University of Alberta

Rising from the ashes; an unanticipated failure with the engorgement factor voraxin leads to advances in three areas of tick biology: Developmental abnormalities and parthenogenesis, identification of a *Coxiella*-like symbiont and the molecular characterization of vitellogenesis in the southern African bont tick, *Amblyomma hebraeum* Koch (1844)

by

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Abstract

Weiss and Kaufman (2004, PNAS 101: 5874-5879) demonstrated that injections of fed male gonad homogenates stimulate engorgement in virgin female *Amblyomma hebraeum* ticks, due to the presence of the engorgement factor voraxin. They also showed voraxin's potential as part of an anti-tick vaccine: 74% of mated females fed on a rabbit immunized against voraxin failed to engorge. The original aim of this thesis was to further characterize and better evaluate the potential of voraxin as a component of a novel anti-tick vaccine. However, I was unable to confirm Weiss and Kaufman's results. Injections of male gonad homogenates or recombinant voraxin produced in two different bacterial expression systems into virgin females, both on and off the host, failed to stimulate engorgement. Immunization of rabbits against the recombinant voraxin proteins also failed to inhibit tick feeding. Silencing of voraxin via RNAi was unsuccessful, but I was able to silence subolesin, an important modulator of tick feeding and reproduction.

I also observed a large number of morphological abnormalities in our ticks, as well as several virgin females that engorged and laid viable eggs. Although low levels of parthenogenesis have been reported in many other normally bisexual tick species, it has not been previously reported in *A. hebraeum*. The causes of these abnormalities are unknown, but could be due, in part, to the high degree of inbreeding in our lab colony.

In addition to the numerous bacterial pathogens transmitted by ticks, many tick species also harbour a number of potentially mutualistic endosymbiotic

bacteria. Here I have determined that out tick colony possess both *Coxiella*-like and *Rickettsia*-like symbionts. Their impact on the biology of the tick is unknown.

Although much is known about vitellogenesis and its regulation in insects, our knowledge in this area is much more limited in ticks. I have sequenced the full-length cDNAs encoding two vitellogenins and the vitellogenin receptor from *A. hebraeum*, which are similar to those of other arthropods. RT-PCR analysis of gene expression showed that the vitellogenin receptor is expressed only in the ovary of fed females, whereas vitellogenin is produced in the fat body and midgut.

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List of Abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
5'/3/ RACE	5'/3' Rapid amplification of cDNA ends
20E	20-hydroxyecdysone
aa	Amino acid
AP	Alkaline phosphatase
AUAP	Abridged universal adaptor primer
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BSAS	Biological sciences animal services
Bla	β-lactamase
bp	Base pair
bw	Body weight
CBS	Centre for Biological Sequence Analysis
CDD	Conserved Domain Database
cDNA	Complementary DNA
CP	Carrier protein
CW	Critical weight
d0pE	Zero days post-engorgement
d4pE	Four days post-engorgement
d10pE	Ten days post-engorgement
dA	Deoxyadenosine
dATP	Deoxyadenosine triphosphate

DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
dT	Deoxythymidine
dTTP	Deoxythymidine triphosphate
dsRNA	Double stranded RNA
EF	Engorgement factor
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
gDNA	Genomic DNA
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JH	Juvenile hormone
LB	Lysogeny broth
LBD	Ligand-binding domain
LC	Liquid chromatography
LDLR	Low-density lipoproteins receptor
LDLRA	Low-density lipoproteins receptor class A
LL	Dileucine
MAG	Male accessory gland
MEGA	Molecular evolutionary genetics analysis
MF	Male factor

mRNA	messenger RNA
MS	Mass spectroscopy
N/A	Not applicable
NC	New colony
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
nt	Nucleotide
OC	Old colony
OD	Optical density
OGP	Ovarian growth phase
OLSD	*O*-linked sugar domain
ORF	Open reading frame
PC	Pro-protein convertase
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PVDF	polyvinylidene difluoride
Q	Query
rDNA	Ribosomal-RNA encoding DNA
RH	Relative humidity
RNAi	RNA interference
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Salivary glands
SPG	Spotted fever group
SR	Seminal receptacle
TEM	Transmission electron micrography
TVD	Testis/vas deferens
UAP	Universal adaptor primer
UTR	Untranslated region
Vg	Vitellogenin
VgR	Vitellogenin receptor
VIF	Vitellogenin inducing factor
VLDLR	Very-low-density lipoproteins receptor
Vn	Vitellin
VSF	Vitellogenesis stimulating factor
VUF	Vitellogenin uptake factor
vWD	von Willebrand factor type D domain

Chapter 1. General introduction

1.1 General Tick Biology

Ticks are obligate haematophagus arthropods (Arthropoda; Arachnida; Acari; Parasitiformes; Ixodida) that are known to infest all classes of terrestrial vertebrates (Sonenshine, 1991). They are also considered to be the second-most important arthropod vector of human disease after mosquitoes, and are known to be vectors of a diverse array of many major animal pathogens (Parola & Raoult, 2001). Tick species transmit a wide variety of viruses, spirochaetes, proteobacteria, protozoa, fungi and filarial nematodes (Sonenshine, 1991; Parola & Raoult, 2001; Tu *et al.*, 2005). *Amblyomma hebraeum* Koch (1844) is the principle vector of *Rickettsia africae* Kelly et al. (1996), the agent of African tick bite fever, in addition to being an important vector of *Ehrlichia* (*Cowdria*) *ruminantium* Cowdry (1925), the causative agent of heartwater disease (Norval *et al.*, 1990; Kelly & Mason, 1991). In Canada, *Dermacentor andersoni* Stiles (1908) is a vector of *R. rickettsii* Wolbach (1919), the causative agent of Rocky Mountain spotted fever, and is known to transmit other bacterial pathogens such as *Francisella tularensis* McCoy and Chapin (1912) and *Coxiella burnetii* Derrick (1939) (Ricketts, 1991; Parola & Raoult, 2001).

In addition to the numerous pathogens ticks transmit, several species, notably *D. andersoni, D. variabilis* Say (1821) and *Ixodes holocyclus* Neumann (1899), secrete a paralytic toxin that is capable of disabling or even killing their human or animal host (Schmitt *et al.*, 1969; Stone *et al.*, 1989; Felz *et al.*, 2000; Mans *et al.*, 2004). Moreover, severe tick infestations may result in host anaemia,

loss of appetite, weakening of the immune system, depletion of the visceral fat stores, disruption of liver metabolism, in addition to extensive hair loss (Nelson *et al.*, 1977; McLaughlin & Addison, 1986; Mooring & Samuel, 1999). It has been estimated that each fully engorged *A. hebraeum* female can contribute to a 10 gram average reduction in the live weight gain of Afrikander steers (Norval *et al.*, 1989), and that the effect of ticks and tick-borne pathogens on cattle production alone contribute to a worldwide annual economic loss estimated at hundreds of millions of dollars (Peter *et al.*, 2005).

