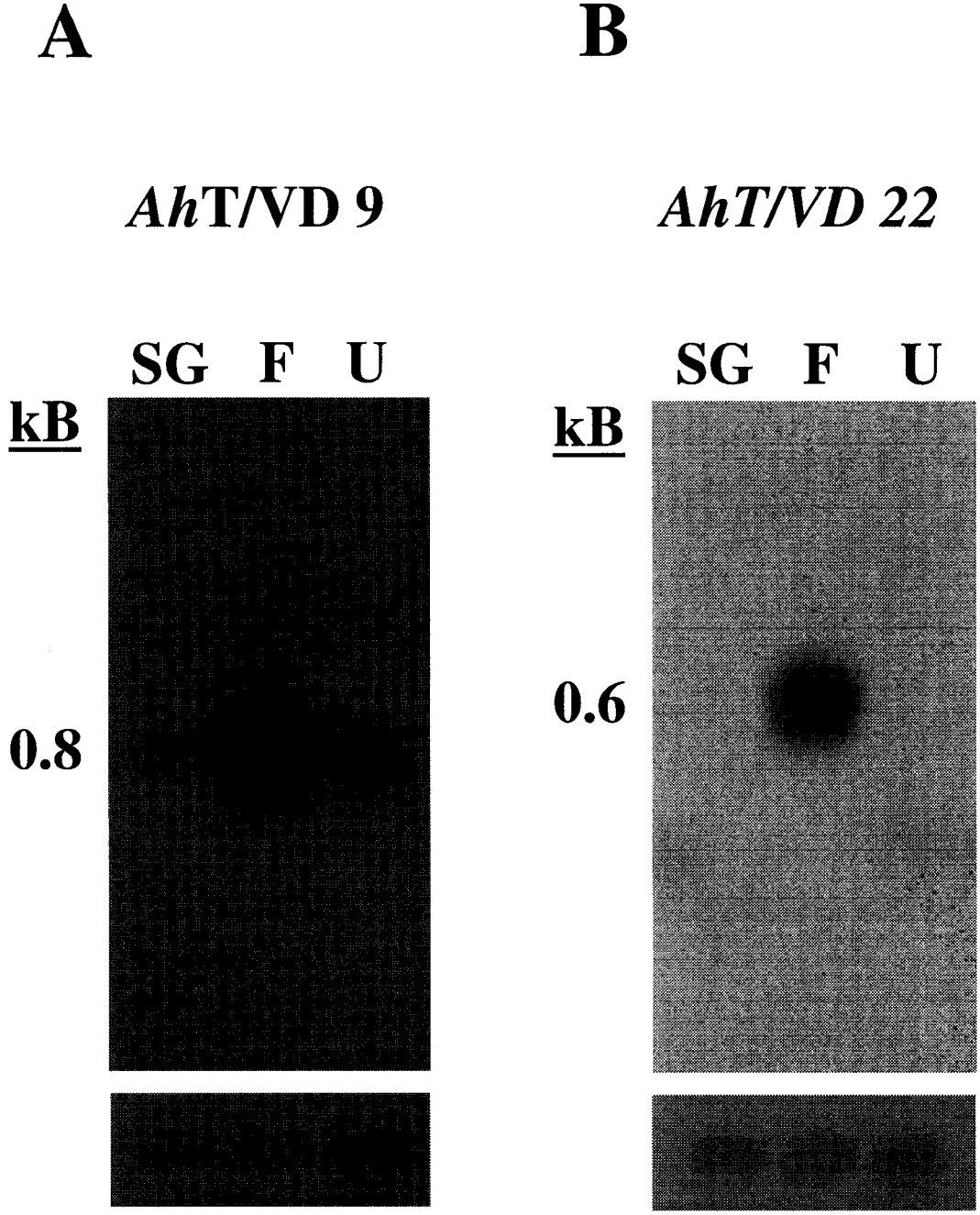

Fig. 4-4. EF bioassay using crude homogenates made from the T/VD of fed males. Virgin females injected with all three doses (0.5, 1.0 and 1.5 equivalents) of T/VD homogenate fed to significantly above the CW (~ 250 mg; indicated by dashed line) after being allowed to feed on fresh hosts for 7 days. However, those females injected with homogenates of T/VD from unfed males (one equivalent) or fed accessory gland (one equivalent) remained below the CW. Controls injected with 1.2% NaCl or nothing also remained below the CW.

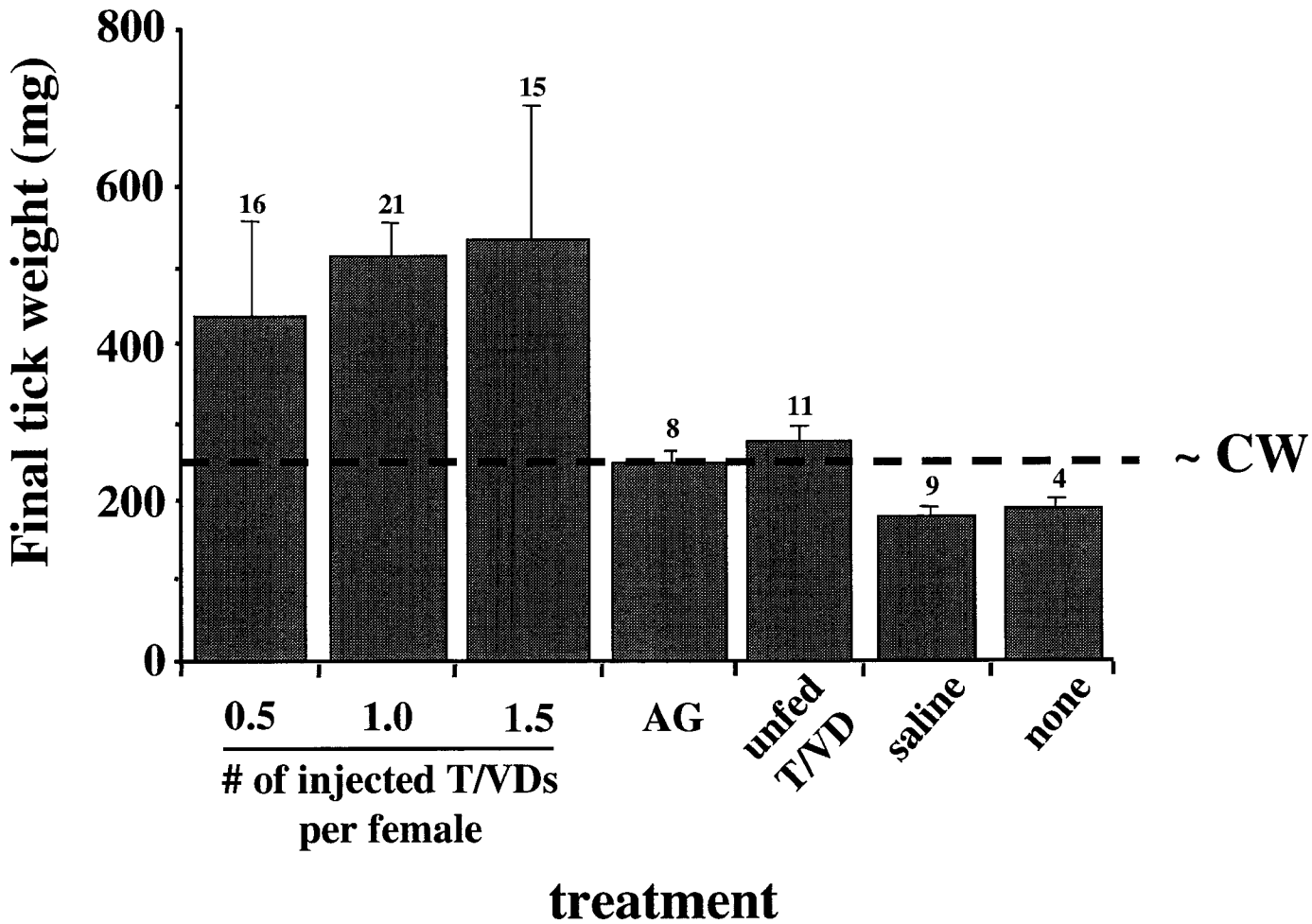

Fig. 4-5. Effects of $_{r}$*Ah*EF on egg production in *A. hebraeum*. Females injected with $_{r}$*Ah*EF were monitored to determine 1) the number of days post-engorgement which elapsed before the beginning of oviposition (latency), and 2) egg clutch size. These data were compared to that of normally mated, engorged ticks (data from Friesen and Kaufman, 2002).

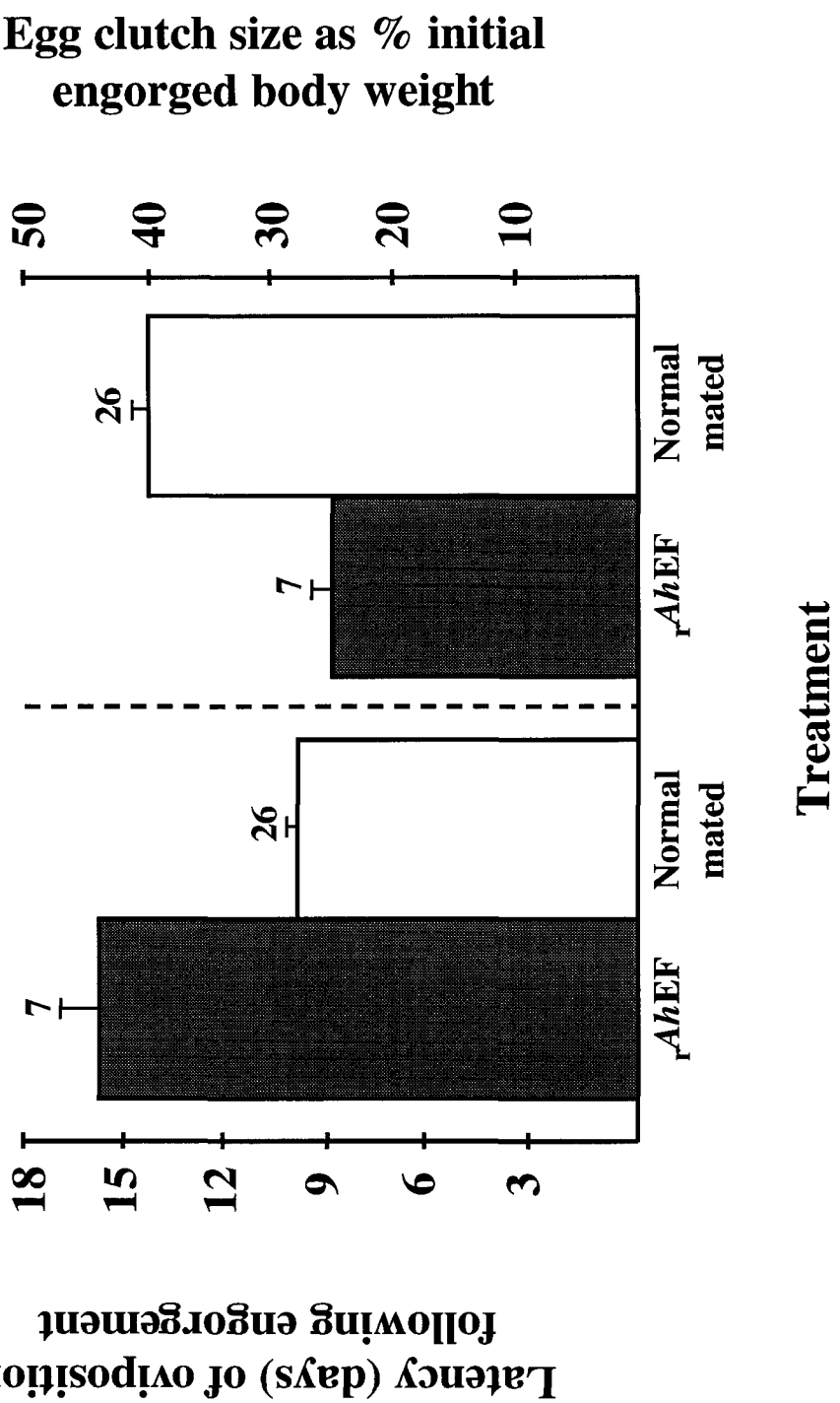

Fig. 4-6. Nucleotide and putative amino acid sequences of (A) *Ah*T/VD 9 (580 bases) and (B) *Ah*T/VD 22 (509 bases). The start codon (atg), stop codons (tag, tga) and polyadenylation signals are bolded and the Kozak consensus sequence (in B) is bolded and underlined (Kozak, 1990). Upper numbers adjacent to each sequence indicate nucleotide position and bolded numbers indicate amino acid position. Below each nucleotide sequence is a diagrammatic representation of the corresponding ,protein following expression. Recombinant *Ah*EFα, which was produced in the pIB/V5-His expression vector, has an C-terminal 6x histidine detection tag. Recombinant *Ah*EFβ was produced in the pIB/His C expression vector and has an N-terminal 6x histidine detection tag. Shaded boxes represent binding sites for other commercially available antibodies (anti-Xpress and anti-V5 monoclonals; Invitrogen Corp.) spacer regions and an enterokinase cleavage site (EK).

A

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gggagctgcgtcgctttgttccacgttgacctcgaggattcgacgggcaa 50
ctgcagcaacgcgaacacgagaaagttcgcccgccttgcggctgtggccg 100
agaacctcgtcaataacgtcaccc atg ttgatcaccaaggacctgatgca 150
                        M  L  I  T  K  D  L  M  Q  9

gaaaagtacggagaacaaaacgttctgcatcagcatcaacctggccgtcc 200
  K  S  T  E  N  K  T  F  C  I  S  I  N  L  A  V   25

tgaaattcgcaactgatgctggaaaccctggtgaccgctgcgactcggag 250
L  K  F  A  T  D  A  G  N  P  G  D  R  R  D  S  E  42

gacgaggtcgcctactcggaggtgtgccagttgaacagcgcggttccagt 300
 D  E  V  A  Y  S  E  V  C  Q  L  N  S  A  V  P  V 59

gtacgacatgaactggatgaccgcgtcactgagtgacagccggcagttct 350
  Y  D  M  N  W  M  T  A  S  L  S  D  S  R  Q  F   75

acacgttcgagaaggctgaaatgctactatctaaggtccttttcctcaag 400
Y  T  F  E  K  A  E  M  L  L  S  K  V  L  F  L  K  92

gcgtggttcccgtcgctctgcgtcgccactttccacgccgagcttgacac 450
 A  W  F  P  S  L  C  V  A  T  F  H  A  E  L  D  T 109

tggtcga tag agaggcctggcccgcattccgatcactacgcccaggctga 500
  G  R                                              111

ctcaatgtgcgcaaaggactccaatcctgctgttacatgggcgacgaaac 550
ctctgggag aataaa cgccctaaaattctc                    580
```

B

```
cctcgtgccgaattcggcacgagggatgaatccggaaatcctgcaaagta 50
cgcgaacgaggaaactctcggagtagccagctccgagcagtggctacctg 100
a ag atgg cgaaacagggacttctgaagaaagtgcgttcaccgctgccgct 150
    M  A  K  Q  G  L  L  K  K  V  R  S  P  L  P  L 16