Ticks are also known to harbour a number of non-pathogenic, transovarially transmittted, *Rickettsia*-like bacteria (Balashov, 1972; Diehl *et al.*, 1982). Initial descriptions of these symbionts were based strictly on ultrastructural observations (Suitor Jr. & Weiss, 1961; Balashov, 1972; Reinhardt *et al.*, 1972), but with the advent of DNA sequencing and other molecular techniques, their identities and the functional roles they play in ticks are now being elucidated more precisely (Niebylski *et al.*, 1997; Noda *et al.*, 1997; Jasinskas *et al.*, 2007; Machado-Ferreira *et al.*, 2011).

Ticks comprise three main families: (1) the Ixodidae, or ‘hard ticks’, of which there are ~692 species, (2) the Argasidae, or ‘soft ticks’, containing ~186 species, and (3) the Nuttalliellidae, a little researched monotypic family represented by the single species, *Nuttalliella namaqua* Bedford (1931) (Nava *et al.*, 2009). Ixodid ticks are often divided into two groups: the Prostriata, consisting solely of the genus *Ixodes* and the Metastriata, comprising all remaining ixodid genera (Fig. 1.1). The designations 'hard' and 'soft' refer to the

characteristics of their cuticle. The ixodid ticks possess a hard, sclerotized, dorsal plate called a scutum, as well as an apically positioned gnasthosoma, whereas the argasids have a soft, leathery integument lacking a scutum, with the mouthparts located on the anterior venter surface (Sonenshine, 1991). *Nutalliella namaqua* possesses characteristics of both the ixodids and the argasids: a leathery integument, an apically positioned capitulum, and a partially sclerotized pseudo-scutum (Fig. 1.2; Bedford, 1931; Keirans *et al.*, 1976; el Shoura, 1990). Recent work by Mans *et al.* (2011) has determined that *N. namaqua* is likely basal to the other tick families and is the closest existent species to the last common ancestor of the Ixodida.

Argasid and ixodid ticks employ distinct reproductive strategies in order to complete their life cycles (Diehl *et al.*, 1982; Sonenshine, 1991). Argasid ticks tend to be nidicolous, dwelling in the nests and burrows of potential host animals and their life cycle generally comprises the egg, larva, 2-8 nymphal stages and a sexually dimorphic adult stage. Following hatching, each instar must imbibe a single blood meal to complete development and moult to the next stage, although a few exceptions to this pattern are recognized (Oliver Jr., 1989). After feeding, each stage normally drops off the host to moult, after which they seek out a new host for the next blood meal. Adult argasids usually feed several times, the females imbibing relatively small blood meals and engorging to ~3 – 12X their unfed weight over a period of several minutes to a few hours (Diehl *et al.*, 1982; Sonenshine, 1991). Following mating, which occurs off the host, either before or after the first adult blood meal, the female produces a batch of a few hundred

eggs, and then seeks another meal (Sonenshine, 1991). Whereas most adult argasid females require a blood meal before they are able to oviposit, several species are autogenous: able to produce eggs prior to feeding as an adult (Feldman-Muhsam, 1973).

A majority of the Metastriata, are not nidicolous, parasitizing a variety of free-ranging host species. Many of the Prostriata (i.e. *Ixodes*) are nest or burrow parasites (Diehl *et al.*, 1982; Sonenshine, 1991; McCoy & Tirard, 2002). Like argasids, ixodids also go through four stages: egg, larva, nymph and adult. However, unlike the argasids, they go through only one nymphal stage. The majority of ixodid ticks require three hosts to complete their life cycles, each active stage taking in a single large blood meal, dropping off the host, and moulting into the next instar (Sonenshine, 1991). A few species have a truncated '2-host' or '1-host' life cycle, in which fed juveniles remain and develop to the next stage on the host after feeding (Sonenshine, 1991). All stages of ixodid ticks feed very slowly compared to argasids, often taking more than a week to feed to repletion.

The long feeding period of ixodids can be divided into three phases: 1) a 24 – 36 hour preparatory phase, during which the tick establishes a feeding lesion and secretes a ‘cement’ to attach it to the host epidermis; 2) a 7 – 10 day slow feeding phase, during which the female's weight increases approximately 10-fold; and 3) a ~12 – 36 hour rapid feeding phase, during which the weight increases up to a further 10-fold (Balashov, 1972). Mating occurs on the host, the ticks usually requiring ~3 days of feeding prior to copulation (Khalil, 1970). The vast majority

of *Ixodes* spp. can copulate prior to feeding (Kiszewski *et al.*, 2001). Although the females take in a huge blood meal, the males rarely increase their unfed weight by more than ~50%, and may remain on the host for weeks to several months (Oliver Jr., 1989; Norval *et al.*, 1991). After engorging, the female drops from the host, oviposits a large number of eggs (up to 20 000 in *A. hebraeum*), and then dies (Sonenshine, 1991). In many species the oviposited eggs weigh about half the engorged body weight of the tick (Friesen & Kaufman, 2002).

1.2 Male reproductive biology

1.2.1 Spermatogenesis and spermiogenesis

The gonads of adult male metastriate ticks consists of elongated paired tubular testes, and a large multi-lobed accessory gland, both of which empty into the ejaculatory duct (Fig. 1.3; Oliver Jr., 1982; Sonenshine, 1991). The process of sperm development in ticks occurs in two stages: (a) spermatogenesis, the series of mitotic and meiotic divisions that lead to the formation of haploid spermatids; and (b) spermiogenesis, the growth and differentiation of the spermatids into fully mature, elongate, aflagellate spermatozoa (Oliver Jr., 1982; Sonenshine, 1991). This process begins during nymphal ecdysis but is arrested in most metastriate species following the formation of the primary spermatocytes (Dumser & Oliver Jr., 1981; Sonenshine, 1991; Kiszewski *et al.*, 2001). Following nymphal ecdysis and feeding of the adult, spermatogenesis resumes: the primary spermatocytes increasing in size, then undergoing reductional and equational divisions, leading to the formation of the spermatids (Oliver Jr., 1982; Sonenshine, 1991). Spermiogenesis begins immediately following spermatid formation. The cells

undergo numerous physiological and morphological changes (described in detail in Oliver Jr., 1982), and the resulting prospermia (mature elongated spermatids) accumulate in the vasa deferentia and seminal vesicle.