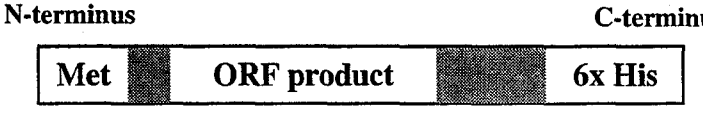
acagaaggggaagtcgaagccgagggcgctgcagcaaatgaggagccgac 200
  Q  K  G  K  S  K  P  R  A  L  Q  Q  M  R  S  R   32

tagtgcgggggccacaacgggcacttcatcccatggcgaagcagtggctg 250
L  V  R  G  P  Q  R  A  L  H  P  M  A  K  Q  W  L  49

agcaaggggaaccgcgggctcgacgtagtttcgaagcagctggcgagggt 300
 S  K  G  N  R  G  L  D  V  V  S  K  Q  L  A  R  V 66

cctggggagcctgcgg tga tccacgtcgggacagccgaggcgaatggagc 350
  L  G  S  L  R                                     71

ctttgacgccttcaacatggcccggaagatggccgaggcactcttcaaca 400
ataa gagcagtactcccagcgccgcaaccgggaaggttcccaacttgatt 450
gtgcccagcgcagtccaaaagttcgactcctttcctgggcttttggtgat 500
aagaaaaag                                          509
```



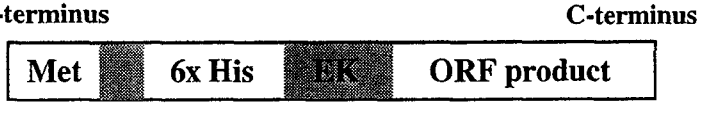


Fig. 4-7. Dose response curve using purified $_r$*Ah*EF. A) Virgin females injected with 0.03-1.0 µg of pure $_r$*Ah*EF fed to healthy engorged weights, while 0.01 and 0.003 µg of pure $_r$*Ah*EF was unable to stimulate a similar response. B) Those virgins injected with 0.03-1.0 µg of pure $_r$*Ah*EF underwent a significant degree of SG degeneration and ovary development. SG degeneration and ovary development did not occur in their counterparts injected with the lower doses of $_r$*Ah*EF. Controls are: C1, normally mated females, and C2, normally mated females injected with 500 mM imidazole (a potentially toxic antifungal agent found in the 6x histidine binding-column elution buffer).

TABLE 4-1

Bio-assay of recombinant proteins ($_r$proteins) derived from blood meal-induced mRNA transcripts expressed in the T/VD of male *A. hebraeum.*

experiment #	group # (n)	$_r$proteins injected[a,b]	mean weight of virgins (mg) at time of injection (± SEM)	mean weight of virgins (mg) at detachment by day 14 (± SEM)[c]	fluid secretory competence (mg/gland/15 min) on day 4 post-removal[d]	ovary weight (mg) on day 10 post-removal[e]
1	1 (14)	1 - 14	156 ± 8.9	182 ± 7.8	--	--
	2 (14)	15 - 28	191 ± 13.3	214 ± 6.6	--	--
2	3 (14)	1 - 7,15 - 20	206 ± 5.1	211 ± 10.2	4.0 ± 0.6 (n=4)	--
	4 (14)	1 - 7, 21-28	219 ± 16.1	237 ± 10	3.9 ± 0.9 (n=6)	--
	5 (14)	8 - 14, 15 - 20	183 ± 11.1	194 ± 11.1	3.6 ± 0.8 (n=6)	--
	6 (14)	8 - 14, 21 - 28	169 ± 10.1	**1070 ± 54.8**	**0.4 ± 0.1** (n=13)	**15.91 ± 1.4**
	7 (7)	control 1	219 ± 14.3	214 ± 8.8	4.2 ± 0.3 (n=8)	--
3	8 (7)	8 - 14	221 ± 21.0	253 ± 8.5	4.1 ± 0.3 (n=4)	1.6 ± 0.43
	9 (7)	21 - 28	178 ± 18.2	199 ± 17.4	4.7 ± 0.7 (n=6)	1.7 ± 0.47
	10 (7)	8 - 14, 21 - 24	236 ± 16.4	**1651 ± 159**	**0.4 ± 0.1** (n=10)	**18.12 ± 1.8**
	11 (7)	8 - 14, 25-28	200 ± 28.1	208 ± 18.2	3.7 ± 0.5 (n=4)	2.0 ± 0.47
	12 (7)	control 2	207 ± 22.3	227 ± 12.9	4.5 ± 0.4 (n=8)	2.1 ± 0.17
4	13 (7)	8 - 10, 21, 22	185 ± 11.7	**1979 ± 210**	**0.3 ± 0.1** (n=8)	**12.5 ± 1.6**
	14 (7)	11 - 14, 21, 22	202 ± 20.9	221 ± 17.2	4.7 ± 0.5 (n=4)	1.6 ± 0.44
	15 (7)	8 - 10, 23, 24	245 ± 22.7	194 ± 16	4.5 ± 0.3 (n=4)	1.8 ± 1.3
	16 (7)	11 - 14, 23, 24	192 ± 17.2	210 ± 15.7	4.0 ± 0.4 (n=4)	1.4 ± 0.22
5	17 (7)	8, 21	183 ± 14.8	234 ± 23.1		
	18 (7)	8, 22	214 ± 15.1	206 ± 13.4		
	19 (7)	9, 21	170 ± 26.4	206 ± 8.2		
	20 (7)	9, 22	191 ± 22.9	**1508 ± 81.0**		
	21 (7)	10, 21	241 ± 12.5	202 ± 9.3		
	22 (7)	10, 22	139 ± 9.3	230 ± 12.2		

[a] The indicated $_r$proteins are a fraction of the total protein (10μg of which was injected) present in the *Sf*21 cell lysates.

[b] Control 1 = non-transfected cell lysates: control 2 = 7.5 μg vector DNA (equal to amount used for transfection reactions).

[c-e] The value of all parameters measured (b-d) for groups (6, 10, 13 and 20) injected with $_r$*Ah*EF was significantly higher ($P < 0.0001$ in all cases, ANOVA) then the same values for groups not injected with $_r$*Ah*EF.

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Chapter 5 – General Discussion and Conclusions

Adult female ixodid ticks encounter numerous challenges. These include avoiding predation and desiccation, locating a suitable host, finding a mate and producing sufficient offspring to ensure continuation of her genome. The female has the capability to ensure success with regard to some of these stresses. For example, ticks have the capacity to secrete a hygroscopic liquid onto the mouthparts that facilitates water vapor uptake when the ambient relative humidity is low (Rudolph and Knulle, 1974). However, male influences are equally important. For example, at least in some species, females are reluctant to attach and feed unless males are already attached (Balashov, 1972). In the case of *A. hebraeum* (and many other ixodid ticks), this behavior is coordinated by a highly volatile, multi-component aggregation-attachment pheromone (Rechav *et al.*, 1977; Norval *et al.*, 1991). This pheromone induces females, and in some cases unfed males and even nymphs (Rechav *et al.*, 1976), "to ardently seek out, and attach in close proximity to, emitting males" (Sonenshine, 1991).

Infested hosts spend a significant amount of time attempting to remove ticks. For example, certain species of wild African bovids perform 600-2000 'grooming episodes' per 12 h observation period (Hart, 2000). Females may attach to a host in unsuitable locations, such as keratotic areas (hardened, scar-like tissue resulting from previous injury), areas with poor blood supply, or areas covered with thick hair, abundant grease or sweat secretions (Balashov, 1972). Furthermore, the host may mount an immune response that interferes with tick feeding (Norval *et al.*, 1988), or the host may die from any number of causes. In any of these situations, the female may find herself looking for a new host.

Under these circumstances the concept of CW becomes very important, and the female may find herself in one of several situations. 1) A mated or virgin female dislodged from the host below the CW will seek out, and attach to, a new one. 2) If a

virgin or mated female is dislodged having surpassed the CW, it will not seek out a new host, but goes on to lay a clutch of eggs, the size of which is limited by the amount of blood consumed (Kaufman and Lomas, 1996). These observations led Kaufman and Lomas (1996) to designate ticks below the CW to be in the 'host-seeking phase' of the feeding cycle, and ticks above the CW to be in the 'reproductive phase' of the feeding cycle. Whereas mated females above the CW are capable of laying at least a small batch of fertile eggs if removed from the host prematurely, any eggs laid by virgin females above the CW are infertile (Kaufman and Lomas, 1996). It thus seems adaptive that very few virgin females will feed beyond the CW, because if dislodged while below, they can still seek out another host (and possibly find a mate). Anderson *et al.* (1998) showed that when partially-fed ticks in the host-seeking phase were removed from the host they responded to high ambient CO_2 with frequent movement, vigorous questing behavior and a relatively short latency between CO_2 exposure and these behaviors. Females in the reproductive phase of adulthood were unresponsive to CO_2. While we are unsure why a small percentage of virgins do exceed the CW, we do know that they too are unresponsive to CO_2 (Anderson *et al.*, 1998).

Previous studies indicated that: 1) females must mate before they are able to engorge (Pappas and Oliver, 1971), and 2) an EF contained in the spermatophore may be the specific stimulus responsible for inducing engorgement (Pappas and Oliver, 1972). This information prompted me to focus the majority of my thesis work on trying to determine the identity of this substance.

I) Questions derived from results presented in this thesis

The following questions arise from the data presented in this thesis.

1) The results from chapter 2 (Weiss and Kaufman, 2001) indicate that CW differs slightly depending on the parameter used to measure it (9x, 10x, 10x, 12x, 12x, 13x the unfed weight). What might be the physiological reason for these differences?

Harris and Kaufman (1985) indicate that SGs are highly sensitive to ES. At 10x the unfed weight, hemolymph ES-titres increase 3-fold to a level previously shown to induce SG degeneration (~100 ng/ml; Kaufman, 1991). This may explain why the CW threshold for SG degeneration was also 10x the unfed weight.

However, numerous studies indicate that the reproductive parameters measured (ovary weight, oocyte length and oocyte haem content) are less sensitive to ES. Kaufman (1991) determined that following SG degeneration, hemolymph ES-titres continue to rise 8-fold higher than the level necessary for SG degeneration (to ~ 800 ng/ml). Connat *et al.* (1985), and Kaufman (1991), further demonstrated that developed ovaries contain high concentrations of ES (90-340 ng/ovary), perhaps for use during embryogenesis and egg hatching (Hagedorn, 1983). Finally, Friesen and Kaufman (2002) induced a significant degree of vitellogenesis in partially fed females by injecting them with 20E ($\geq$ 430 ng/g body weight; lower doses did not stimulate vitellogenesis significantly). This requirement for higher ES-titres for reproductive processes than needed for SG degeneration may explain why the CW for ovary development (12-13x the unfed weight) is higher than the CW for SG degeneration.

2) In this thesis I speculate that EF and MF are the same substance. Previous studies provide conflicting information that may be interpreted to imply that the above-mentioned statement is an anomaly. 1) Lomas and Kaufman (1992a) found that MF hastens SG degeneration by 4 days by inducing an early release of ES (the SGs of virgin females above the CW will degenerate, but in 8 days). 2) Weiss and Kaufman (2001) determined that increasing ES titres indirectly inhibit feeding by suppressing

reattachment to the host. Thus, if these two factors are the same substance, how can they stimulate engorgement (EF) and inhibit feeding (MF, via the induction of 20E release)?

I speculate that the two factors have different modes of action that operate via distinctly separate pathways. First, EF/MF enters the female's hemocoel and stimulate engorgement well within 36 h, likely via a neurosecretion from the synganglion. Second, EF/MF induces 20E synthesis 3-4 days later either by acting at the synganglion (Lomas *et al.*, 1997) or directly on the epidermis (Zhu *et al.*, 1991).

Based on the above information, I have developed a model to summarize the physiological events leading to engorgement, SG degeneration and ovary development in female *A. hebraeum* (Fig. 5-1). Some portions of the model are speculative while others have been verified (indicated by the appropriate references in the figure legend).

II) Potential of $_r$*Ah*EF as an anti-tick vaccine

The importance of this work with regard to tick reproduction and the transmission of tick-borne diseases cannot be overstated. Ticks serve as vectors for a diverse variety of pathogens, and the worldwide incidence of tick-borne diseases has increased significantly in the past 25 years (Orloski *et al.*, 2000; Gayle and Ringdahl, 2001). Thus, the implications of inhibiting tick feeding, which include less time for pathogen development, less salivation (thus less pathogen transmission to the host) and less oocyte development, are substantial.

Current methods for controlling the rapid growth of tick populations in areas where they transmit pathogens to humans and domestic animals include acaricides (Frisch, 1999), biological control agents (Samish and Rehacek, 1999; Samish, 2000) and recombinant anti-tick vaccines (Willadsen, 2001). These vaccines, some of which are under development and some of which are currently in use, are based on gut (Rand *et al.*, 1989; Patarroya *et al.*, 2002), salivary gland (Wang and Nuttall, 1999; Tsuda *et al.*, 2001;

Trimnell *et al.*, 2002) and reproductive proteins (vitellin; Tellam *et al.*, 2002). The effects of all three methods are marginal for numerous reasons. 1) Ticks rapidly acquire resistance to previously available and newly developed acaricides. Evolution then selects for these resistant strains. Furthermore, acaricides pollute the general environment and build up toxic residues in commercially available meat (Frisch, 1999). 2) An inadequate number of natural enemies have been identified to make them viable as large-scale tick biological control agents (Samish, 2000). 3) The practicality of recombinant anti-tick vaccines is still in question principally due to a lack of information regarding cross-reactivity and the exorbitant amount of time and money necessary to bring them to market (Willadsen *et al.*, 1995). However, with more research, recombinant anti-tick vaccines may prove to be the most desirable method for controlling ticks because of their relatively cheap production costs subsequent to development (Willadsen, 2001).

Recombinant *Ah*EF may serve as a more suitable anti-tick vaccine antigen because it targets directly the degree of tick engorgement that in turn has an impact on reproduction. We are currently in the initial stages of testing *Ah*EFs efficacy for this purpose.

III) Future directions

The discovery of EF provides the opportunity to further characterize a major developmental pathway in ticks. To conclude, I would like to make some suggestions that could help upcoming researchers interested in this system further enhance our knowledge of EF.

1) Production of an EF antibody. A polyclonal antibody raised against EF will be an extremely valuable tool for obtaining a thorough understanding of EF physiology. The antibody will serve as a diagnostic tool for conducting many of the experiments listed below.

2) Characterization of EF in the male. We currently know that EF is produced in the T/VD portion of the male gonad, EF bio-activity is absent from unfed males (Fig. 4-2; Pappas and Oliver, 1972) and EF is passed to the female via the spermatophore during copulation. However, which cell type(s) in the T/VD produce EF is unknown. Immunocytochemical techniques and *in situ* hybridization could be used to pinpoint EF site of production. Furthermore, development of an EF radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) would allow for its quantification in the spermatophore and at various stages of development within the T/VD. *In situ* hybridization may be used to determine at what point during male gonad maturation EF synthesis begins, and by using an ELISA or RIA, how much is produced.

3) Characterization of EF in the female. EF is a protein passed to the female, via the spermatophore, during copulation. We must now determine what happens to EF after it has entered the female reproductive tract. Monitoring immuno-reactivity with the polyclonal antibody may be used to track EF's progress once inside the female. Also, a variety of female tissues (reproductive tract, hemolymph, synganglia, epidermis, fat body) could be electrophoresed on polyacrylamide gels and the proteins transferred to nylon membranes. Allowing these blots to hybridize with $_{r}$*Ah*EF may reveal it's target tissues (antigen-binding assay). A loss in immuno-reactivity, or changes in molecular weight at any point during these experiments, would indicate that EF becomes chemically altered. Such results might indicate that EF gets cleaved once inside the female (possibly before crossing the wall of the reproductive tract), or triggers another substance that has EF bio-activity. Under these circumstances it will be necessary to identify that substance.

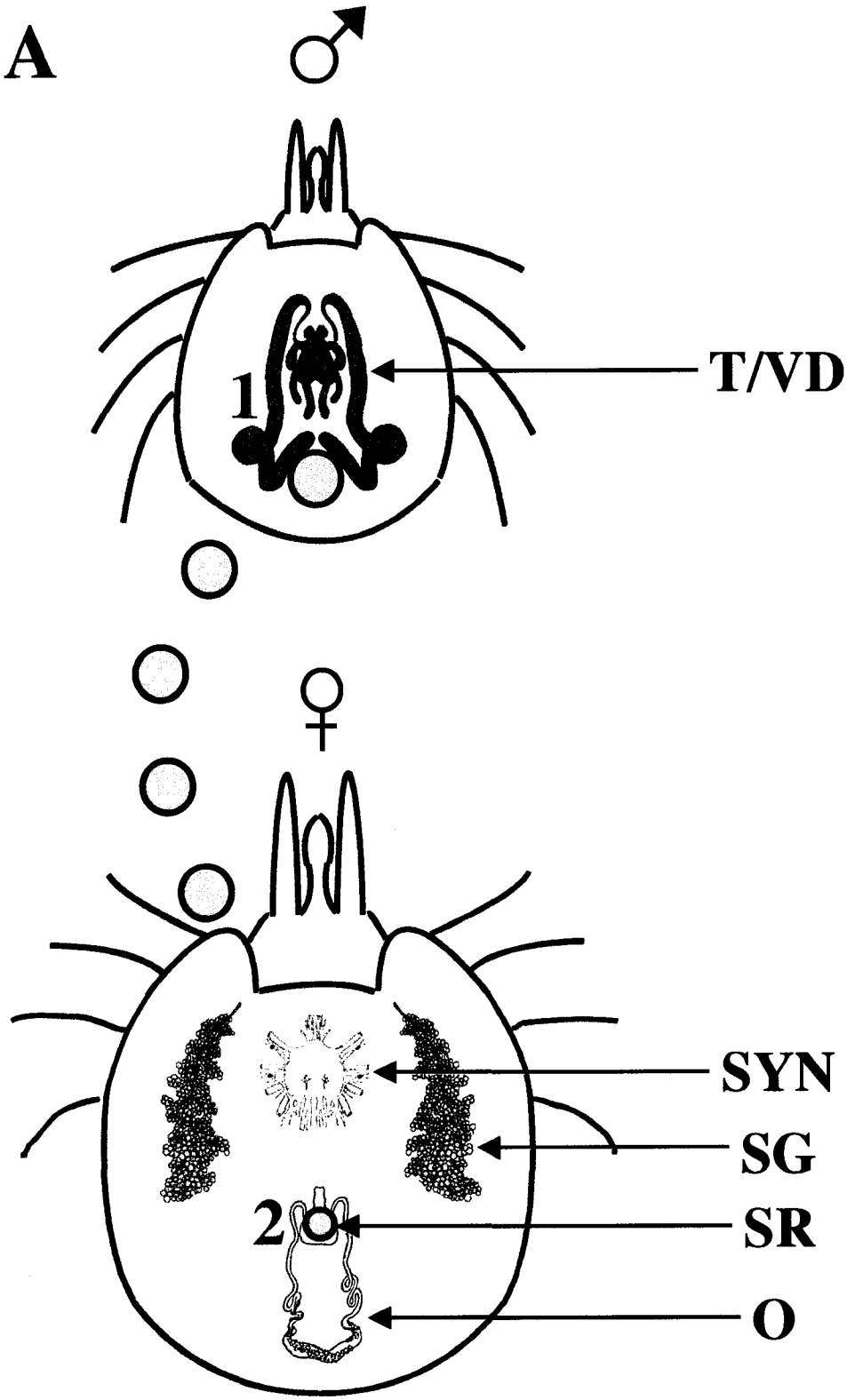
4) Inactivation of EF *in vivo* using the polyclonal antibody. EF is vital for normal growth and development of female *A. hebraeum*. Two experiments should be performed to determine whether the $_{r}$*Ah*EF antibody can inhibit feeding *in vivo*. The first is to inject

$_r$*Ah*EF antibody into partially fed virgin females and then feed them, along with males, for two weeks on fresh rabbits, to determine if this treatment attenuates the activity of native EF in females. The second experiment is to inject a host with $_r$*Ah*EF to see if the resulting immune response is capable of inhibiting the engorgement of normal mated females. If the results of these two experiments are positive, this raises great promise for developing and anti-tick vaccine.

5) Determine efficacy of *A. hebraeum* $_r$EF in other medically and economically important tick species. Some currently available anti-tick vaccines do show cross-reactivity in species other than those from which they were derived (deVos *et al.*, 2001). While we know that $_r$EF triggers virgin female *A. hebraeum* to engorge, we do not know if it will have the same effect on other tick species. Unfed adults of some potential target species (e.g., *D. andersoni, A. americanum, I. scapularis, I. holocyclus, Boophilus microplus, Rhipicephalus appendiculatus*) should be acquired and $_r$*Ah*EF tested on virgin females to determine if it promotes engorgement. Furthermore, T/VD from fed males of each of the above-mentioned species should be tested on virgin females of all the other species. The confirmation of cross-reactivity will further confirm $_r$*Ah*EFs potential utility for producing an anti-tick vaccine antigen.

Alternatively, RT-PCR could be used as a method to obtain information on EF in other ixodid tick species. Primers used to PCR amplify cDNAs encoding EF from *A. hebraeum* could be used for the same purpose with cDNAs made from the T/VD of fed males from the aforementioned species. The nucleotide sequences of isolated cDNA fragments could then be compared to one another and to the sequences of the cDNAs encoding EF from *A. hebraeum*. High sequence homology would confirm the presence of EF in these other species.

Fig. 5-1. Model indicating the proposed mode of action of EF. This model follows our assumption that EF and MF are the same substance and is referred to here only as EF. Abbreviations are as follows: T/VD, testis/vas deferens; SYN, synganglia; SG, salivary gland; I, integument; SR, seminal receptacle; O, ovary. A) EF, indicated by yellow circles, is produced by the T/VD (step 1) and passed into the female's SR (step 2) via the spermatophore during copulation. B) Once inside the female, EF likely passes unaltered through the wall of the SR and into the hemocoel (step 3) where it's target tissue is the SYN. C) EF stimulates engorgement within 36 h by an unknown mechanism (step 4) and D) causes the release of a neuropeptide(s) (indicated by black circles) from the SYN (step 5). Lomas *et al.* (1997) hypothesized that synganglial neuropeptide induces the integument to synthesize and secrete ES (indicated by red circles; step 6) which causes SG degeneration in 4 days (step 7; Harris and Kaufman, 1984) and egg development by 10 days (step 8; Kaufman, 1991; Friesen and Kaufman, 2002).



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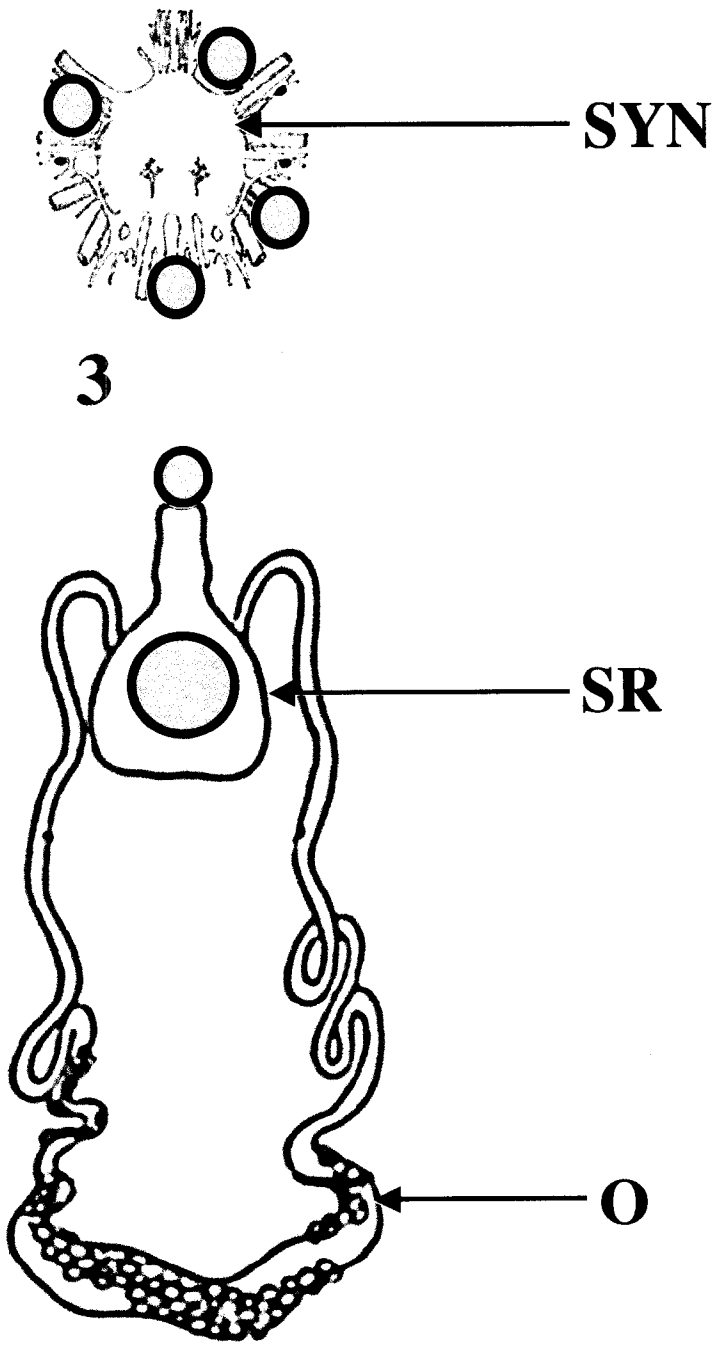

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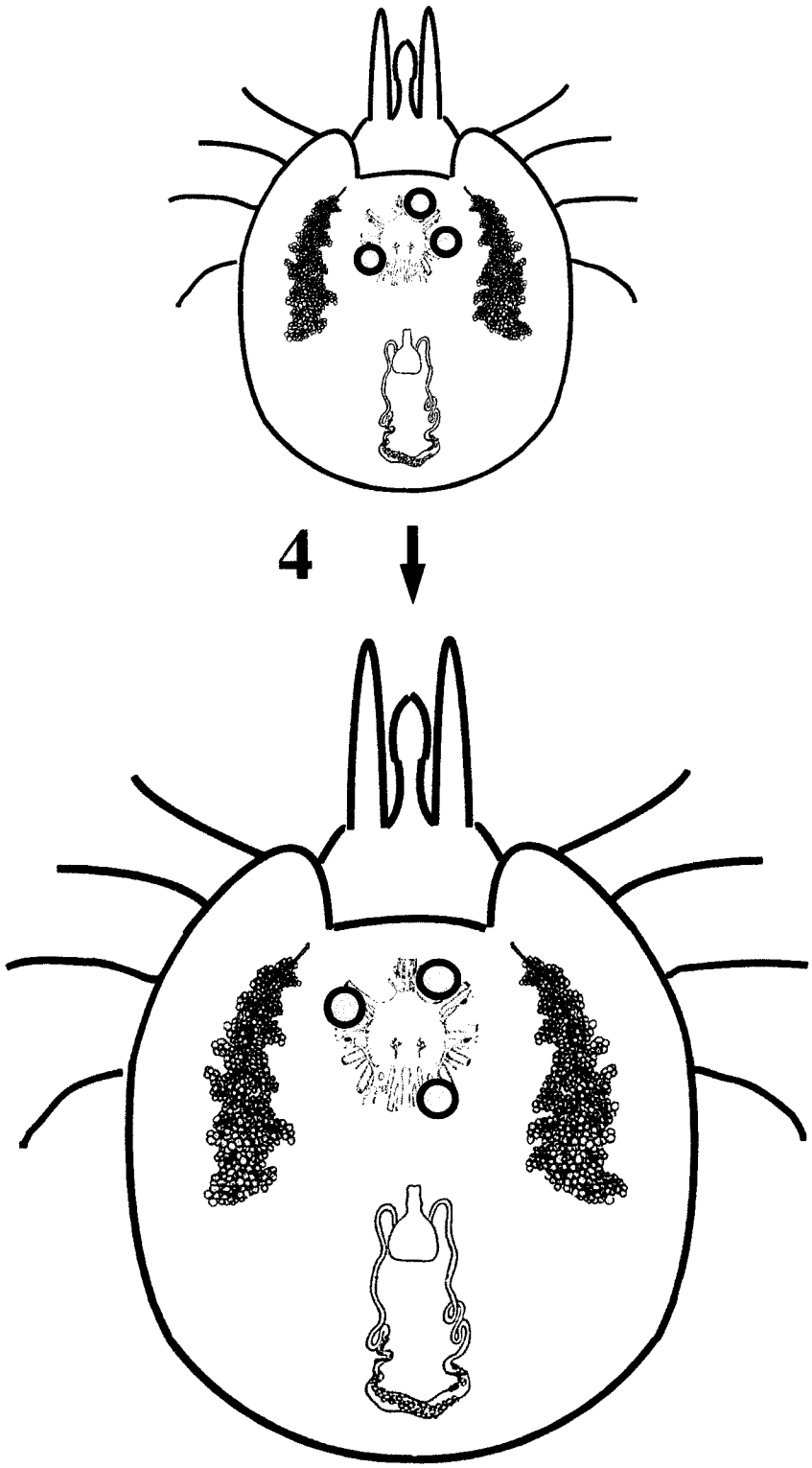

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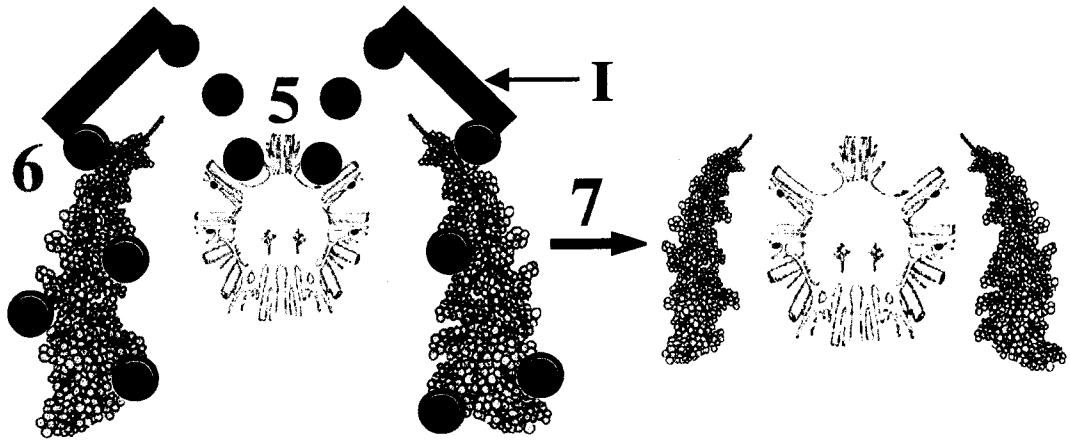

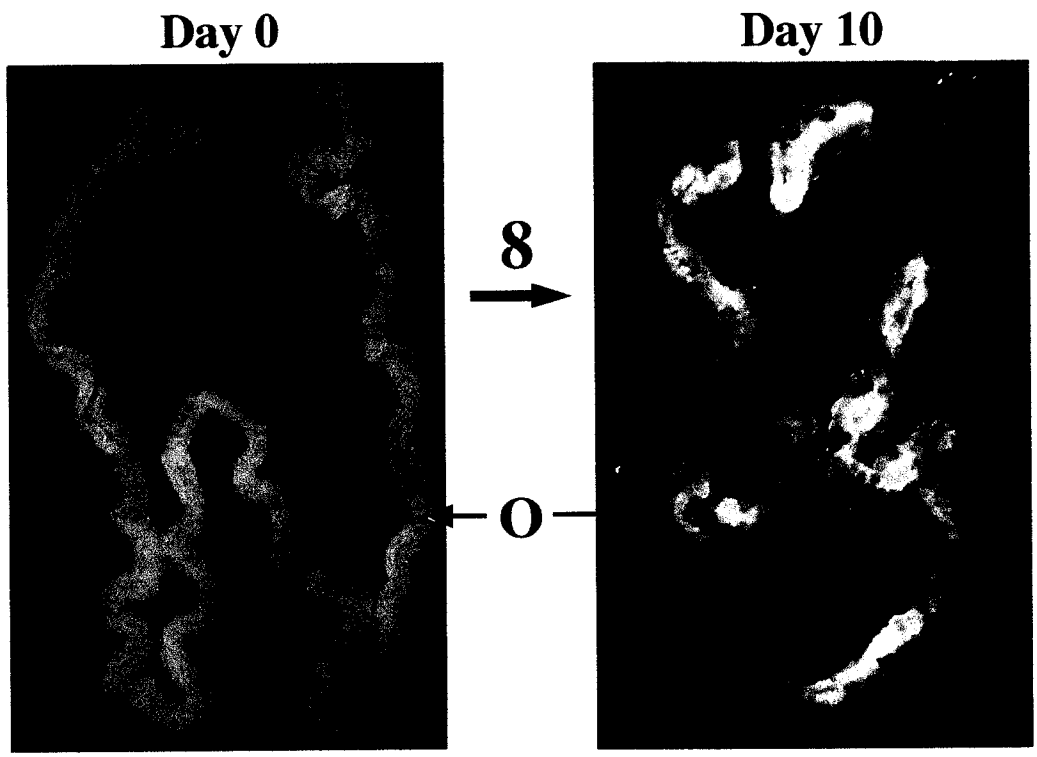

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Appendices

Appendix 1 – Abbreviations

Acp – accessory sex gland protein
AG – accessory gland
cDNA – complimentary DNA
CW – critical weight
ddH_20 – deionized, distilled water
DEPC – diethylpyrocarbonate
DMSO – dimethylsulfoxide
dNTP – deoxynucleoside (5'-) triphosphate
EF – engorgement factor
ELISA – enzyme-linked immunosorbent assay
ES – ecdysteroid
EST – expressed sequence tag
FBS – fetal bovine serum
FES – fecundity-enhancing substance
FR – female reproductive system
HL - hemolymph
MF – male factor
MMLV-RT – Moloney-Murine leukemia virus-reverse transcriptase
MR –male reproductive system
mRNA – messanger RNA
Mt – Malpighian tubule
MW – molecular weight
ORF – open reading frame
PCR – polymerase chain reaction
$_r$*Ah*EF – recombinant *Amblyomma hebraeum* engorgement factor
RIA – radioimmunoassay
RIS – receptivity-enhancing substance
$_r$protein – recombinant protein
RT-PCR – reverse transcriptase polymerase chain reaction
SDS – sodium dodecyl sulfate
SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF – spermathecal factor
SFM – serum-free medium
SG – salivary gland

SSC – sodium chloride tri-sodium citrate
SYN – synganglia
TSGDF – tick salivary gland degeneration factor
T/VD – testis/vas deferens
UV - ultraviolet
VSF – vitellogenesis-stimulating factor
20E – 20-hydroxyecdysone

Pergamon

Appendix 2

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The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick, *Amblyomma hebraeum*

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Abstract

The feeding cycle of adult female ticks (Acari: Ixodidae) is divided into preparatory, slow and rapid feeding phases. At the transition from slow to rapid feeding, *Amblyomma hebraeum* (Koch) females reach a 'critical weight' (CW; approx. 10× the unfed weight) that is characterized by several behavioural and physiological changes. Five of these changes were used as criteria to establish a more precise estimate of CW than we have to date. The CW as defined by re-attachment to the host was 9× the unfed weight, while for haemolymph ecdysteroid titre, salivary gland degeneration, ovary weight, oocyte length and oocyte vitellin content the CW was 10×, 10×, 12×, 12× and 13×, respectively. CW thus varies depending on the parameter measured. Although previous studies have established the influence of ecdysteroids on salivary gland degeneration and vitellogenesis, here we demonstrate a further effect of ecdysteroids: inhibiting re-attachment to the host.

Keywords: Ixodid ticks; Critical weight; Ecdysteroids; Tick salivary glands; Vitellogenesis

1. Introduction

Adult female *Amblyomma hebraeum* (Koch) require 10–14 days to engorge completely (approx. 100× the unfed weight). When ticks are held at 26°C, oviposition usually begins 10–12 days later and continues for 3–4 weeks. The feeding period comprises three distinct phases: (1) a 24–36 h *preparatory phase*, during which the female inserts its mouthparts, establishes a feeding lesion and secretes cement to secure attachment to the host epidermis, (2) a 7–10 day *slow feeding phase*, during which the female gradually expands to approx. 10× its unfed weight, and (3) a 24 h *rapid feeding phase*, during which the female increases its weight another 10-fold (Balashov, 1972).

Species of the genus *Ixodes* can copulate prior to finding a host, but in all other genera, copulation can occur only during the feeding cycle. Most feeding virgins (approx. 85%; Kaufman and Lomas, 1996) remain attached to the host for several weeks at a small, partially-fed state (under 10× the unfed weight). Once fed males are introduced, copulation occurs and the females engorge rapidly (Snow, 1969). Approximately 15% of feeding virgins may achieve 10–20× the unfed weight, but full engorgement never occurs (Kaufman and Lomas, 1996).

If females are removed from the host prematurely, their subsequent behaviour is influenced mostly by two factors: (1) how much blood they have imbibed to that point, and (2) whether or not they have mated. If either virgin or mated females are removed from the host before they have achieved approx. 10× the unfed weight, they will readily re-attach and resume feeding if presented to a host once again. If mated females are removed from the host during the rapid phase of feeding, however, they will generally not re-attach if presented to a host. Instead, their salivary glands (SG) degenerate within about 4 days, and an egg mass is laid subsequently, the size being approximately proportional to the amount of blood consumed. The hormone directly responsible for SG degeneration is 20-hydroxyecdysone (20E; Kaufman, 1991). Vitellogenesis in *Dermacentor variabilis* is also triggered by 20E (Sankhon et al., 1999). The source of hormonal ecdysteroid (ES) is the epidermis, and synthesis of ES by this tissue is controlled by

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a neuropeptide (Lomas et al., 1997). If virgin females are removed at a weight exceeding 10× the unfed weight, SG degeneration occurs within about 8 days, and any eggs laid are infertile (Kaufman and Lomas, 1996). Because this transition from the slow to rapid phase of feeding at about 10× the unfed weight results in such major changes in physiology and behaviour, it has been termed a 'critical weight' (CW) in the feeding cycle (Harris and Kaufman, 1984; Lomas and Kaufman, 1992a,b).

When compared to mated females, virgins take longer to degenerate their SG (8 days vs 4 days) because of an equivalent delay in the secretion of ES (Lomas and Kaufman, 1992b). A protein in the spermatophore transferred to the female during copulation is responsible for hastening the secretion of ES and thus hastening the process of SG degeneration (Lomas and Kaufman, 1992a,b). The site and mechanism of action of this 'male factor' (MF) are not yet known.

Although the concept of CW has been recognized for over 15 years, its value has never been measured precisely. In previous studies we have not known the individual unfed weights of the partially-fed ticks removed from the host, and the range of unfed weights in our colony of *A. hebraeum* can vary considerably (~15–45 mg, with an approximate average of 20–30 mg). Furthermore, we have assumed, but never demonstrated, that the CW is a unique value for all of the physiological and behavioural changes which become evident at this point in the feeding cycle. The purpose of this study was to establish a precise weight range for CW in individual females based on four measures: haemolymph ES-titre, SG degeneration, ovary maturation and re-attachment to the host. Our results indicate that, contrary to earlier assumptions, the CW varies somewhat according to the parameter measured.

2. Materials and methods

Adult *A. hebraeum* (Koch) were obtained from our laboratory colony which is maintained in darkness at 25°C and >95% relative humidity. Ticks were fed on rabbits as described by Kaufman and Phillips (1973) and removed for experimentation within a weight range known to overlap the CW considerably (~5.0–20.0× unfed weight). For each set of experiments, 24 unfed adult females were weighed and individually coded using a coloured thread tied to a distinct leg. An equal number of males and females were confined together on the rabbits. Fig. 1 shows the relative sizes of an unfed female, a female at approximately the CW, and an engorged female. The complete experimental protocol is shown in Fig. 2.

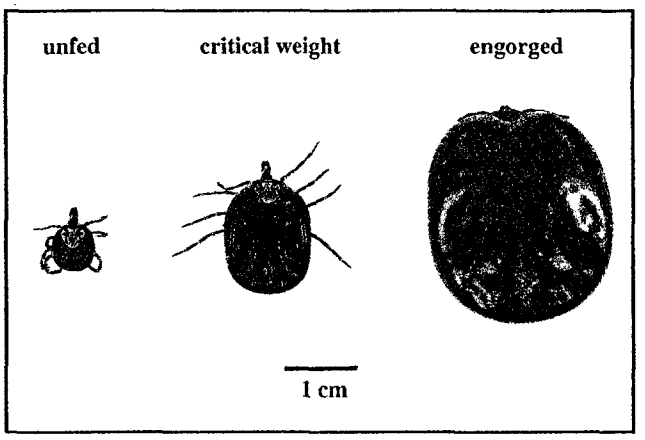


Fig. 1. Female *A. hebraeum* ticks at three defined stages of the feeding cycle (unfed, CW and fully engorged).

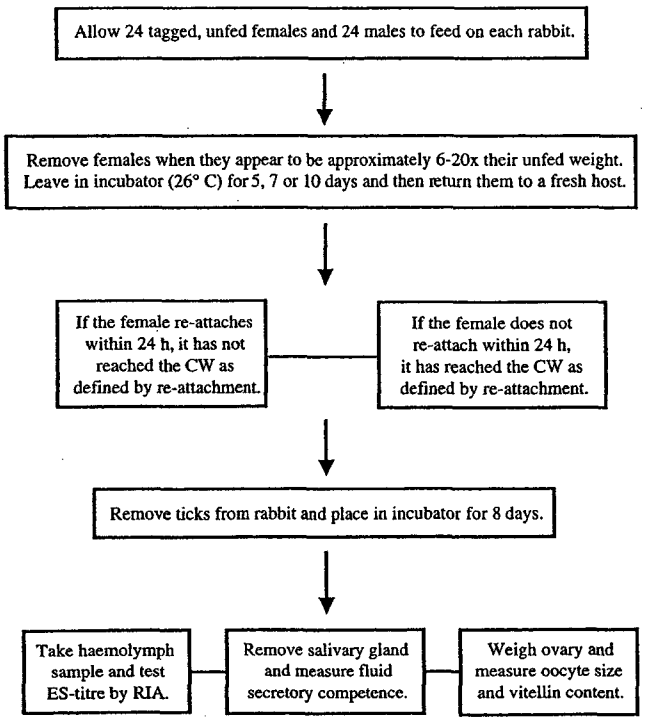


Fig. 2. Flow chart of the complete experimental protocol to determine CW.

2.1. Haemolymph ES-titre

Haemolymph was collected 8 days after ticks were removed from the host. Radioimmunoassay (RIA) was performed as described by Kaufman (1990). Because the antibody used (directed against ecdysone-22-succinylthyroglobulin) cross-reacts with some other side-chain modified ES, results are expressed as 20E equivalents/μl. The protein A used to separate free from bound ligand was prepared according to Kessler (1981). A marked increased in haemolymph ES concentration was used as one criterion for determining CW.

2.2. Assay for salivary fluid secretory competence

Ticks were glued ventral surface down to a Petri dish and bathed in ice-cold TC medium-199 (Gibco, Long Island, NY), supplemented with 2.09 g 3-N-morpholinopropanesulfonic acid (MOPS; Sigma Chemical Co., St Louis, MO) and 2.1 g NaCl/l, pH 7.3. The SGs were excised and the main duct of each was ligated with one strand peeled from 8-0 surgical silk thread (Davis & Geck, Pearl River, NY). Glands were gently blotted, weighed to the nearest 0.01 mg, and allowed to incubate for 12 min in fresh TCM-199 containing 10 μM dopamine (Sigma Chemical Co.). Glands were then re-weighed after gentle blotting. Net weight gain of the tissue was used as a measure of fluid secretory competence, which is inversely proportional to the degree of SG degeneration (Harris and Kaufman, 1984). A marked reduction in fluid secretory competence was used as another criterion for determining CW.

2.3. Ovary maturation assays

Ovaries were removed, gently blotted, weighed to the nearest 0.01 mg and the length of the 10 largest oocytes measured using a compound microscope fitted with an ocular micrometer. Ovaries were then homogenized in 3 ml distilled water, centrifuged @ 20,000*g* for 10 min and the supernatant frozen for later spectrophotometric analysis. The absorbance at 400 nm (specific for the haem moiety of vitellin) and 500 nm (non-specific to vitellin) was recorded and the difference was taken as a measure of total ovary vitellin content (Kaufman et al., 1986). Marked increases in ovary wet weight, oocyte length and ovary vitellin content were used as further criteria for the determination of CW.

2.4. Re-attachment assay

Partially fed females, approaching and exceeding the CW, were removed from the host, weighed and left in the colony incubator for 5, 7 or 10 days. After the predetermined time had elapsed, females were returned to a fresh host and allowed up to 24 h to re-attach (re-attachment was monitored every hour for the first 12 h). Those which reattached were considered to be below the CW based on the criterion of re-attachment.

2.5. Effect of 20E on re-attachment to the host

From the results presented in Fig. 4 we hypothesized that an increase in haemolymph 20E-titre may be the factor which ultimately inhibits re-attachment in ticks exceeding the CW. To test this, we injected seven groups of partially fed ticks weighing 194±16 (SEM) mg with 0.2 (n=14), 0.6 (n=10), 1.3 (n=10), 2.1 (n=14), 6.4 (n=11), 10.4 (n=19) or 31.2 (n=7) μmol 20E/kg body weight (20E was purchased from Sigma Chemical Co.), all delivered in 10 μl/tick. An eighth group of ticks (n=15) were injected with 10 μl 1.2% NaCl containing 1.7% ethanol to serve as vehicle injected controls. Following injection, all ticks were returned to fresh hosts and monitored hourly (for the first 12 of 24 h) until re-attachment. All ticks that had re-attached, or were clearly in the process of re-attachment, were removed immediately and a haemolymph sample taken for ES-RIA.

2.6. Statistics

All data are expressed as mean ± SEM (n). Statistical significance was determined by Student's t test using Microsoft Excel software on a Macintosh computer.

3. Results

Fig. 3 shows CW as defined by haemolymph ES-titre (3A), SG degeneration (B), ovary weight (C), oocyte length (D) and absorbance (400Δ500 nm) of ovary homogenates (E). The CW was identified as a marked discontinuity in the values of the dependent variables, and occurred at 10×, 10×, 12×, 12× and 13× the unfed weight, respectively.