1.2.2 Mate pairing and formation of the spermatophore

Males, attracted by conspecific female sex pheromones, will seek out females and probe the female's genital pore with their mouthparts (Feldman-Muhsam & Borut, 1971; Sonenshine *et al.*, 1982; Feldman-Muhsam, 1986). While probing the female, the male forms the spermatophore externally by extruding various mucopolysaccharides and proteins to form a membranous, multilayered sac-like ectospermatophore (Feldman-Muhsam & Borut, 1971; Feldman-Muhsam, 1986; Matsuo *et al.*, 1998). The sac is then filled with spermatids and seminal fluids and sealed with a mucopolysaccharide droplet, the whole process requiring only ~30 s (Feldman-Muhsam, 1986). The male then contorts its body to bring the spermatophore closer to the female gonopore and, salivating copiously, it deposits the spermatophore onto the female gonopore. Using its chelicerae, it then implants the neck of the spermatophore into the female genital aperture and the contents of the spermatophore are everted into the female genital opening due to internally generated CO_2 pressure (Feldman-Muhsam & Borut, 1971; Feldman-Muhsam *et al.*, 1973).

1.3 Mating factors

In addition to the spermatids, the spermatophore contains a number of other factors and secretions from the male accessory gland (MAG) and testis/vas

deferens (TVD). In insects, these secretions have a number of functions (reviewed by Gillott, 2003; Kaufman, 2004; Avila *et al.*, 2011) including the maintenance of the spermatozoa within the female reproductive tracts (den Boer *et al.*, 2009a, 2009*b*; King *et al.*, 2011), secretion of the spermatophore (Gerber *et al.*, 1971), alteration of female physiology and behaviour (Heifetz *et al.*, 2000; Ravi Ram & Wolfner, 2007; Shutt *et al.*, 2010), formation of a mating plug to enable sperm storage or inhibit further copulations (Orr & Rutowski, 1991; Rogers *et al.*, 2009), to provide the female with a nutritional supplement (Voigt *et al.*, 2008; South & Lewis, 2011), and to increase sperm viability (Simmons & Beveridge, 2011; King *et al.*, 2011).

Capacitation of the prospermia follows the transfer of the spermatophore to the female. Shepherd *et al.* (1982) demonstrated that capacitation is induced by a trypsin-sensitive 12.5 kDa protein secreted by the MAG of *D. variabilis* and *Ornithodoros moubata* Murray (1877). Notwithstanding the size similarity of the factors present in the two species, they are not cross-reactive, each having no effect on the spermatids of the other. The authors hypothesized that the capacitation factor was a proteolytic enzyme, the activity of which led to the rupture of the operculum present on the outer sheath of the spermatid. However, neither trypsin nor pronase were able to trigger capacitation (Shepherd *et al.*, 1982). Only a short period of exposure to MAG extract was necessary to induce capacitation, suggesting that the capacitation factor acts like a signalling molecule as opposed to directly acting on the spermatids as an enzyme (Shepherd *et al.*, 1982).

In *D. variabilis*, copulation is required for the females to feed to repletion (Pappas & Oliver Jr., 1971). Females co-fed with males that had blocked genital apertures failed to engorge, suggesting that the spermatophore contains an 'engorgement factor' (EF) that stimulates feeding to repletion (Pappas & Oliver Jr., 1972). This factor was not associated with the spermatids/spermatozoa, because males irradiated with cobalt-60 (to kill the spermatids and germinal cells of the males) were still able to induce engorgement in females, despite the complete absence of spermatids within the spermatophore (Pappas & Oliver Jr., 1972). In *A. hebraeum*, very few virgin females ever exceed ~10X the unfed weight, a threshold termed 'the critical weight' (CW), marking the transition between the slow and rapid phases of feeding (Harris & Kaufman, 1984; Lomas & Kaufman, 1992*a*). However, copulation stimulates full engorgement. Weiss & Kaufman (2004) found that injection of TVD homogenates into the haemocoel stimulate partially fed virgin females to engorge, but that MAG homogenates do not. Following the identification of 35 transcripts upregulated in the fed male TVD (Weiss *et al.*, 2002), recombinant versions of these proteins were produced in an insect cell expression system. Two of these proteins (voraxinα and voraxinβ) must be co-injected for full engorgement to occur.

Following engorgement and detachment from the host, the salivary glands (SG) of mated female *A. hebraeum* degenerate within about 4 days (Harris & Kaufman, 1984), and those of virgins above the CW within about 8 days (Lomas & Kaufman, 1992*a*). Surgical removal of the seminal receptacle of engorged females prevented SG degeneration, but SG degeneration resumed on either the

replacement of the organ or the injection of male gonad homogenates into the haemocoel (Harris & Kaufman, 1984). Injection of male gonad homogenates into virgins above the CW also hastened SG degeneration, causing the glands to lose 95% of their fluid secretory competence within 4 days as opposed to the 8 it would normally take (Lomas & Kaufman, 1992*a*). The so-called "male factor" (MF) responsible for the latter effects is produced in the TVD only during feeding (Lomas & Kaufman, 1992*a*; Kaufman & Lomas, 1996). It acts by triggering the secretion of an ecdysteroid hormone into the haemolymph, possibly via the mediation of an ecdysteroidgenic neuropeptide from the synganglion (Harris & Kaufman, 1985; Lindsay & Kaufman, 1988; Lomas & Kaufman, 1992*b*; Lomas *et al.*, 1997).

Connat *et al.* (1986) described another mating factor, the 'vitellogenesis stimulating factor' (VSF), in *O. moubata*. Following their first adult blood meal, virgin *O. moubata* start the process of vitellogenesis. However, if they remain virgin, whatever yolk was produced is then reabsorbed, a process termed 'abortive vitellogenesis'. Subsequently, ~100 days after feeding, the ovary again resembles that of an unfed virgin (Sahli *et al.*, 1985; Connat *et al.*, 1986). Mechanical stimulation of the female genital tract by the insertion of small metal beads into the gonopore is sufficient to induce vitellogenesis and oviposition in females following abortive vitellogenesis (Ducommun, 1984; Connat *et al.*, 1986). Injection into virgin females of homogenized endospermatophores taken from the uteri of mated females, also stimulates vitellogenesis and oviposition (Connat *et al.*, 1986). Vitellogenesis was not triggered by injections of MAG, TVD or

spermatophores that had not been introduced to the uterus (i.e. prior to capacitation). However, mature spermatazoa, even after 20 h of washing in a physiological medium, retained VSF activity. To determine the source of the VSF activity, Sahli *et al.* (1985) dissected endospermatophores taken from mated females 1 – 48 h post-copulation. They washed the endospermatohores and assayed the wash media for VSF activity. VSF activity was detected 12 – 48 h post-copulation, the highest VSF activity occurring ~12 h post-copulation, by which time capacitation is complete (Sahli *et al.*, 1985). The foregoing, along with the observation that VSF activity was abolished by proteinase K treatment, led Sahli *et al.* (1985) to conclude that the VSF is a protein (or proteins) synthesized by the prospermia and released into the female genital tract only following capacitation.