Fig. 4 shows the CW as defined by re-attachment to the host, as a function of the number of days spent off the host. In all cases there was some overlap in the weight range defining the CW, but the overlap diminished appreciably as a function of duration off the host. The overlap range for 5, 7 and 10 days was 7.3–12.5× unfed weight, 8.0–10.5× unfed weight and 7.8–8.8× unfed weight, respectively (more limited data for 2 days off the host suggested an overlap range similar to that of 5 days). If one accepts the lowest value on day 10 above which there was no overlap, the CW based on the criterion of re-attachment was 9× the unfed weight.

Regarding the criterion of re-attachment to the host as a measure of CW: Fig. 4 demonstrates a clear increase in precision as a function of time off the host. This observation suggested that the increase in haemolymph ES-titre that occurs above the CW, and which triggers SG degeneration and vitellogenesis, may also be the factor which inhibits re-attachment to the host. To test this hypothesis we injected partially fed ticks below the CW with increasing amounts of 20E, returned them to the host, and checked hourly for re-attachment (see Section 2). Fig. 5 demonstrates clearly that 20E inhibited re-attachment. Vehicle injected controls re-attached within 2.8±0.6 h (n=15), significantly sooner then ticks receiving 1.3 μmol 20E/kg body weight (7.3±0.6 h, n=10; $p<0.0001$). The latency for re-attachment did not change significantly thereafter up to 10.4 μmol 20E/kg body weight (7.1±1.21 h, n=19; p=0.67).

Fig. 5 also demonstrates the equilibrated haemolymph

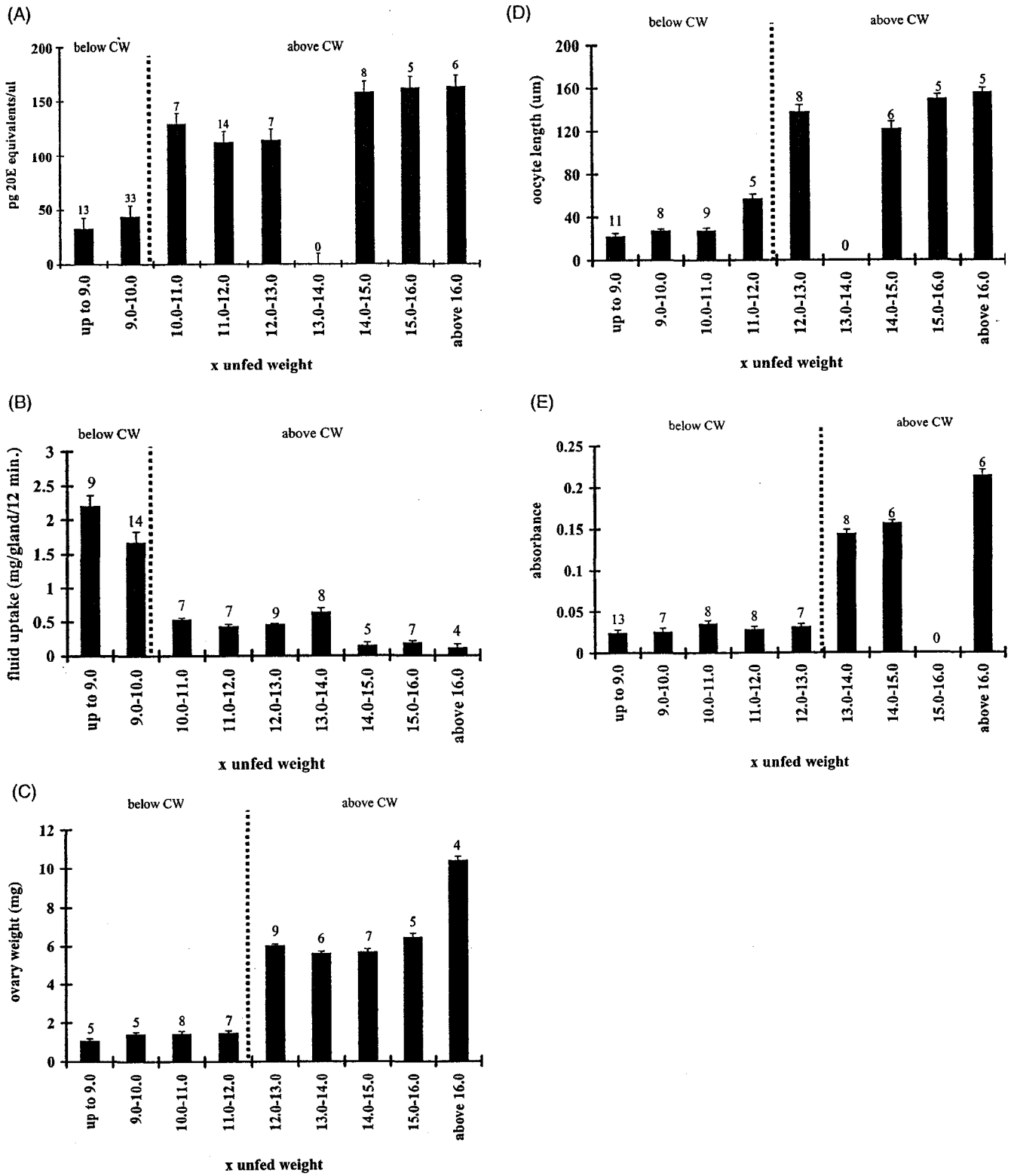
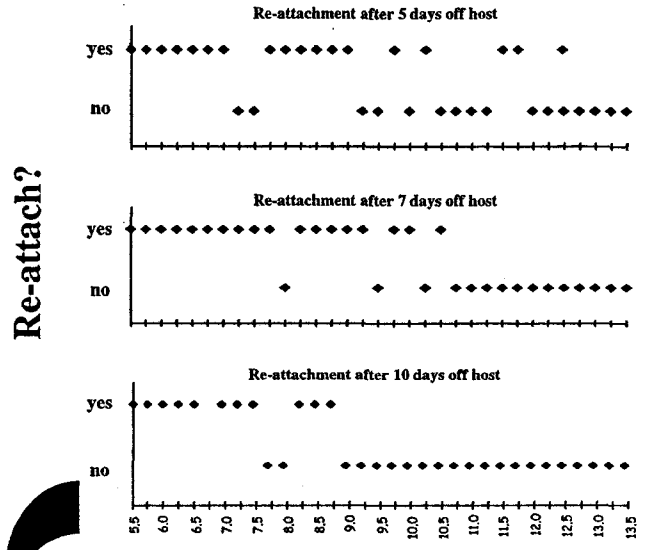


Fig. 3. Estimating CW by (A) haemolymph ES-titre, (B) rate of salivary fluid transport, (C) wet weight of ovary, (D) length of largest oocytes, and (E) oocyte vitellin content. See Section 2 for the respective techniques. In all panels, the vertical dotted line indicates the threshold of CW according to the parameter measured.



Days off host	CW as defined by re-attachment
5	12.5 x unfed weight
7	10.5 x unfed weight
10	8.8 x unfed weight

Fig. 4. CW as estimated by re-attachment to the host. Partially-fed ticks were removed between 5.0 and 20.0× the unfed weight and left off the host at 26°C for 5, 7 and 10 days before being presented to a new host. The range of overlap between those individuals which re-attached and those which did not re-attach narrowed as a function of time off the host. CW was determined as the lowest value above which no overlap was evident. By day 10 the CW was 8.8× the unfed weight.

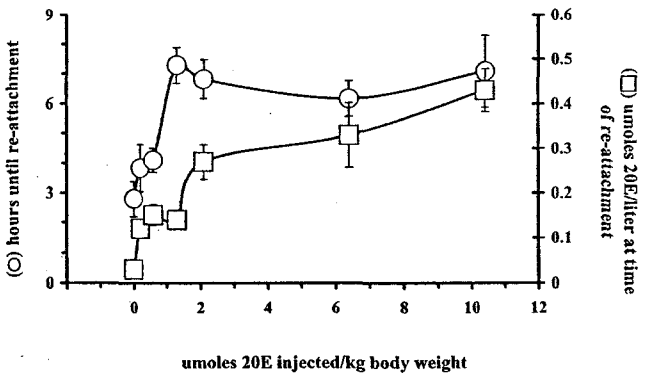


Fig. 5. The effect of exogenous 20E on delaying re-attachment. Small, partially-fed ticks (194±16 mg) were injected with increasing concentrations of 20E, returned to fresh hosts, and monitored hourly until re-attachment. Hours until re-attachment increased in a dose-dependent manner up to 1.3 μmol 20E/kg body weight. In all cases, most of the exogenous ES (>85%) was cleared from the haemolymph before the ticks re-attached. Seven ticks injected with 31.2 μmol 20E/kg body weight died within 10 h, without re-attaching (data not shown). SEM are shown wherever they exceed the dimension of the symbol, and n=10–19.

ES-titre in the same ticks at the time of re-attachment. Comparable concentration units are used for both axes (μmol/kg body weight for the abscissa and μmol/l for the ordinate). Because haemolymph volume accounts for approx. 25% of body weight (Kaufman et al., 1980), the initial concentrations in the haemolymph following injection would be approx. 4-fold higher than those indicated on the abscissa. Table 1 indicates that most of the RIA-detectable ES disappeared rapidly from the haemolymph during the 4–7 h required for re-attachment; the rate of disappearance increased almost 32-fold (from 0.18 to 5.8 μmol/h) over the total range of haemolymph ES-titre at time of injection (0.8–41.6 μmol/kg body weight; Table 1).

4. Discussion

While conducting experiments with large populations of *A. hebraeum*, Harris and Kaufman (1984) established the CW as 'approx.' 10× the unfed weight. This study reveals that the value for CW differs significantly according to the parameter measured.

SG degeneration in *A. hebraeum* (Harris and Kaufman, 1985) and yolk synthesis by the fat body in *D. variabilis* (Sankhon et al., 1999) can be triggered in vitro by similarly low doses of 20E (~30–40 ng/ml). Our results, however, indicate that the CW for haemolymph ES-titre and SG degeneration is 10× the unfed weight whereas that for ovary maturation is higher (12×–13× the unfed weight). The full expression of oocyte development depends on vitellogenesis followed by yolk uptake. Although vitellogenesis is triggered by 20E (Sankhon et al., 1999), this hormone is not sufficient to stimulate yolk uptake in vivo whether injected alone or in combination with juvenile hormone (Lunke and Kaufman, 1993; Kaufman, 1997). So if vitellogenesis was triggered in our ticks at 10× the unfed weight, a higher weight is required for sufficient yolk uptake to be detected in our spectrophotometric assay.

Re-attachment to the host is a less well-defined measure of CW because its value changed with duration off the host. This finding suggested that rising haemolymph ES-titre between 5–10 days post-removal may inhibit re-attachment, and the data presented in Fig. 5 support this hypothesis. Although the specific mechanism for this effect is unknown, ES inhibit feeding in some insects. The 5th larval stage of *Manduca sexta* experiences a brief rise and fall in haemolymph ES-titre during the 4th to 5th days of the instar. This commitment peak causes the larva to stop feeding, void its gut contents and wander in search of a suitable location for pupation (Nijhout, 1994). A similar brief pulse of ES also occurs during the rapid phase of engorgement in *A. hebraeum* (Mao and Kaufman, 1999), although its physiological significance has not been clearly established yet.

Table 1
Clearance of 20E equivalents from haemolymph ihn ticks injected with exogenous 20E

Amount 20E injected (μmol/kg body weight)	(A) Approximate haemolymph ES-titre at time of injection (μmol/l)[a]	(B) Time until re-attachment (h)	(C) Haemolymph ES-titre at time of re-attachment (μmol/l)	%ES metabolized or cleared[b]	Approximate rate of ES disappearance (μmol/h)[c]
0.2	0.8	3.8	0.12	85	0.18
0.6	2.4	4.1	0.15	94	0.55
1.3	5.2	7.3	0.14	97	0.69
2.1	8.4	6.8	0.27	97	1.20
6.4	25.6	6.2	0.33	99	4.08
10.4	41.6	7.1	0.43	99	5.80

[a] Calculated as 4× the amount injected.
[b] Calculated as [(A−C)/A]×100.
[c] Calculated as (A−C)/B.

In our experiments, the inhibitory effect of 20E was recorded as merely a delay in re-attachment (Fig. 5). We assume this was because of the rapid clearance of exogenous 20E that occurred following a bolus injection. Among numerous insects, the half-life of injected ES ranges from 4 min to 35 h (Koolman and Karlson, 1985). In vivo, however, haemolymph titres probably remain elevated in ticks above the CW due to ongoing ES-synthesis (Kaufman, 1991). Initially we were surprised that the latency to re-attachment did not continue to rise at doses over 1.3 μmol 20E/kg body weight (Fig. 5). The reason appears to be the marked increase in clearance rate that occurs at higher injected doses (Table 1). Several processes may contribute to the clearance of ES from haemolymph, but the relative importance of these routes is unknown. ES may be transported actively or passively to various tissues, or excreted by the Malphigian tubules. ES may be inactivated by a number of catabolic pathways. Finally, long chain fatty acids form apolar conjugates with ES in ticks (Diehl et al., 1985). Such conjugates may have greatly reduced hormonal activity or immuno-reactivity in our RIA. Conjugation, even if not followed by excretion or storage (in the ovary, for example), would represent a component of the clearance recorded in Table 1. Using the numbers generated in Table 1, we attempted to calculate an 'apparent K_m' and an 'apparent V_{max}' for the clearance process, but the experimental design clearly did not satisfy the criteria for Michaelis–Menten kinetics (initial rates of reaction with no significant reduction in substrate concentration). Nevertheless, the 32-fold rise in clearance rate occurred over the sub-micromolar to low-micromolar range (Table 1). Perhaps more than coincidentally, the K_m values for a number of enzymes that metabolize E and 20E fall over a similar range in a variety of insects (Webb et al., 1996; Williams et al., 2000; Young, 1976; Smith et al., 1979). For a better understanding of the physiological regulation of haemolymph ES-titres in ticks, we shall have to evaluate the relative importance of the factors responsible for clearance from the haemolymph.

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Appendix 3

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Identification and characterization of genes differentially expressed in the testis/vas deferens of the fed male tick, *Amblyomma hebraeum*

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Abstract

Most ixodid ticks must feed for at least a few days to complete gonad maturation. Substances produced by the mature male gonad, and carried in the spermatophore, induce physiological changes in the female that lead to engorgement and oviposition. To begin defining the molecular phenotype at this stage of male development, we differentially cross-screened a cDNA library made from the testis and vas deferens of fed ticks (*Amblyomma hebraeum* Koch) and isolated 35 genes that were putatively up-regulated in tissues of fed compared to unfed animals. While the majority of these were novel, two clones, AhT/VD16 and AhT/VD146, yielded homologies (53 and 44%) to known genes (acylphosphatse and 9.0 kD *Drosophila melanogaster* basic protein, respectively). Results of Northern blot analysis of AhT/VD16 and AhT/VD146 demonstrate that both clones hybridized with mRNA transcripts that were up-regulated in the testis/vas deferens of fed compared to unfed males. In addition, hybridization of clone AhT/VD16 to water strider (*Gerris argentatus*) genomic DNA, and sequence similarities to mammalian acylphosphatase, suggest that it represents an evolutionarily conserved sequence.

Keywords: *Amblyomma hebraeum*; Male gonad; Acylphoshatase; Gene regulation; Differential screening

1. Introduction

Female ticks of the family Ixodidae usually require 7–10 days to engorge fully. During this period, numerous developmental changes occur, the most dramatic and best studied of which occur in the female salivary gland (SG; Sauer et al., 1995). Within 4 days following engorgement, however, the SG degenerate, a process triggered by the hormone 20-hydroxyecdysone (20E; Kaufman, 1991).

Although gonadal development begins during metamorphosis between the nymphal and adult stages, in most ixodid ticks it remains arrested at the primary oocyte stage (female) and late prophase primary spermatocyte stage (male) until the adult begins feeding (Kiszewski et al., 2001). The onset of feeding in females is characterized by the 'great cytoplasmic growth' phase during which the oocyte cytoplasm increases substantially in volume. Following engorgement, oocytes begin to migrate out of the ovary wall, thus increasing the surface area in contact with hemolymph where vitellogenin accumulates. At this stage oocytes begin to enlarge considerably and fill with brown yolk granules (Brinton and Oliver, 1971; Diehl et al., 1982).

Substances produced by the male gonad [testis (T), vas deferens (VD) and accessory gland (AG)] are transferred to the female via the spermatophore and cause physiological and behavioral changes in the female. Pappas and Oliver (1972) reported that the male gonad of *Dermacentor variabilis* produces an 'engorgement factor' (EF) that triggers full engorgement in the female (virgin females rarely exceed 10–20% of the normal engorged weight even after a lengthy sojourn on the host). Homogenates of gonad from unfed males do not stimulate the engorgement response, suggesting that EF is synthesized during feeding.

Previous work in our laboratory on the control of SG degeneration established that large partially fed virgin females require 8 days to degenerate their SG, whereas

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weight-matched mated females require only 4 days (Lomas and Kaufman, 1992a). The factor responsible for this is a protein from the male gonad (which was named 'male factor'; MF; Harris and Kaufman 1984) that acts somewhere in the female to trigger an early release of 20E (Lomas and Kaufman, 1992b). Little MF bioactivity is present in crude gonad homogenates from unfed males, suggesting that synthesis of MF is induced by feeding. MF is probably a gonad-specific product, as it cannot be detected in SG homogenates from fed males (Harris and Kaufman 1984; Lomas and Kaufman, 1992a).

Feeding-induced male gonad development is necessary for successful reproduction. Although specific aspects of the physiology involved are known (Kaufman, 1997), the gene expression underlying these changes has not yet been explored. Using a differential approach to screen a fed tick T/VD cDNA library, we have characterized the molecular phenotype of the gonad in fed male *A. hebraeum* and identified changes in gene expression in this tissue that are induced by feeding. Thirty-five genes were confirmed to be differentially expressed in the T/VD of fed compared to unfed males. Single-pass sequencing and comparison with the Genbank database revealed that the majority of clones are novel. Two significant homologies with confirmed expressed sequence tags were however present. We further characterized these two genes using Northern and Southern blot analysis, and discuss their potential biological relevance within the male gonad.

2. Materials and methods

2.1. Ticks

Male *A. hebraeum* were taken from a laboratory colony maintained in the dark at 26°C and at a relative humidity of >95%. To allow for sufficient salivary gland and genital tract maturation, 30 male ticks were fed per rabbit for >4 days in a foam and cloth backpack as described by Kaufman and Phillips (1973). A total of 2500 male ticks were used in this study.

2.2. Tissue/RNA isolation

Males were stuck ventral surface down to a petri dish using a cyanoacrylate glue (Loctite, Rocky Hill, NJ), flooded with DEPC treated water and the T/VD, AG, SG, Malphigian tubules (Mt), synganglion (SYN) and gut were dissected out. Tissues were frozen immediately on dry ice. Total cellular RNA was extracted by grinding tissues with a mortar and pestle and then further homogenizing in a glass tissue homogenizer in the presence of TRIzol reagent (Gibco–BRL, Rockville, MD). Poly (A)+ RNA was extracted using an Oligotex mRNA mini kit (Qiagen, Carlsbad, CA.) according to the manufacturer's protocol.

2.3. cDNA library construction

A cDNA library was constructed from 4 μg fed tick T/VD poly (A)+ RNA's using a Uni-ZAP XR cDNA library synthesis kit and the Gigapack II Gold Packaging Extract (Strategene, La Jolla, Ca.) according to the manufacturer's protocol. The fed-T/VD library contained between 1×10^6 and 2×10^6 independent cDNA clones. Twenty randomly chosen clones were amplified by PCR, and then were subjected to electrophoresis on a 1% agarose gel for 2 h at 80 v. The gel was stained with ethidium bromide and viewed over UV light to determine average insert size.

2.4. Preparation of DNA probes

Poly (A)+ RNA was prepared from fed and unfed testis as described above. One microgram of mRNA was reverse transcribed using a Timesaver cDNA synthesis kit (Amersham Pharmacia, Piscataway, NJ) to produce a mixed population of double-stranded cDNA probe representative of the mRNA population in each of the tissues. Insert DNA from selected clones were prepared by PCR amplification as described below in the section 'PCR and secondary screening'.

Probes for all experiments were labelled using random primers and a mixture of dNTP's and Klenow fragment (Random Primers DNA Labeling System; Gibco-BRL, Rockville, MD). Probes made for the primary and secondary differential screens were triple-labeled ([^{32}P]αdATP, [^{32}P] αdCTP and [^{32}P] αdGTP) while those made for Northern and Southern blots were single labeled ([^{32}P] αdCTP). Unincorporated nucleotides from each reaction were removed by Sephadex G-50 chromatography.

2.5. Differential cross-screening of fed T/VD cDNA library

The library was screened unamplified. Differential screening was performed as described by Benton and Davis (1977). Clones from the fed-T/VD library, using XL1-Blue *E. coli* cells as a host, were plated at a density of 1500 pfu/150mm plate. Nylon colony plaque screen hybridization transfer membranes were marked for later re–orientation with plates and screened as defined by the manufacturer (NEN-Dupont, Boston, MA). The first of each duplicate set of plaque lifts was screened with [^{32}P]-labelled fed-T/VD mixed cDNA probe and the second with [^{32}P]-labelled unfed-T/VD mixed cDNA probe. Lifts were hybridized with the respective T/VD cDNA probe and processed under stringent conditions (final wash with 0.1×SSC/0.1% SDS for 10 min at 65°C)

in Hybrisol II (Intergen Co., Purchase, NY). Screened blots were exposed for 1–3 days at −70°C to Kodak X-O Mat film. Unless otherwise noted these conditions were used for all hybridization experiments performed for the current study. In the case of the library screening, plaques with different intensities of hybridization signal between the two probes were identified and isolated (Sambrook et al., 1989).

2.6. PCR and secondary screening

PCR was performed on all putative feeding-induced clones isolated after primary screening. A 5 µl sample of each plaque was added to a 95 µl reaction mixture containing ddH_2O, dNTPs (200 µM), PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl, 50 mM $MgCl_2$), T3 primer (0.5 µM; 5′-ATT AAC CCT CAC TAA AGG GA-3′), T7 primer (0.5 µM; 5′-TAA TAC GAC TCA CTA TAG GG-3′; BioServe, USA) and 10 units of Taq DNA polymerase. PCR was conducted using an Eppendorf (Westbury, NY) thermal cycler. The amplification program consisted of a three min hotstart at 94°C, followed by 30 cycles at 94°C for 1 min (DNA denaturation), 50°C for 1 min (annealing of primers), 72°C for 3.5 min (DNA elongation) and a final elongation/extension at 72°C for 7 min. Amplified products were verified by agarose gel electrophoresis.

For secondary screening, 0.2 µl of PCR product from each putative feeding-induced clone isolated after primary screening was arrayed onto three gridded nylon membranes (secondary blot). Each membrane was then allowed to hybridized with either [^{32}P]-labeled fed-T/VD mixed cDNA probe or [^{32}P]-labeled unfed-T/VD mixed cDNA probe. Prehybridization, hybridization, wash conditions and the final processing of the blots for the secondary screen were the same as those used for the primary screen.

2.7. Sequencing and sequence analysis

cDNA clones which passed the secondary screening process were purified using either the QIAquick Gel extraction kit or the QIAquick PCR purification kit (Qiagen, Mississauga, Ontario). Clones isolated from the secondary screen were submitted to single pass sequencing using a DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia, Piscataway, NJ) in order to generate an expressed sequence tag for each gene in question. Sequenced inserts were run on a PE Applied Biosystems 377 automated sequencer. Sequence data were analyzed using Genetool™ (Biotools Inc., Edmonton, Canada) and comparisons with the Genbank database performed by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

2.8. Northern blots

Three micrograms of total RNA was subjected to electrophoresis on an agarose gel and transferred overnight to Genescreen Plus nylon membranes (NEN-Dupont, Boston, MA) following the protocol of Sambrook et al. (1989). Blots were screened with the relevant radio-labeled probe under stringent conditions (as described for the library screens) and then exposed to Kodak X-O Mat film between two intensifying screens.

The intensity of bands on autoradiographs was quantified using the Kodak Digital Science ID image analysis system (Eastman Kodak Co., Rochester, NY). In order to normalize the band intensities to possible variations in RNA loading, we also quantified the relative level of 18S RNA in each lane of the gel used to generate the Northern blot analyzed. The normalized value of any transcript is the intensity of the corresponding band on the autoradiograph divided by the intensity of the 18S RNA band in the photograph of the corresponding sample in the original agarose gel photograph (Coorrea-Rotter et al., 1992). Statistical analysis was performed using Microsoft Excel software (Microsoft, WA).

2.9. Southern blots

Genomic DNA was extracted from seven species of arthropod (Acarina: *A. hebraeum, Dermacentor andersoni;* Diptera: *Glossina morsitans morsitans, Drosophila melanogaster;* Orthoptera: *Acheta domestica;* Blattodea: *Blaberus gigantia;* Hemiptera: *Gerris argentatus*) using the procedure of Sambrook et al. (1989). Separate *Cla*I and *Sau*3AI restriction endonuclease digests were set up for each genomic DNA sample according to the manufacturers of the enzyme (Gibco–BRL, Rockville, MD). Southern blot analysis using 3 µg of digested genomic DNA from each species considered was performed under reduced stringency (final wash in 0.1×SSC/0.1×SDS at 57°C for 10 min) for AhT/VD16 and AhT/VD146 as described by Sambrook et al. (1989). Autoradiography was done using Kodak X-O Mat film between two intensifying screens.

3. Results

3.1. cDNA library and screening

Fig. 1A and B displays the considerable development occurring within the T/VD and AG following 5 days of feeding. On average, the gonad weight of fed males exceeded that of unfed males by six–seven fold (Fig. 1C). In order to gain some insight into the genes which underlie this feeding-induced change, we made and screened a T/VD cDNA library from tissues taken from fed male ticks.

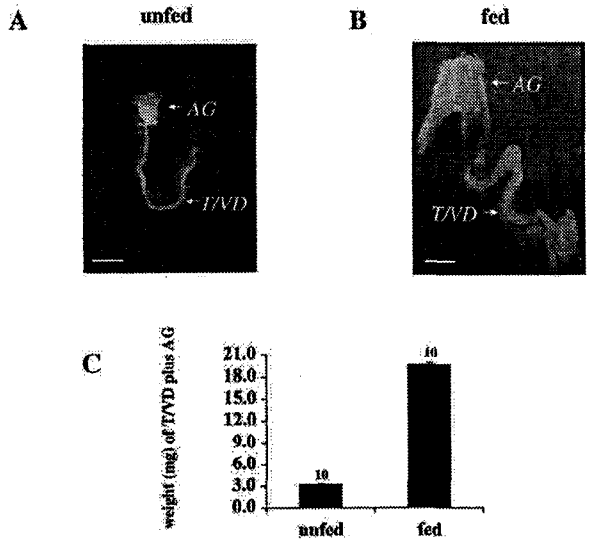

Fig. 1. Feeding-induced growth of male gonad in *A. hebraeum*. (A) unfed tick; (B) tick fed for 5 days. In A and B, only one of the testes is shown. Scale bar for A and B=150 μm. (C) Weight change of gonad after 5 days of feeding. Mean±SEM and n are shown for each group. Gonads were dissected out under 1.2% NaCl (isosmotic with tick haemolymph).

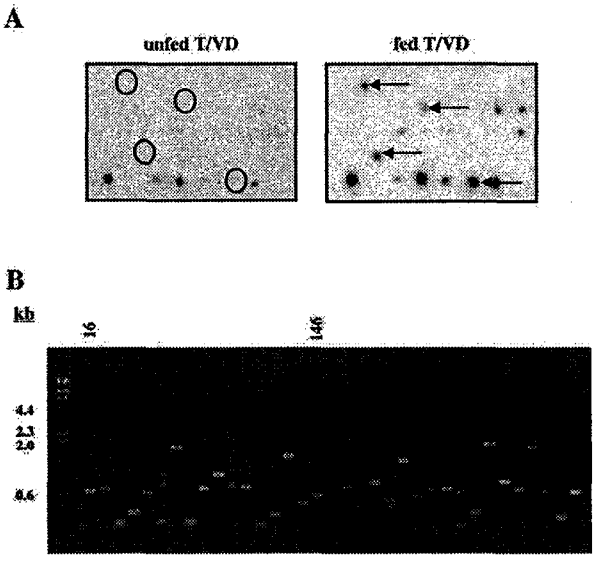

Fig. 2. Differential screening and PCR analysis of fed T/VD cDNA library clones. (A) Secondary screening of putative fed testis cDNA clones. Each PCR-amplified cDNA clone isolated from the primary screen (not shown) was spotted onto two nylon membranes. The first membrane was screened with a mixed cDNA unfed T/VD probe and the second with a mixed fed T/VD cDNA probe. Clones up-regulated by feeding were kept for bioinformatic, Northern and Southern blot analysis. Four such examples are indicated by arrows. (B) PCR-amplification of the 35 putative feeding induced clone inserts following the secondary differential screen. Amplified products were electrophoresed on a 1.2% agarose gel at 80 v for 2 h. Amplification of clone inserts revealed 89% were pure (contained only one insert). Those with more then 1 insert were purified as stated in Section 2.7.

A cDNA library of 10^6 clones has a >99% probability of including a copy of all rare transcripts (<10 copies per cell) at a detectable frequency (Sambrook et al., 1989). Thus, our library, which contains 1×10^6–2×10^6 independent cDNA clones, is likely to contain representatives of abundant, medium and low abundance transcripts from the fed T/VD.

Analysis of the primary differential screen of 15,000 clones on duplicate plaque lifts, using [^{32}P]-labeled fed-T/VD cDNA as probe on the first lift and [^{32}P]-labeled unfed-T/VD cDNA as probe on the duplicate plaque lift, allowed us to isolate 247 clones which apparently displayed higher levels of hybridization with the fed testis compared to unfed testis probe (results not shown). Analysis of the secondary screen confirmed 35 putative differentially expressed sequences (Fig. 2A). PCR analysis was used to amplify out the inserts from the 35 above-mentioned clones. Insert sizes range from 300 to 2150 bp (average insert size was 650 bp) and 31 (89%) contained only one insert (Fig. 2B).

3.2. Sequence analysis

Sequence comparison of up-regulated genes with the Genbank database revealed that two are significantly similar to known entries and the remaining 33 represent unique, previously unidentified transcripts. Clone AhT/VD16 contains a 460 nucleotide insert with a 288 nucleotide open reading frame (ORF) followed by a polyadenylation (polyA+) signal at position 413. The ORF, with a start codon (atg) at position 28 and a stop codon (tga) at position 316, encodes a 96 amino acid protein (10.4 kD; Genbank/EMBL accession number AF31662; Fig. 3A). The putative polypeptide has sites for protein kinase C, cAMP-dependent protein kinase A and myosin I heavy chain protein. This amino acid sequence is 53% similar to an acylphosphatase muscle type isozyme found in a number of vertebrate species (Stephani et al., 1997; Fig. 4A). Further bioinformatic analysis of clone AhT/VD16 is presented in Table 1. This information indicates our putative protein is hydrophilic, as are the acylphosphatases of the other species examined. The molecular weight and isoelectric point of acylphosphatase in all species examined fall within a narrow range (10.5–11.3 kD and 8.6–9.7 *p*I respectively). Furthermore, the tertiary structure of these amino acid sequences are highly conserved (based on predictions of estimated radius of folded proteins and % alpha helix, beta sheet and coil).

Clone AhT/VD146 contains a 370 nucleotide insert with a 255 nucleotide ORF. This ORF, which encodes an 85 amino acid protein (9.8 kD; AF316621), has a start codon (atg) at position 33 and a stop codon (tga) at position 288. The polyA+ signal in this nucleotide

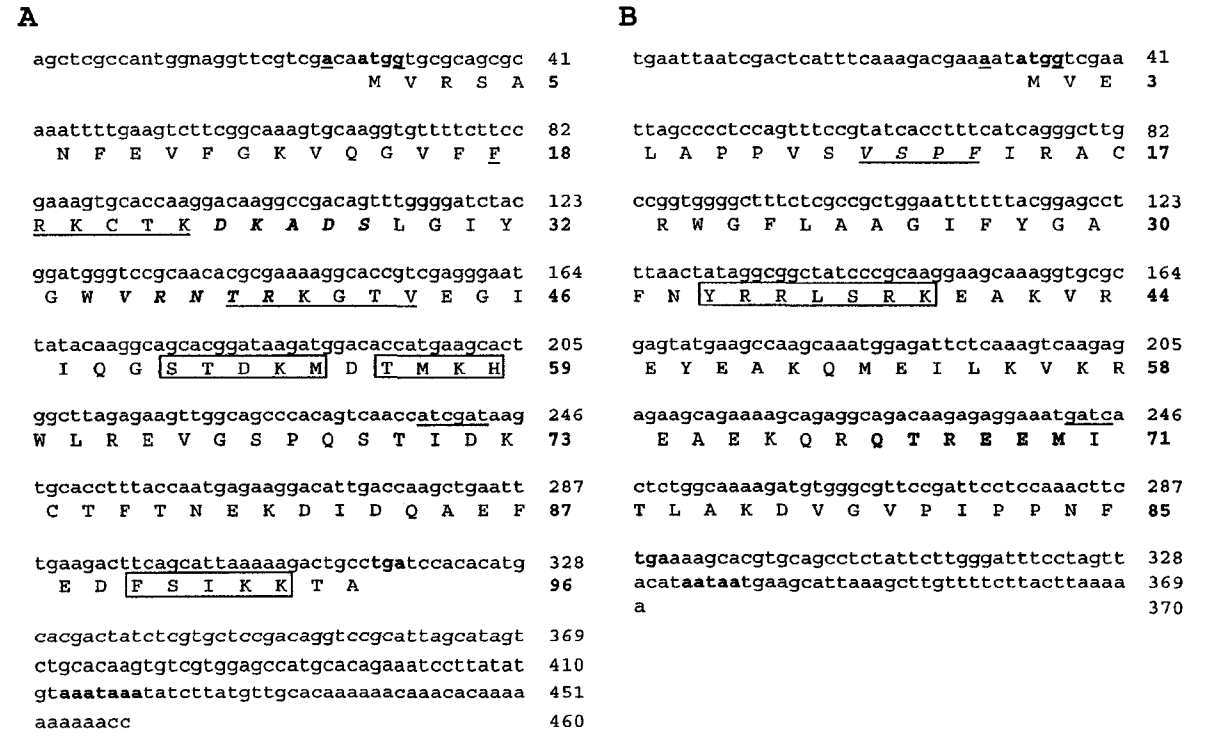

Fig. 3. Nucleotide and putative amino acid sequence of (A) AhT/VD16 (460 kb) and (B) AhT/VD146 (370 kb). The start codon (atg), stop codon (tga) and polyadenylation signals (aataa) are bolded and Kozak consensus sequences are bolded and underlined (Kozak, 1990). *Cla*I (A) and *Sau*3AI (B) restriction sites are underlined. Potential amino acid sequence post-translational modifications are as follows: protein kinase C phosphorylation sites are boxed, cAMP-dependent protein kinase A phosphorylation site, ***myosin I heavy chain phosphorylation site***, *proline-dependent phosphorylation site* and **caesin kinase II phosphorylation site**. Upper numbers adjacent to each sequence indicate nucleotide position and bolded numbers indicate amino acid position.

sequence is at position 333 (Fig. 3B). This putative protein has sites for protein kinase C, caesin kinase II and proline-dependent kinase protein. The putative protein is 44% similar to a *Drosophila melanogaster* 9 kD basic protein (Ghabrial et al., 1998; Fig. 4B).

3.3. Northern blot and Southern blot analysis

Northern blot analyses of clones AhT/VD16 and AhT/VD146 are shown in Fig. 5. Clone AhT/VD16 (Fig. 5A) hybridized to 3 fed-T/VD transcripts and 1 unfed-T/VD transcript. A 1.5 kb transcript is up-regulated in the fed-T/VD as the result of feeding (Fig. 5A). This 1.5 kb transcript was also detected in a number of other organs, although expression was highest in the fed-T/VD. Additional 3.6 and 6.0 kb transcripts were observed only in the fed-T/VD. Clone AhT/VD146 hybridized with two transcripts (2.7 and 0.6 kb) from the fed-T/VD (Fig. 5B). The 0.6 kb transcript is expressed in all tissues examined. Expression of this transcript is highest in the Mt, fed-T/VD and gut. The 2.7 kb transcript was expressed only in the fed-T/VD. In the course of our study we confirmed the differential expression pattern of a total of 35 of the clones identified (data not shown). Thus, 0.2% of the 15,000 fed-T/VD clones originally screened represent differentially up-regulated genes.

Southern blot analysis was performed using genomic DNA from seven species of arthropod to determine whether their genomes contained sequences similar to clones AhT/VD16 and AhT/VD146. The restriction endonucleases used to cut the genomic DNA were chosen based on their cut sites within each clone (AhT/VD16, ClaI, 5′-AT|CGAT-3′; AhT/VD146, Sau3AI, 5′-|GATC-3′). Clone AhT/VD16 hybridized to two *Cla*I genomic DNA fragments (6.0 and 2.8 kb) from *A. hebraeum*. This clone further hybridized to four genomic DNA fragments from *G. argentatus*, each 12.2, 6.6, 6.1 and 2.2 kb in size (Fig. 6). Clone AhT/VD146 hybridized to two *Sau*3AI genomic DNA fragments from *A. hebraeum*. These fragments were 9.4 and 8.5 kb in size (Fig. 6). No hybridization was observed between clone AhT/VD146 and genomic DNA from the other species examined (data not shown).

A AhT/VD16

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R. norvegicus -------AEPLKSVDYEVF GTVQGVC F R MYTEGEAKKRGLVGWVK N TSKGTVTGQVQGPEEK
M. musculus        MTELLKSVDYEVF GTVQGVC F R MYTEGEAKKRGLVGWVK N TSKGTVTGQVQGPEEK
S. scrofa     -----STARPLKSVDYEVF GRVQGVC F R MYTEDEARKIGVVGWVK N TSKGTVTGQVQGPEEK
M. gallopavo  -SALTKASGALKSVDYEVF GRVQGVC F R MYTEEEARKLGVVGWVK N TRQGTVTGQVQGPEDK
O. cuniculus  -----STAGPLKSVDYEVF GRVQGVC F R MYTEGEAKKIGVVGWVK N TSKGTVTGQVQGPEDK
H. sapiens    ----------LKSVDYEVF GRVQGVC F R MYTEDEARKIGVVGWVK N TSKGTVTGQVQGPEDK
A. hebraeum          MVRSANFEVF GKVQGVF F R KCTKDKADSLGIYGWVR N TRKGTVEGIIQGSTDK

R. norvegicus VNSMKSWLSKVGSPSSRIDRADFSNEKTISKLEYSNFSIRY
M. musculus   VDAMKSWLSKVGSPSSRIDRADFSNEKTISKLEYSDFSIRY
S. scrofa     VNSMKSWLSKIGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
M. gallopavo  VNAMKSWLSKVGSPSSRIDRTNFSNEKEISKLDFSGFSTRY
O. cuniculus  VNSMKSWLSKVGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
H. sapiens    VNSMKSWLSKVGSPSSRIDRTNFSNEKTISKLEYSNFSIRY
A. hebraeum   MDTMKHWLREVGSPQSTIDKCTFTNEKDIDQAEFEDFSIKKTA
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B AhT/VD146

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D. melanogaster    MSQAPVRVSPLIKFGRWSLLLVGIAYGAAHQSRLSKKEEKLREIEAQQKAVRDAKLAEEK
A. hebraeum        MVELAPPVSVSPFIRACRWGFLAAGIFYGAFNYRRLSRKEAKVREYEAKQMEILKVKREAEK

D. melanogaster    KRSAEAEARALAELSKPTPKH
A. hebraeum        QRQTREEMITLAKDVGVPIPPNF
```