1.4 Female reproductive biology

1.4.1 Anatomy of the female reproductive system

The female gonads of all ticks show a generally similar morphology, consisting of a single hollow horseshoe shaped ovary (Fig. 1.4) comprising a lumen surrounded by a thin wall of epithelial cells, oogonia and primary oocytes in various early stages of development (Diehl *et al.*, 1982; El Shoura *et al.*, 1984). Each end of the ovary empties into an oviduct; the oviducts enter a common uterus, leading to a vagina that comprises cervical and vestibular regions (Diehl *et al.*, 1982). Metastriate ixodids have a sac-like seminal receptacle that extends from the cervical vagina in which the endospermatophore is stored following mating, whereas argasid and prostriate ixodids store the endospermatophore in the

uterus or an expanded portion of the cervical vagina, respectively (Diehl *et al.*, 1982). In all female ticks, paired, tubular accessory glands are located at the dorsal junction of the cervical and vestibular regions, but only ixodids also posses a tri-lobed lobular accessory gland that forms on the dorsal hypodermis of the vestibular vagina during feeding (Diehl *et al.*, 1982; Sonenshine, 1991).

Distinct from the genital tract is Géné's organ, an eversible glandular tissue possessing finger-like ‘horns’ that protrude through the camerostomal cavity during oviposition (Diehl *et al.*, 1982; Sonenshine, 1991). As each egg exits the genital aperture, Géné's organ everts and covers it with a lipid-rich secretion that waterproofs the eggs (Lees & Beament, 1948; Balashov, 1972; Diehl *et al.*, 1982). This secretion from Géné's organ also contains antimicrobial activity (Arrieta *et al.*, 2006; Yu *et al.*, 2012). Two porose areas found externally on the dorsal surface of the basis capitulum also produce secretions that are smeared onto the eggs by Géné's organ during oviposition (Diehl *et al.*, 1982).

1.4.2 Oogenesis in ixodid ticks

Oogonia are present in the ovarian primoridia of unfed larvae with continued oogonial divisions occurring as the tick goes through the larval and nymphal stages (Balashov, 1972). In unfed nymphs, the ovary is a simple tube-like structure consisting of oogonia, undifferentiated interstitial cells and a simple epithelium (Brinton & Oliver Jr., 1971*a*; Balashov, 1972). In the newly moulted unfed female, the ovary contains numerous small oocytes and is surrounded by a single cell layer of epithelial cells, occasional smooth muscle cells and the acellular tunica propria, a sheath of fibrillar connective tissue (Sonenshine, 1991).

A longitudinal groove containing primary oocytes in the earliest stages of development, runs the length of the ovarian surface (Diehl *et al.*, 1982). Unlike the germarium of insect ovaries, the longitudinal groove contains no oogonia, all oogonial divisions having occurred during the larval and nymphal stages of the adult female (Brinton & Oliver Jr., 1971*a*). In the engorged female, the oocytes rapidly enlarge, protrude into the surrounding haemolymph, and migrate outward from the longitudinal groove as they expand (Fig. 1.5). The mature oocytes remain connected to the ovary via the funiculus, a thin stalk of elongated epithelial cells; and the tunica propria (Diehl *et al.*, 1982; Sonenshine, 1991).

Balashov (1972) divided the growth and development of the primary oocytes into five distinct stages, consisting of two initial pre-vitellogenic stages, followed by two vitellogenic stages and ending with oviposition. Stage I primary oocytes first appear following nymphal feeding and the subsequent transformation of oogonia to primary oocytes; at this time the small oocytes enter prophase of the first maturation division, bypassing interkinesis. This period of so-called 'small cytoplasmic growth', ends when the meiotic prophase is arrested in diakinesis. Further oocyte development is arrested in most metastriate ticks until feeding begins, although it continues in both the Prostriata and Argasidae (Sonenshine, 1991). Stage II, the period of 'great cytoplasmic growth', begins with the adult blood meal and ends with the first appearance of yolk granules in the cytoplasm (Balashov, 1972). This stage is characterized by the marked enlargement of both the nucleus and cytoplasm, resulting in the protrusion of the oocyte into the surrounding haemolymph. Stages III and IV are marked by the enormous

expansion of the oocytes as they accumulate yolk proteins and expand the surrounding vitelline envelope (Sonenshine, 1991). Stage III ends once the oocyte cytoplasm has become completely filled with large yolk granules. During stage IV, the nucleolus and nuclear membrane dissolve and the chromosomes condense into a dense karyosphere. By the end of this stage, the oocytes bulge out into the haemolymph, remaining connected to the ovary wall by the funicular cells and possess a complete vitelline envelope (Balashov, 1972). Entry of the oocytes into the ovarian lumen and their subsequent oviposition marks stage V of development. The site and timing of oocyte fertilization is still a point of contention, with some authors suggesting that fertilization occurs in the ovary (Brinton & Oliver Jr., 1971*b*), whereas others claim that it takes place in the anterior part of the oviducts (Balashov, 1972).

Development of oocytes in the tick ovary is an asynchronous process, particularly once yolk uptake has begun. Even during oviposition, oocytes at all stages of development are still found in the ovary, with the least developed oocytes found in the longitudinal groove. Using *A. hebraeum* as a model, Seixas *et al.* (2008) developed a system that combined an index of ovary size with the Balashov oocyte stages. Ovaries 0 – 2 days post-engorgement generally correspond to ovarian growth phase 1 (OGP 1), and are typically very small, thin, translucent white thread-like structures, with the oocytes <150 µm in length and possessing visible nuclei. During OGP 2, ~2 – 5 days post-engorgement, the ovaries become significantly larger and at least some oocytes have increased to ~250 µm in length, are opaque but have not yet taken up yolk. Ovaries in OGP 3,

~5 – 6 days post-engorgement, have again increased in overall size and show considerable growth, with many oocytes ~400 µm and filled with reddish-brown yolk granules, although oocytes from all earlier stages of development are still present. OGP 4 begins 6 – 7 days following engorgement and ends with the onset of ovulation. At this point the ovary is the second largest organ after the midgut, and is completely covered in large yolk-filled oocytes, with distinct yolk spheres visible in the oocytes. Ovaries in OGP 5, ~10 days post-engorgement, appear similar in size and morphology to those in OGP 4, with the exception that many large yolk-filled oocytes are now present in the ovary lumen and oviduct, and oviposition may have commenced.