Fig. 4. Putative amino acid sequence of (A) AhT/VD16 compared to that of a number of other species (Genbank/EMBL accession numbers: *R. norvegicus*, P35745; *M. musculus*, P56375; *S. scrofa*, P24540; *M. gallopavo*, P00821, *H. sapiens*, NP001099), and (B) AhT/VD146 compared to *D. melanogaster* 9.0 KD basic protein (AAC42662). The residues involved in the formation of the active site are boxed. Bold residues are identical in all sequences and underlined residues indicate the positions at which conservative substitutions are found. Sequence alignment and bioinformatic analysis was obtained by using the Peptool™ software.

Table 1
Protein statistics for the putative amino acid sequences of clones AhT/VD16 and AhT/VD146 from *A. hebraeum* in comparison to similar sequences found in other species[a,b]

Species[c]	Molecular Weight (kD)	Hydrophobicity (kcal/mol)	Estimated isoelectric point (*p*I)	Estimated radius of folded protein (Å)	Expected % alpha helix content	Expected % beta sheet content	Expected % coil content
(1) *R. norvegicus*	10.7	−6.3	9.4	17.7	26.0	30.2	43.8
(2) *M. musculus*	10.9	−5.4	9.0	17.8	25.8	32.0	42.2
(3) *S. scrofa*	11.3	−6.1	8.6	18.0	14.0	35.0	51.0
(4) *M. gallopavo*	11.3	−5.6	9.7	18.1	14.7	30.4	54.9
(5) *O. cuniculus*	10.9	−5.7	9.7	17.9	11.2	39.8	49
(6) *H. sapiens*	10.5	−6.2	9.6	17.6	11.8	41.9	46.2
(7) ***A. hebraeum***	10.9	−6.2	9.0	17.7	24.0	28.1	47.9
(8) *D. melanogaster*	9.0	−6.1	10.1	16.8	75.3	0.0	24.7
(9) ***A. hebraeum***	9.8	−3.6	10.0	17.0	75.3	0.0	24.7

[a] Acyphosphatase and *D. melanogaster* predicted 9.0 kD basic protein amino acid sequences for the above-mentioned species were obtained for protein statistic analysis from the Genbank database.
[b] Protein statistic analysis was done using Peptool software (Biotools Inc., Edmonton, Canada).
[c] Species 1–7, acylphosphotase protein statistics (AhT/VD16); species 8–9, predicted 9.8 kD basic protein (AhT/VD146) statistics.

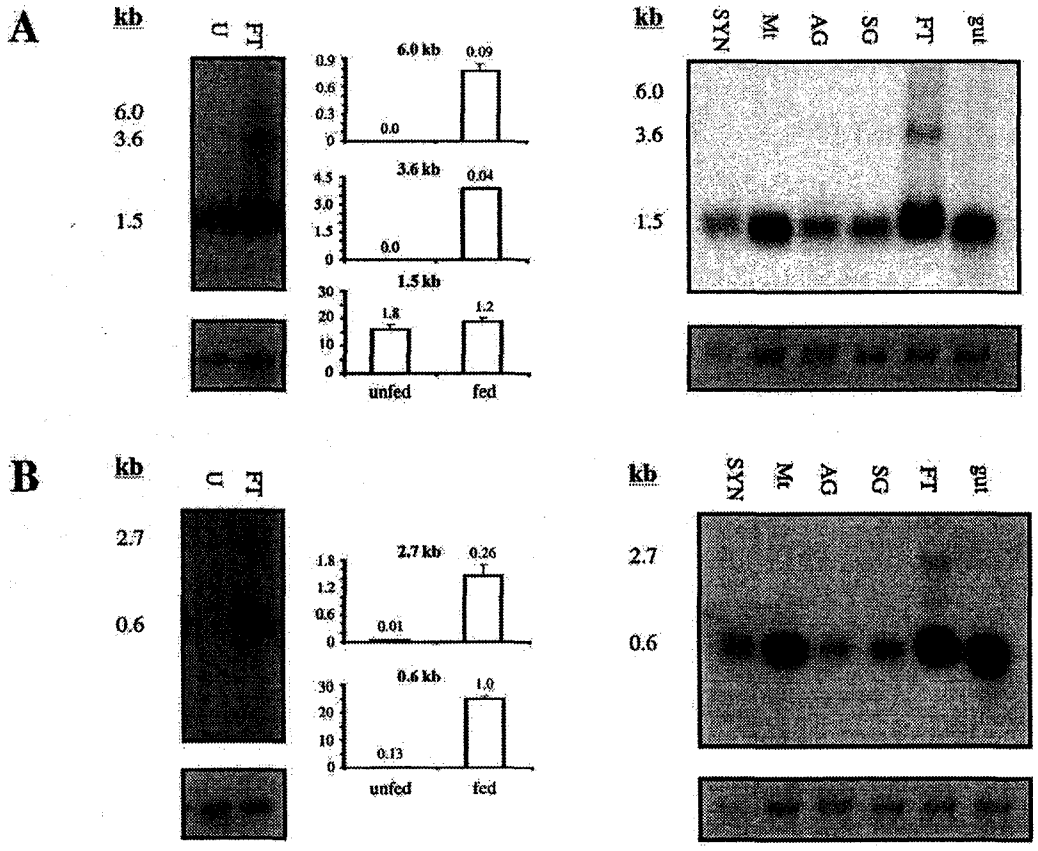

Fig. 5. Northern blot analysis of differentially expressed clones. (A) Radio-labeled clone AhT/VD16 insert DNA was used to probe 3 μg/lane of total RNA from the following tissues: unfed testis/vas deferens (U), fed testis/vas deferens (FT), fed synganglion (SYN), fed Malphigian tubule (Mt), fed accessory gland (AG), fed salivary gland (SG) and fed gut. RNA was electrophoresed on 1.0% agarose–formaldehyde gels and subsequently transferred to nylon membranes. (B) The same procedure using PCR product of clone AhT/VD146 as a probe. 18S ribosomal RNA was used as a loading standard. Three independent densitometry readings from two autoradiographs of all hybridization signals from clones AhT/VD16 and AhT/VD146 were taken. Ordinate units for each densitometry graph are arbitrary. The standard deviation for each set of densitometry determinations is provided above each column.

4. Discussion

We have used a differential screening technique to begin characterizing the molecular phenotype of the T/VD from fed males. This study represents the first mass screening for clones which are differentially expressed in the gonad of ticks. In this study we identified 35 genes that are induced by feeding. The fact that 33 out of 35 clones contain novel sequences suggests that the tick T/VD cDNA library is a valuable resource for defining new genes.

Two of these clones, AhT/VD16 and AhT/VD146, have amino acid sequences which are similar to previously described sequences. The Genbank database currently contains 242 protein sequences and 2983 gene sequences from the family Ixodidae. Of these catalogued protein sequences, none come from *A. hebraeum*. Furthermore, all four of the catalogued *A. hebraeum* nucleotide sequences correspond to ribosomal RNA sequences. Therefore, the contribution of the sequence data contained in this paper, as well as likely future submissions, adds significantly to the collection of known *A. hebraeum* ESTs.

Possible orthologues of AhT/VD16 and AhT/VD146 are produced in the reproductive tissues of other species and play roles in cell differentiation, cell membrane permeability and ribonucleotide production. The amino acid sequence corresponding to clone AhT/VD16 from our cDNA library is significantly similar (53%) to acylphosphatase, a small cytosolic enzyme widely distributed in the tissues of numerous animals (Thunnissen et al., 1997; Pieri et al., 1998). Southern blot analysis revealed that clone AhT/VD16 hybridized to genomic DNA fragments from *A. hebraeum* and *G. argentatus*, supporting the concept that AhT/VD16 represents an evolutionarily conserved DNA sequence. This enzyme is reported to serve multiple functions. Acylphosphatase is capable of hydrolyzing the phospho-aspartyl intermediate formed

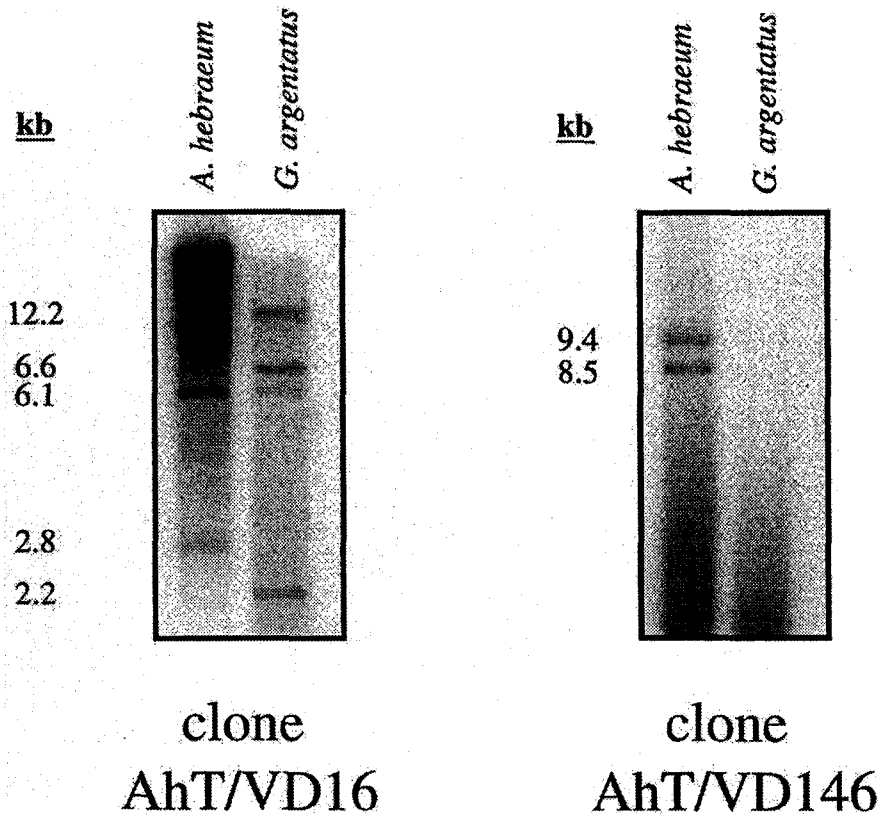

Fig. 6. Two preparations of genomic DNA, from seven species of arthropod, were digested to completion with either *Cla*I and *Sau*3AI. Samples were subjected to electrophoresis on 0.8% agarose gels and subsequently transferred to nylon membranes. Preparations were probed with insert DNA from clones AhT/VD16 and AhT/VD146, respectively, to determine the extent of sequence similarity within closely related animals. Clone AhT/VD16 hybridized with two bands in *A. hebraeum* and four bands in *G. argentatus* (water strider), while clone AhT/VD146 hybridized with two bands from *A. hebraeum*. Although some smearing was evident in the samples from the other species examined (*Dermacentor andersoni, Glossina morsitans morsitans, Drosophila melanogaster, Acheta domestica, Blaberus gigantia*), no significant hybridization was detected (data not shown).

during the action of membrane pumps such as Na^+/K^+-ATPase and Ca^{2+} -ATPase. This action could influence the ion concentrations across cell membranes by increasing the turnover rate of these enzymes (Nassi et al., 1991, 1993; Thunnissen et al., 1997). Kaufman et al. (1976) demonstrated that Na^+/K^+-ATPase activity in the salivary glands of female *A. hebraeum* increased with feeding duration. Although undocumented in the reproductive system of ticks, we speculate that ATPase activity in the male gonad may also increase as the result of feeding and in conjunction with spermatozoa maturation. This putative feeding-induced increase in reproductive system ATPase activity may correlate with the up-regulation of acylphosphatase.

Recent studies demonstrate that increases in acylphosphatase expression are accompanied by ribonucleotide production (Paoli et al., 2000) and cell differentiation in many cell lines including germ cells (Chiarugi et al., 1997). In ticks, germ cell differentiation is complete after spermatogenesis and spermiogenesis (Oliver and Brinton, 1972, 1973; Feldman-Muhsam and Filskie, 1976). In ticks AhT/VD16 hybridized to a 1.5 kb transcript that was observed in all tissues examined, and a pair of transcripts (6.0 and 3.6 kb) which represent fed T/VD specific messages (Fig. 5A). The two fed T/VD specific transcripts may underlie a specific function pertaining to T/VD and germ cell development.

The amino acid sequence of clone AhT/VD146 is similar (44%) to a predicted 9.0 kD basic protein from *D. melanogaster*. In *D. melanogaster* the 550 bp gene encoding this protein is located in region c550 at position 88B on the polytene chromosome (Ghabrial et al. 1998). Analysis of the putative amino acid sequence revealed that both proteins are hydrophilic. Furthermore, the predicted tertiary structure of these two proteins are virtually identical (Table 1). The function of these proteins is unknown.

Our cDNA library is the first one reported from any tissue of *A. hebraeum* and it's screening represents the initial steps in the characterization of the molecular

phenotype of the fed T/VD. As mentioned earlier, MF bio-activity has been found only in the fed T/VD (Harris and Kaufman, 1984; Lomas and Kaufman, 1992a). Thus, transcripts hybridizing to clones AhT/VD16 and AhT/VD146 in tissues other then the T/VD are not likely to be candidates for EF and MF. The three transcripts exclusive to the fed T/VD (6.0 and 3.6 kb transcripts detected by clone AhT/VD16 and the 2.7 kb transcript detected by clone AhT/VD146), and the 33 other differentially expressed transcripts, will be investigated to determine their possible relevance to factors secreted by the tick T/VD and their subsequent effects on female engorgement, reproduction and SG degeneration.

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Appendix 4

Tissue dissection and total RNA isolation

Total RNA was extracted using TRIzol reagent (Gibco-BRL, Rockville, MD). Males were stuck ventral surface down to a petri dish using a cyanoacrylate glue (Loctite, Rocky Hill, NJ), flooded with DEPC treated water and the T/VD, AG, SG, Malphigian tubules (Mt), synganglion (SYN) and gut were dissected out. Tissues were frozen immediately on dry ice. Total cellular RNA was extracted by grinding tissues with a mortar and pestle and then further homogenizing in a glass tissue homogenizer in the presence of 1 ml of TRIzol reagent. Homogenized samples were incubated at room temperature for 2 min to allow for complete dissociation of nucleo-protein complexes. Following the addition of 200 µl of chlorophorm, each sample was shaken vigorously and again placed at room temperature for 2 min. A 15 min centrifugation at 12,000 x g (4-8° C) separated the samples into a phenol-chloroform organic phase containing protein, an interphase containing DNA and a top aqueous phase containing RNA. The RNA-containing aqueous layer was isolated and an equal volume of isopropyl alcohol added to precipitate the RNA. Precipitations were left for 15 min at room temperature. Following a 15 min centrifugation at 4^0C the RNA pellet was washed with 70% cold ethanol and re-suspended in DEPC-treated water. Finally, RNA was then flash frozen on dry ice and stored at –70°C.

Quality of extracted RNA was determined by running 3 µl of each sample on a 1% agarose-formaldehyde gel (refer to section '*Northern blot analysis, Sample preparation*' for more details). The RNA was verified as undegraded by observing the sharpness of the 18s and 28s ribosomal bands on the formaldehyde gel.

Poly(A+) messenger RNA isolation

Although technically unnecessary, mRNA was isolated from transfer RNA and ribosomal RNA to increase cloning efficiency. mRNA was isolated using a Qiagen mRNA isolation kit according to the manufacturers protocol. Prior to mRNA purification, total RNA was quantified using a GeneQuant machine (Stratagene). Approximately 5 μg of mRNA was needed for cDNA library and probe production. mRNA comprises 1-5% of total RNA, thus I began with 500 μg of total RNA. Total RNA was incubated at 65°C for 3 min to denature any secondary structures. The RNA was then added to a 500 μl cocktail containing DEPC-treated water, binding buffer and an oligo dT_{30} resin (which binds the poly A tail of mRNA) and incubated at 65 °C for 5 min. The mixture was centrifuged and the supernatant discarded. The mRNA is now bound to the oligo dT_{30} resin that is linked to latex particles. Following a wash, the mRNA pellet was transferred to a spin column that bound the latex particles linked to the mRNA through the oligo dT_{30} primer. All RNA lacking a poly(A+) tail did not bind the oligo dT^{30} resin and thus flowed through the column when centrifuged. Finally, mRNA was eluted from the column via an elution buffer of increased salt concentration. mRNA was quantified on an ethidium bromide agarose plate.

Construction of the fed testis/vas deferens cDNA library

The Stratagene ZAP cDNA synthesis and ZAP cDNA Gigapack III Gold Cloning kits were used in the construction of the fed T/VD cDNA library.

Synthesis of first strand cDNA

Purified mRNA was used as template for first strand cDNA synthesis by adding to it Moloney-Murine leukemia virus reverse transcriptase (MMLV-RT), nucleotides and a 50 base pair primer containing a *Xho*I restriction endonuclease recognition site and a

poly dT region. Template mRNA was removed with the addition of RNase H to the first strand reaction mix.

Synthesis of second strand cDNA

Digested mRNA fragments generated by RNAse H served as primers for second strand cDNA synthesis. [^{32}P]dATP was incorporated into second strand cDNA. Radioactive second strand cDNA was used in a subsequent step as a marker for size fractionation purposes when run through a sepharose column.

cDNA termini blunting and EcoRI adapter ligations

Double-stranded cDNAs were blunt-ended with the addition of *Pfu* DNA polymerase and blunting dNTPs. *Eco*RI adapters (9- and 13- mer oligonucleotides), containing *Eco*RI restriction endonuclease recognition sites, were ligated to both the 5' and 3' blunt ends of the newly synthesized cDNA. These adapters add a second restriction endonuclease site (a *Xho*I site was included in the first-strand cDNA synthesis primer) to the cDNA. cDNA was then digested with *Xho*I, this producing an *Eco*RI site at the 3' end and a *Xho*I site at the 5' end. The cDNA fragments then contained 'sticky ends' for directional cloning into the Uni-ZAP XR vector.

Size fractionation of the cDNA

Size exclusion chromatography increased ligation efficiency of the cDNA into the Uni-ZAP XR vector by removing both unincorporated nucleotides and *Eco*R1 adapters released by *Xho*I digestion. A gravity-flow column packed with Sepharose CL-2B gel filtration resin was used to recover cDNA fragments > 400 bp in length. cDNA was then precipitated using 100% ethanol.