1.4.3 Vitellogenesis

Vitellogenesis, the synthesis and accumulation by the developing oocytes of the major yolk protein vitellogenin (Vg), begins at the end of Balashov's stage II (OGP 2/3) and continues until oviposition has ended. Vg is a complex haemoglycolipoprotein composed of large glycosylated lipoprotein subunits and haem moities, the latter of which are responsible for the golden brown colour of the eggs (Boctor & Kamel, 1976; Sonenshine, 1991). In most insects, Vg synthesis occurs in the fat body, but in ticks, the midgut is also a significant source of this protein (Thompson *et al.*, 2007; Tufail & Takeda, 2008; Horigane *et al.*, 2010; Boldbaatar *et al.*, 2010; Khalil *et al.*, 2011). Following synthesis, Vg undergoes various post-translational modifications, such as glycosylation, phosphorylation, and sulphation, and is cleaved into large and small subunits before being released into the haemolymph (Taylor & Chinzei, 2002). The

developing oocytes take up Vg via receptor-mediated endocytosis and is then further processed and stored in large homogenous yolk granules (Coons *et al.*, 1989; Sonenshine, 1991).

In most insects vitellogenesis is regulated primarily by juvenile hormone (JH), with ecdysteroids also playing an essential role in dipterans (Tufail & Takeda, 2008). In ticks, however, JH does not play a role in the regulation of vitellogenesis; instead, the vitellogenic hormone is an ecysteroid (probably 20-hydroxyecdysone) (reviewed by Rees, 2004). Following engorgement and detachment from the host, an ecdysteroidogenic neuropeptide, the 'vitellogenin inducing factor' (VIF) is released from the synganglion (Chinzei & Taylor, 1990; Chinzei *et al.*, 1992; Lomas *et al.*, 1997; Friesen & Kaufman, 2009), which then acts on the epidermal cells to initiate ecdysteroid synthesis (Zhu *et al.*, 1991; Lomas *et al.*, 1997; Roe *et al.*, 2008). This stimulates the fat body and midgut to begin Vg synthesis and, in *D. variabilis*, prompts Vg uptake into the oocytes (Friesen & Kaufman, 2002, 2004; Thompson *et al.*, 2005). Interestingly, *A. hebraeum* requires an additional 'vitellogenin uptake factor' (VUF) to permit Vg-uptake into the oocytes (Lunke & Kaufman, 1992; Friesen & Kaufman, 2004; Seixas *et al.*, 2008).

1.5 Thesis objectives

The original aim of this thesis was the further elucidation of the engorgement factor, voraxin. As has been stated previously, voraxin appeared to be a promising target for the development of a novel anti-tick vaccine, as immunization of a rabbit against recombinant voraxin produced in an insect cell

expression system resulted in a 74% of mated females failing to feed beyond one tenth the normal engorged weight (Weiss & Kaufman, 2004). To this end, I attempted to confirm the results of Weiss & Kaufman (2004) using a bacterial expression system to produce recombinant voraxin (Chapter 2).

Weiss and Kaufman (2004) had previously shown that injection of both voraxinα and voraxinβ were sufficient to induce engorgement in partially fed virgins. However, it remained unclear if these proteins were necessary for engorgement, or if they were merely sufficient to stimulate it to occur. To determine if both components of voraxin were required for EF activity, I attempted to silence voraxin using RNA interference (Chapter 3).

While I was attempting to replicate the work of Weiss and Kaufman, I noticed many of our ticks appeared to be deformed, and we had several virgin female ticks engorge, in direct opposition to all data collected to date stating that mating must occur in order to stimulate female engorgement. This prompted me to undertake a more thorough examination of our tick colony for morphological abnormalities (Chapter 4).

At the same time as my work on voraxin, during routine feeding of nymphal ticks for the purpose of colony maintenance, we had several rabbits suddenly, and unexpectedly die. This prompted me to examine our tick colonies for the presence of potential bacterial pathogens. Although no putative pathogens were found, I was able to detect the presence of two bacterial symbionts in our tick colonies, identifying both a *Coxiella* sp. and a *Rickettsia* sp. that are similar to other tick endosymbionts and appear to be transovarially transmitted (Chapter 5).

Due to the difficulties I encountered attempting to further characterize voraxin, as well as the myriad issues associated with our tick colony described in Chapters 4 and 5, I decided to switch the main focus of my research to another aspect of tick reproductive biology, namely the process of vitellogenesis (Chapter 6). I hypothesized that the genes involved in this process in *A. hebraeum* were likely similar to those present in insects and other tick species. To determine if this was the case, I used degenerate primers to amplify the cDNA sequences of the vitellogenin receptor and two vitellogenins. I then established the approximate spatial and temporal patterns of expression of each gene within the tick and confirmed their identities via RNAi and sequence analysis.