Ligating cDNA into the Uni-ZAP XR vector

The Uni-ZAP XR vector is designed to accommodate 0 to 10 kb DNA inserts. To facilitate directional cloning of the cDNA, the vector is double digested with *Eco*RI and *Xho*I. The cDNA insertion site is flanked by T3 and T7 promoter sequences (the poly (A+) tail of the insert is adjacent to the T3 primer site), thus allowing for PCR amplification of the inserted cDNA fragment using T7 and T3 primers (Invitrogen Corp., Carlsbad, CA). Size-fractioned cDNA was ligated into the Uni-ZAP XR vector using 4 µl T4 DNA ligase (4 Weiss units). The reaction was incubated at 12°C overnight.

Phage packaging

Gigapack III Gold packaging extract contains all of the proteins coded for by the phage. These proteins are synthesized using the host bacteria's transcriptional and translational machinery. This step is necessary for the generation of recombinant phage capable of infecting the host bacteria.

Preparation of the host bacteria and tittering of the library

RecA$^-$ *E. coli* XL1-Blue MRF' was used as the host bacterial strain. Bacterial cells in LB broth (supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$) were grown overnight in a 30°C shaking incubator. The cells were spun at 2800 rpm and resuspended in 10 mM $MgSO_4$ (to an O.D. of 1.0).

To titre the library, 1 µl and 4 µl of fed T/VD cDNA library was added to 200 µl of the above-mentioned bacterial cell culture. Cells and phage were the incubated at room temperature for 5 min to allow the phage to attach to the bacterial cell and at 37°C for 15 min to permit the cells to finish one life cycle. The infected bacteria was then added to 15 ml of NZY top agar (≤ 50°C) and poured onto NZY plates. Plates were incubated

overnight at 37°C, and the number of plaques on each plate were counted to determine the titer of the packaged phage.

Primary differential cross-screen of fed T/VD cDNA library

The library was screened unamplified as described by Benton and Davis (1977). Using the method described above (library titring), 3 µl of the fed-T/VD library was plated at a density of 1500 pfu/150 mm plate. Once plaques became visible, plates were placed at 4°C for 1 hr. Nylon colony plaque screen hybridization transfer membranes (NEN-Dupont, Boston, MA.) were used to perform duplicate lifts of each plate. Each membrane was marked for later re-alignment with plates and autoradiographs by cutting one set with two asymmetrical notches and the other set with three asymmetrical notches. Membranes were then placed on each plate for 1 min for the first lift and 2 min for the second. While on the plates, three orientation holes (made with a sterilized probe) were made through the membrane to further the accuracy of later re-alignment. Following removal from the plate, membranes were incubated in 0.5 M NaOH for 4 min and 1.0M Tris-HCl pH 7.5 for 2 min. Membranes were finally placed on Whatman paper to dry and UV cross-linked (Stratagene UV cross-linker).

Preparation and radioactive labeling of cDNA probes

Poly (A)+ RNA was prepared from fed and unfed testis as described in (*Poly(A+) messenger RNA isolation*). One microgram of mRNA from each preparation was used to produce a mixed population of double-stranded cDNA probe (using a Timesaver cDNA synthesis kit, Amersham Pharmacia, Piscataway, NJ) representative of the mRNA population in each of the tissues. Heat-denatured mRNA (65°C for 10 min, then on ice) was added to a first strand reaction mix containing 1 µl DTT, oligo(dT) primer, dNTPs and MMLV-RT and then incubated at 37°C for 1 h. For second strand synthesis, the

above mixture was added to another tube containing RNase H, dNTPs and DNA polymerase I and incubated at 12°C for 1 h and 22°C for another. In this procedure, the RNase H 'nicks' the RNA strand of the cDNA:RNA duplex and the resulting RNA fragments then serve as primers for second strand synthesis (Gubler and Hoffman, 1983; reference listed at end of chapter 3). Klenow fragment was then added to the reaction mixture and allowed to incubate at 37°C for 30 min. Finally, the resulting double-stranded cDNA was phenol/chloroform extracted and purified using Sephacryl S-300 chromatography.

Probes for library screening were triple-labeled ([^{32}P]αdATP, [^{32}P] αdCTP and [^{32}P] αdGTP; 6000Ci/mmol) using a Gibco-BRL Random Primers DNA Labeling System (Rockville, MD). Double-stranded fed and unfed T/VD cDNA was denatured by boiling for 5 min and then placed on ice. Random primers, dTTP, radioactive nucleotides, reaction buffer and Klenow fragment were then added to each tube. The reaction was allowed to incubate at room temperature for 2 h. Unincorporated nucleotides from each reaction were removed by Sephadex G-50 chromatography. Immediately prior to use, 100 µl of sheared salmon sperm DNA (5 Prime 3 Prime Inc., Boulder Colorado) was added to the probe and the mixture boiled for 5 min.

Probe hybridization and analysis

Membranes were pre-hybridized by washing them at 65 °C for 20 min in 2x SSC and 3 h in 10ml Hybrisol II (Invitrogen Corp.).

Hybridizations were carried out overnight at 65 °C. The first of each duplicate set of plaque lifts was allowed to hybridize with [^{32}P]-labeled fed-T/VD mixed cDNA probe and the second with [^{32}P]-labelled unfed-T/VD mixed cDNA probe. Following hybridization, blots were subjected to two 15 min washes in 2x SSC, 1x 30 min wash in 2x SSC/0.1% SDS and a final 10 min wash in 0.1x SSC/0.1% SDS. Blots were given a

final rinse in 2xSSC before placed on pre-wetted (with 2xSSC) Whatman paper and wrapped in Saran wrap. Blots were exposed to Kodak X-O Mat film between two intensifying screens for approximately 2 days.

Following film exposure, both sets of autoradiographs were compared. Plaques with increased signal intensity on the blot screened with fed T/VD probe, as compared to the same plaques on the blot screened with unfed T/VD probe, were identified. Autoradiographs and plates were then re-aligned using the notches and orientation holes made before performing the plaque lifts. Plaques containing feeding-induced cDNA were picked using sterile Pasteur pipettes and placed in SM buffer containing two drops of chloroform. These 'phage stocks' were numbered and stored at 4°C.

Sequencing and sequence analysis

cDNA clones, which passed the secondary screening process, were purified using either the QIAquick PCR purification kit (if only one crisp band was visible following PCR) or the QIAquick Gel extraction kit (if more than one band was visible following PCR) according to the manufacturers protocol (Qiagen, Mississauga, Ontario). Clones isolated from the secondary screen were sequenced using half reactions from a DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia, Piscataway, NJ). Sequencing PCR reactions using SK primer (priming off of the 5' end of cDNA inserts in the lambda Uni-ZAP II vector) or T7 primer (priming off of the 3' end of the cDNA inserts in the lambda Uni-ZAP II vector) consisted of 25 cycles at 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. Reactions were precipitated in 80 µl of 95% EtOH and 1 µl sodium acetate (followed by centrifugation at 10,000x g for 15 min) and washed in 200 µl of 70% EtOH (followed by another 5 min centrifugation). Most of the 70% EtOH was aspirated away with a pipette tip and any remaining was allowed to evaporate by placing the tubes containing the reactions opened into the thermalcycler at

50°C. Sequenced inserts were run on a PE Applied Biosystems 377 automated sequencer.

Sequence data were analyzed using Genetool™ (Biotools Inc., Edmonton, Canada) and comparisons with the Genbank database performed by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Northern blot analysis

Sample preparation

Northern blot analysis was performed following the protocol of Sambrook *et al.* (1989). Samples for Northern blot analysis were prepared by adding 3 µg of total RNA to a master mix containing 4 µl 10x MOPS buffer (Oncor; Gaithersburg, MD), 7 µl formaldehyde, 20 µl deionized formamide and 1 µl ethidium bromide (1 mg/ml; to fluorescently label RNA). Samples were heated at 57°C for 15 min (to denature secondary structure) and 5 µl of 10x sample buffer was added (5 Prime 3 Prime Inc.). The mixture was loaded onto 1% agarose formaldehyde gels (see below) and electrophoresed at 30 V for approximately 18 h.

1% agarose formaldehyde gels were prepared to a volume of 150 ml as follows: 27 ml formaldehyde, 15 ml of 10x MOPS buffer, 108 ml deionized water and 1.5 g agarose. Running buffer used during formaldehyde gel electrophoresis was prepared as follows: 83 ml formaldehyde, 100 ml 10x MOPS buffer and 817 ml deionized water.

Transfer of RNA

Following electrophoresis, RNA was transferred overnight to Genescreen Plus nylon membranes (NEN-Dupont, Boston, MA.) via capillary action using 10x SSC as buffer. To ensure RNA remained attached during subsequent hybridizations and washes, all membranes were UV cross-linked.

Probe labeling and hybridization

Blots were screened with probe produced from relevant insert DNA prepared by PCR amplification. Probes were labeled with [^{32}P] αdCTP using a Gibco random primers labeling kit.

Prior to hybridization blots were washed in 2x SSC for 15 min and pre-hybridized in Hybrisol II for >3 h at 65°C. Probe was then added to fresh Hybrisol II and allowed to hybridize with membrane-bound RNA at 65°C for >14 h. Membranes were then washed at 65°C for 2x 15 min in 2x SSC, 1x 30 min in 2x SSC/0.1% SDS and a final 10 min wash in 0.1x SSC/0.1% SDS. Finally, blots were rinsed at room temperature in 2x SSC, wrapped in Saran wrap and exposed to Kodak X-O Mat film between two intensifying screens.

Southern blot analysis

Sample preparation

Southern blot analysis was performed following the protocol of Sambrook *et al.* (1989). Genomic DNA was extracted from seven species of arthropod (Acarina: *A. hebraeum, D. andersoni;* Diptera: *Glossina morsitans morsitans, Drosophila melanogaster;* Orthoptera: *Acheta domestica;* Blattodea: *Blaberus gigantia;* Hemiptera: *Gerris argentatus*).

Tissue from each species was collected and immediately frozen on dry ice. Using sterile mortar and pestle, tissues were homogenized in liquid nitrogen and the powder resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg proteinase K). Samples were then incubated with shaking at 50°C overnight and the genomic DNA extracted with equal volumes of phenol/chloroform/isoamyl alcohol. Samples were then centrifuged at 1700x g and the aqueous top layer (which contains the DNA) transferred to a new tube containing 1/2

volume 7.5 M ammonium acetate and two volumes 100% EtOH. Precipitated genomic DNA (which appears stringy and white) was recovered by centrifugation at 1700x g for 2 min. Pelleted DNA was washed with 70% EtOH, air dried and resuspended in ddH_2O. Separate *Cla*I and *Sau*3AI restriction endonuclease digests were set up for each genomic DNA sample according to the manufacturers of the enzyme (Gibco-BRL, Rockville, MD). Genomic DNA (2 µg) was added to a 50 µl reaction containing 1.5 µl of each (*Cla*I and *Sau*3AI) restriction endonuclease, 5 µl of 10x reaction buffer and ddH_2O. Samples were incubated at 37°C for 2 h.

DNA was then subjected to electrophoresis in 0.8% agarose gels. Gels were washed in 0.25 M HCl for 10 min, denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 2x 20 min and neutralization buffer (1.5 M NaCl, 0.5 M Tris HCl pH 7.0) for 2x 20 min. DNA was then transferred overnight to Genescreen Plus nylon membranes (NEN-Dupont, Boston, MA.) and UV cross-linked. Following pre-washing and pre-hybridization (57°C for 20 min in 2x SSC and then for 3 h in Hybrisol II respectively), blots were probed at 57°C with [^{32}P]-random primer labeled PCR product from desired clones. Finally, blots were washed, under reduced stringency, at 57°C for 15 min with 2x SSC, 1x in 2x SSC/0.1% SDS and 0.1x SSC/0.1% SDS. Blots were wrapped in Saran wrap and autoradiography was done using Kodak X-O Mat film between two intensifying screens.

Appendix 5

Primer sequences used to PCR amplify all feeding-induced clone inserts.

Clone #	Primer sequence*
1	5'-gggaattcggatgtccgcgccagagtcc-3' 3'-cggcggaacctctagttgtcgggagctcgg-5'
2	5'-gggaattcggatgcagccgcacctagatcc-3' 3'-ggaccgacctttgtcagatctttgagggagctcgg-5'
3	5'-gggaattcgggatgctcctaatcgtcaccg-3' 3'-ccgcacgctattttcctgggagctcgg-5'
4	5'-gggaattcgggatgtccgaagaaccgactaaag-3' 3'-ccgtccatggcgcgatagggagctcgg-5'
5	5'-gggaattcgggatgcacgtgccgaattcg-3' 3'-agctcacaaccccagtttgatcgggagctcgg-5'
6	5'-gggaattcgggatgcagctgacccaacc-3' 3'-gaaatgacgtttgtgttctggggagctcgg-5'
7	5'-gggaattcgggatggtgcgcagcgcaaa-3' 3'-ttctgaagtcgtaatttttctgacgggggggagctcgg-5'
8	5'-gggaattcgggatggtcgaattagcccctcc-3' 3'-gcaaggctaaggaggtttgaaggggagctcgg-5'
9	**5'-gggaattcgggatgttgatcaccaaggacctga-3' 3'-ggctcgaactgtgaccagctgggagctcgg-5'**
10	5'-gggaattcgggatgagcgcgtacaaggc-3' 3'-ctcaccatggcgtgggtggggagctcgg-5'
11	5'-gggaattcgggatgggtggtggcagtgtg-3' 3'-gctcgttagggtgaatgttatcgggagctcgg-5'
12	5'-gggaattcgggatggattgaggctcatctgtc-3' 3'-cgggaccttgaagatgacctgggagctcgg-5'
13	5'-gggaattcgggatgctggaacagacacatctc-3' 3'-gaacggttggcaatcctttgggagctcgg-5'
14	5'-gggaattcgggatggatctgtcctcccagg-3' 3'-gacgagttgaagagtgggaaggggagctcgg-5'
15	5'-gggaattcggggatgaggttacgccccc-3' 3'-cggtttgatccgggaaagggagctcgg-5'
16	5'-gggaattcgggatggctcttgaaaaaactctgg-3' 3'-ttcgtacgaccatgagagaaacgggagctcgg-5'
17	5'-gggaattcgggatggtcattcggatatctct-3' 3'-gatccgagcgcccgggagctcgg-5'
18	5'gggaattcgggatggactccagtgaggaagc-3' 3'-aactgggagctcgggagctcgg-5'
19	5'-gggaattcgggatggtcaaatctcgaagccggc-3' 3'-tgtgagtatcctgataagtagtaactggggagctcgg-5'
20	5'-gggaattcgggatgatccggcggcgttct-3' 3'-gttcataatcggcctattaaccttagggagctcgg-5'
21	5'-gggaattcgcatgggggcgtta-3' 3'-ccctttgcaccccacggggagctcgg-5'
22	**5'-gggaattcgggatggcgaaacagggactt-3' 3'-acccctcggacgccgggagctcgg-5'**
23	5'-gggaattcgggatgattgacagtatttggaccgc-3' 3'-ttcgggctcctaatcctatcgggagctcgg-5'
24	5'-gggaattcgggatgggagaactagtctcgacgg-3' 3'-gcccgcttcactcagcggggagctcgg- 5'
25	5'gggaattcgggatgtgaaagtcggagccgaa3' 3'-taacaggacagggactatggtttgggagctcgg-5'
26	5'-gggaattcgggatggcgacgactactctc-3' 3'-aattatgcaggggatgtcggggagctcgg-5'
27	5'-gggaattcgggatggtgaactacgcctgaaaggaatc-3' 3'-atttgatcctcaggggattatgtgtagggagctcgg-5'
28	5'-gggaattcgggatggcctcgtgccgaattcgg-3' 3'-gagtttgccgagtccgtgttgggagctcgg-5'

* Primers were designed using Genetool software (Biotools Inc., Edmonton, Canada) and made by Invitrogen Co. (Carlsbad, Ca.).

Bolded sequences are those used to amplify inserts encoding proteins found to have EF bio-activity.

Appendix 6

Cell line maintenance

Upon receipt, cryovials containing *Sf*21 and *Sf*9 cells were rapidly defrosted and their contents emptied into a 25 cm^2 culture flask containing 4 ml of 4°C sterile complete TNM-FH medium (Grace's insect medium supplemented with TC yeastolate and lactalbumin hydrolysate, 5% fetal bovine serum (FBS) and 10mg/ml gentamyicin). Cells were given 30-45 min to attach to the flask, at which point the old medium was replaced with 5 ml of fresh, room temperature medium (this is required to remove toxic DMSO contained in the freezing medium).

Cells were passaged every 48-60 h, or after having reached a confluent density of ~$1x10^7$ cells/ml. Once a confluent monolayer had formed, cells were lifted from the surface by tapping the bottom of the flask. Cells were generally divided into three new culture flasks and the volume of media topped off at 5 ml. In this manner cell cultures were maintained for stock (freezing) purpose and experimentation.

The following medium was used to freeze *Sf*21 cells: 60% Grace's Insect Medium, 30% FBS and 10% DMSO. Appropriately labeled sterile cryovials were placed on ice. Cells were removed from the flask surface and spun at room temperature for 10 min at 400-600x g. The supernatant was removed and replaced with the above-mentioned freezing medium at a density of $1x10^7$ cells/ml. Finally, cryovials were placed at -20°C for 1 h, -80°C for 24-48 hr and then liquid nitrogen for long-term storage.