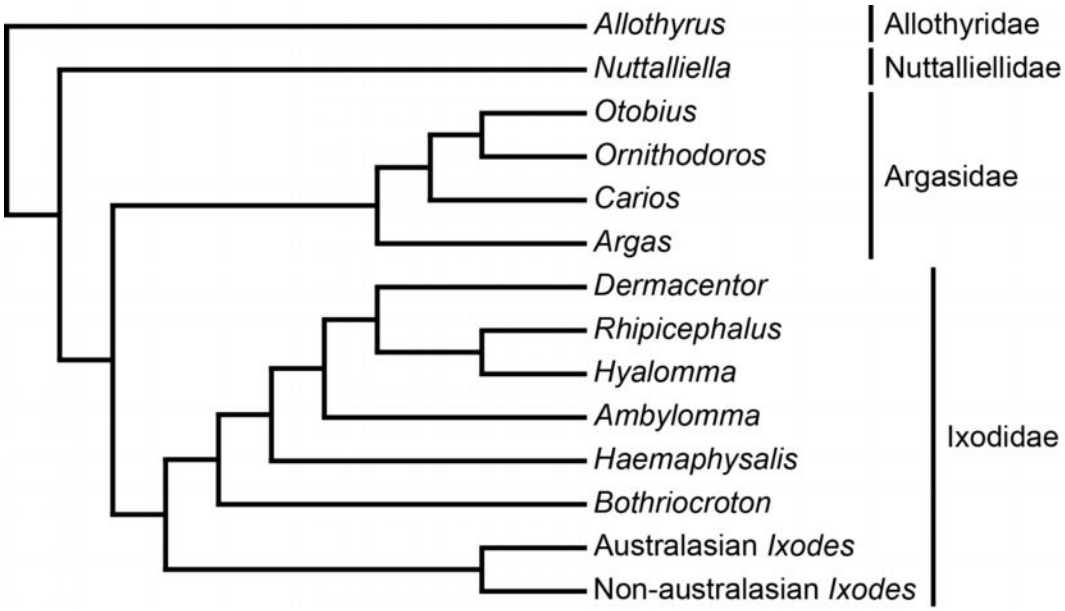

Figure 1.1. A current hypothesis for the phylogenetic relationships of ticks. This diagrammatic representation of proposed phylogeny of ticks is a simplified version of the trees generated from 16S, 18S and 28S ribosomal RNA genes by Mans *et al.* (2011) and Burger *et al.* (2012), with all species branches collapsed and distance information removed. The Holothyrida, a small order of mites, are the sister group to the Ixodida (represented here by genus *Allothyrus* from the family Allothyridae), and the Nuttalliellidae represent the most basal lineage of the Ixodida. The Amblyomminae is likely polyphyletic, containing members more closely related to both the Bothricrotoninae and the Rhipicephalinae. Placement of the Haemaphyaslinae within the Metastriata is still uncertain, there being no clear consensus if it belongs in a position basal to the Amblyomminae.

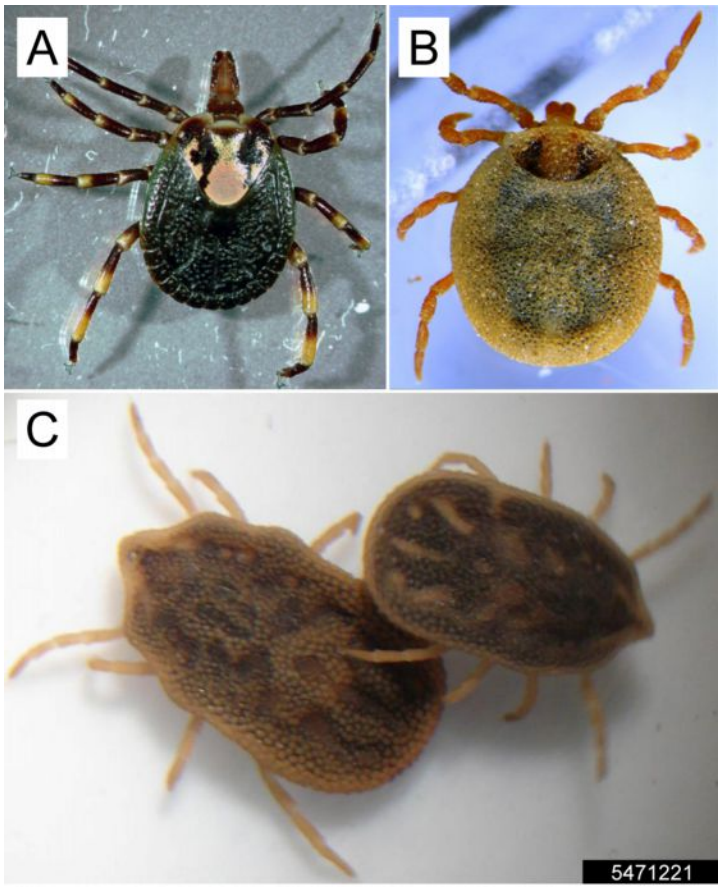

Figure 1.2. Representative members of the three families of Ixodid ticks. (A) The Ixodidae, or hard ticks, are represented by the southern African bont tick, *Amblyomma hebraeum*; (B) the Nuttalliellidae is a monotypic family, whose only member is *Nuttalliella namaqua*; and (C) the Argasidae are represented by the bat tick, *Carios kelleyi*. The image of *N. namaqua* comes from Mans *et al.* (2011) and the photograph of *C. kelleyi* is from Mohammed El Damir, Pest Management, Bugwood.org, available online at http://www.insectimages.org/browse/detail.cfm?imgnum=5471221.

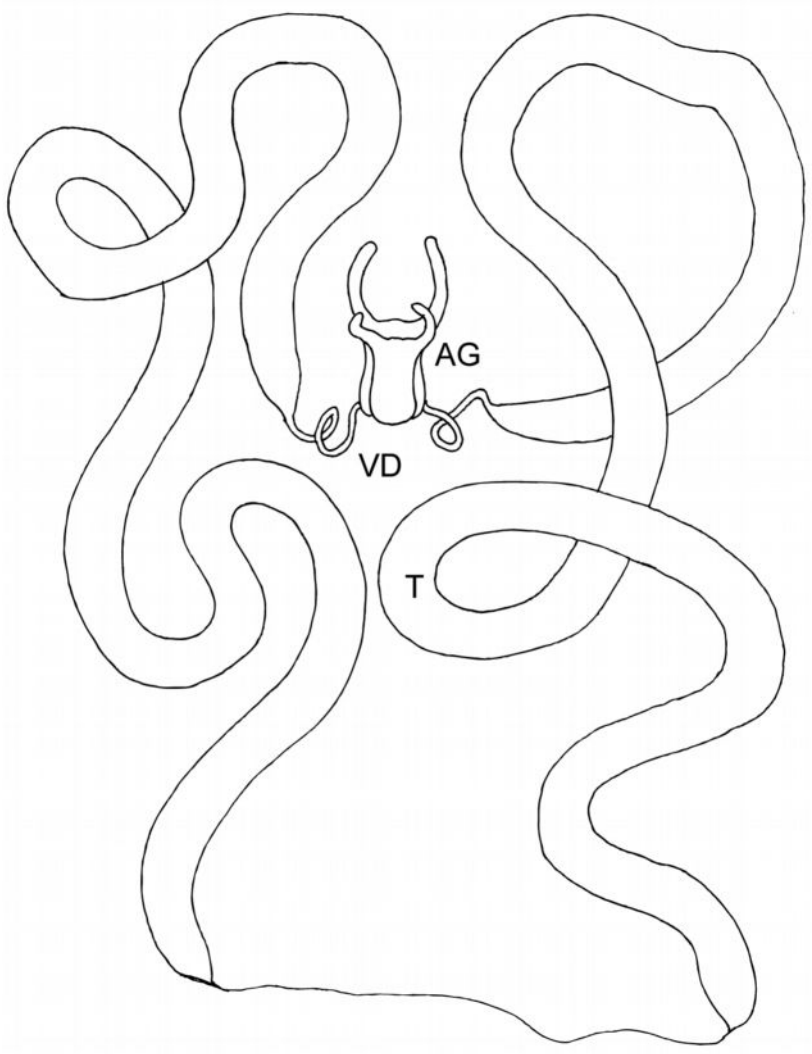

Figure 1.3. Diagrammatic representation of the dorsal view of the fed male gonad of *A. hebraeum*. The gonads of adult male metastriate ticks consist of elongated paired tubular testes, connected by a slender strand of filamentous tissue at the posterior end, and connect anteriorly to the vasa deferentia which fuse into a common seminal vesicle. A large, multi-lobed accessory gland is located dorsally to the seminal vesicle and both it and the seminal vesicle empty into the ejaculatory duct which leads to the genital pore. AG, accessory gland; T, testis; VD, vas deferens.

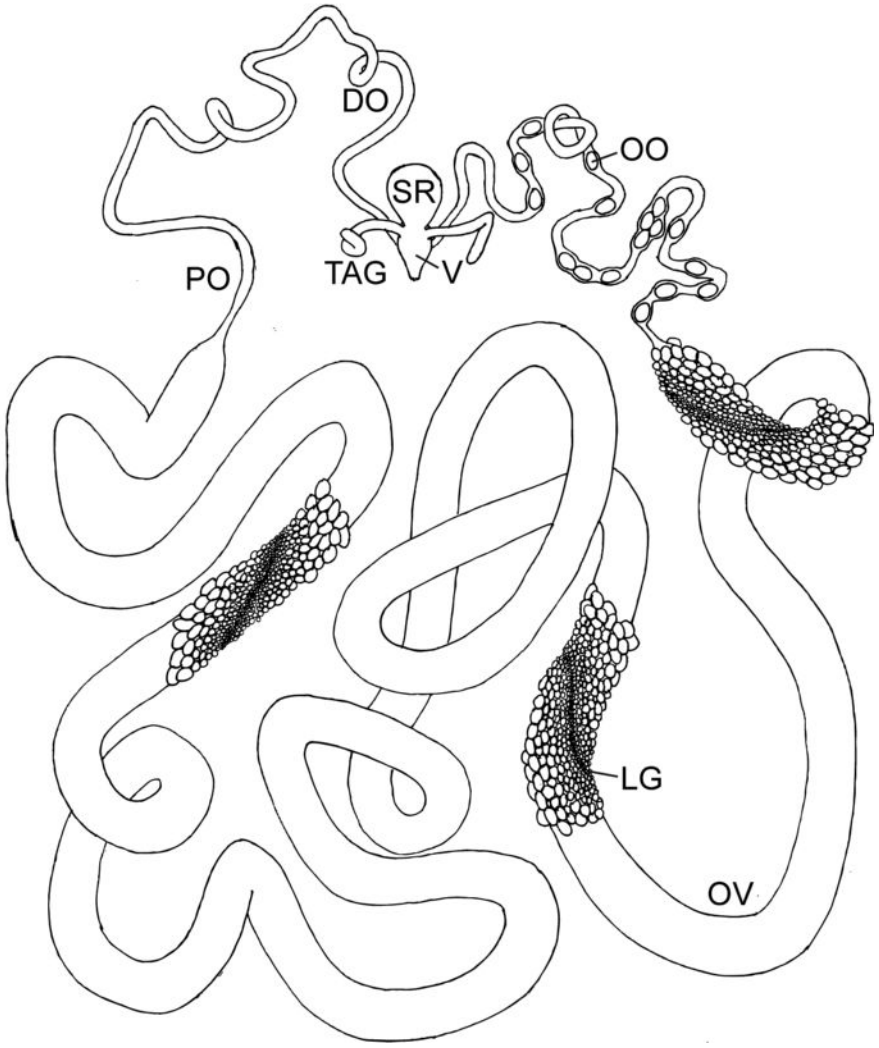

Figure 1.4. Diagrammatic representation of the dorsal view of the *A. hebraeum* female gonad. The single ovary forms a hollow tube, the ends leading into the oviducts, which join to form a common uterus and vagina. A sac-like seminal receptacle extends from the cervical vagina in which the endospermatophore is stored following mating. Paired tubular accessory glands are located at the dorsal junction of the cervical and vestibular regions of the vagina. A longitudinal groove, containing oocytes at the earliest stages of development runs the length of the ovary. DO, distal oviduct; LG, longitudinal groove; OO, oocyte; OV, ovary; PO, proximal oviduct; SR, seminal receptacle; TAG, tubular accessory gland; V, vagina.

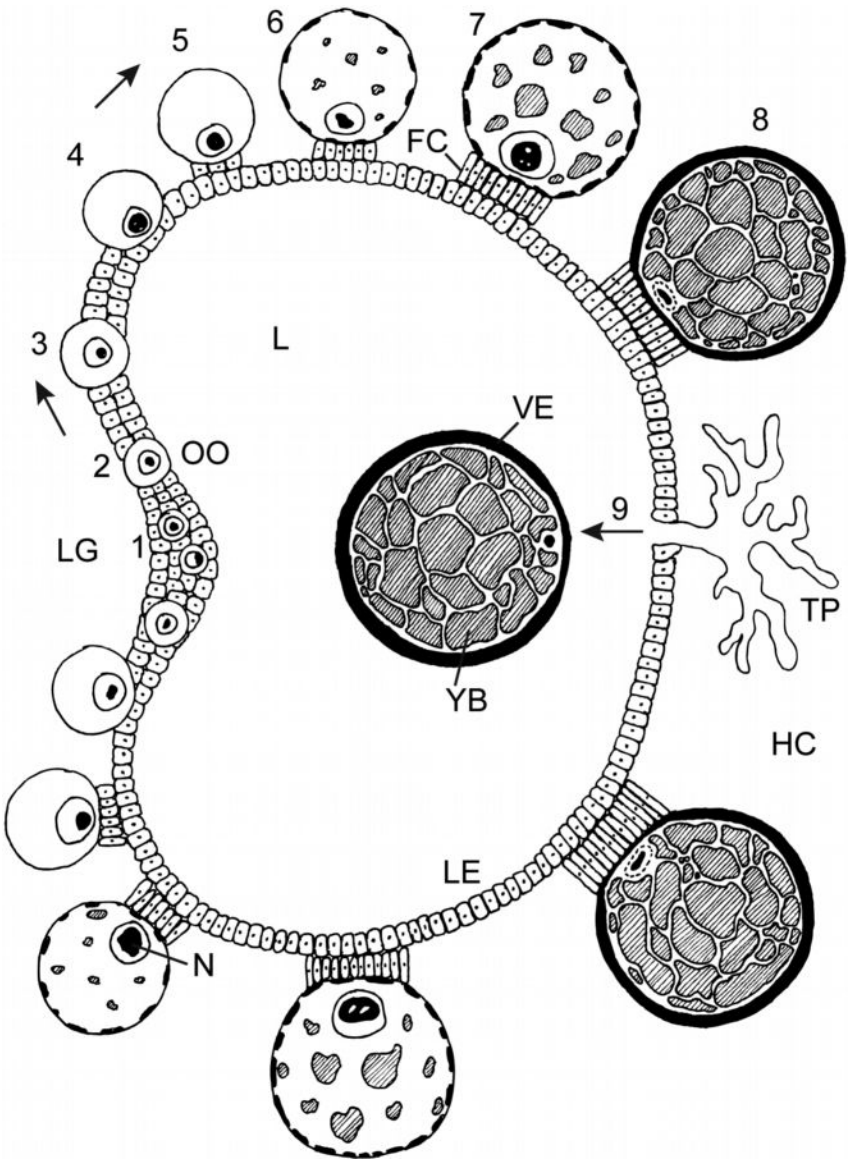

Figure 1.5. Diagrammatic representation of oogenesis, depicted in ovarian cross-section. The earliest stage oocytes (1 – 3) are restricted to the longitudinal groove, and migrate away from the groove as they develop. During Balashov stage II, the oocytes enlarge greatly, held away from the ovary surface by the funiculus (4 – 6). Further growth, characterized by the accumulation of large amounts of yolk proteins and the expansion of the vitelline envelope occurs during stages III and IV (7 – 8). The entry of the mature oocyte into the ovarian lumen and subsequent

oviposition, marks stage V of development (9). Following entry into the lumen, the collapsed tunica propria is visible on the ovary exterior. The figure was drawn to resemble a modified version of Fig. 9.6 from Diehl *et al.* (1982). FC, Funicular cells; HC, haemocoel; L, ovary lumen; LE, lumenal epithelium; LG, longitudinal groove; N, nucleus; OO, oocytes; TP, tunica propria; VE, vitelline envelope; YB, yolk body.

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Chapter 2. Production of the tick engorgement factor, voraxin, in a bacterial expression system

2.1 Introduction

Adult female *Amblyomma hebraeum* Koch require about 8-10 days of on-host feeding in order to fully engorge, feeding up to 100 times the unfed weight. The feeding period comprises three phases: 1) a preparatory phase, lasting 24 – 36 h, during which the tick inserts its mouthparts, establishes a feeding lesion and secretes a 'cement' to fix it firmly to the epidermis, 2) a slow feeding phase, generally lasting up to 10 days, during which its weight increases approximately 10-fold, and 3) a rapid feeding phase, which lasts about 24 h, during which the weight increases up to a further 10-fold (Balashov, 1972).

With the exception of some species of the genus *Ixodes*, ixodid ticks copulate only during the feeding cycle, both sexes requiring several days of feeding to complete gonad maturation (Khalil, 1970). Virgin females, on the other hand, will remain attached to the host indefinitely, rarely feeding beyond 10X their unfed weight. For the 5 – 15% minority of *A. hebraeum* that do feed beyond 10X, they rarely exceed 20X their unfed weight, and never achieve the engorged weight range characteristic of mated females (Kaufman & Lomas, 1996).

The transition between the slow and rapid phases of feeding has been termed the 'critical weight' (CW) in *A. hebraeum*, and is characterized by a number of behavioural and physiological changes (Harris & Kaufman, 1984; Kaufman & Lomas, 1996; Weiss & Kaufman, 2001). If forcibly removed from the host while still below the CW, both virgin and mated females will (i) reattach if

presented to a suitable host, (ii) will not undergo salivary gland (SG) degeneration, (iii) will have a low ecdysteroid titre in their haemolymph and (iv) will not oviposit. However, if removed from the host above the CW, females (i) will not resume feeding when given the opportunity, (ii) will resorb their SGs within 4 days (mated) or 8 days (virgin), iii) will exhibit an elevated haemolymph ecdysteroid titre and (iv) will oviposit a clutch of eggs (infertile, if the female is virgin) and die (Weiss & Kaufman, 2001).

Some of the differences between the mated and virgin female responses to exceeding the CW are due to the transfer to the female of a 'male factor' (MF) in the spermatophore (Harris & Kaufman, 1984). Further work demonstrated that MF is in fact a protein found in the testis/vas deferens (TVD), more specifically in the testicular fluid, rather than being associated with the spermatozoa or with the accessory gland secretions (Lomas & Kaufman, 1992*a*). MF hastens the release of an ecdysteroid hormone into the haemolymph and thereby hastens the onset of SG degeneration and vitellogenesis (Lomas & Kaufman, 1992*b*; Friesen & Kaufman, 2004).

In ticks, there are at least three other mating factors transferred to the female during mating. A 'sperm capacitation factor' (a 12.5 kDa protein) produced by the male accessory glands (MAG) of *Dermacentor variabilis* and *Ornithodoros moubata* stimulates the final phase of sperm maturation once the spermatophore has been transferred to the female (Shepherd *et al.*, 1982). The 'vitellogenesis-stimulating factor', found in the argasid tick *O. moubata*, comprises two large proteins (~100 and ~200 kDa), that are released from recently

capacitated spermatozoa, and that play a role in stimulating vitellogenesis and subsequent oviposition (Sahli *et al.*, 1985). The third was initially described by (Pappas & Oliver Jr., 1971, 1972) from *D. variabilis*. They showed that the stimulus for rapid engorgement was an 'engorgement factor' (EF) produced in the male gonad and transferred to the female during copulation. Subsequently, a similar factor was demonstrated in *Haemaphysalis longicornis* (Matsuo *et al.*, 2002). Recent work on *D. variabilis* indicates that EF activity in this species requires elements from both the TVD and MAG. Full engorgement can be stimulated in feeding virgin females by the injection of both TVD and MAG homogenates into the haemocoel, but injection of either tissue homogenate alone is insufficient (Donohue *et al.*, 2009).

In *A. hebraeum*, EF activity was detected in the TVD of *A. hebraeum*, though not in the MAG, when homogenates of each were injected separately into the female haemocoel (Weiss & Kaufman, 2004). Weiss and Kaufman (2004) hypothesized that MF and EF are the same substance due to the fact that EF stimulates feeding beyond the CW, and MF is responsible for the various behavioural and physiological changes occurring at that time. Following a differential screen of cDNA libraries made from the TVD of fed and unfed males, no fewer than 35 genes were upregulated during feeding (Weiss *et al.*, 2002). Recombinant protein versions of 28 of the upregulated genes were produced in an insect cell expression system; the co-injection of two of these proteins (EFα and EFβ), into partially fed virgin females, resulted in EF and MF activity, but this did not occur when each EF was injected on its own (Weiss & Kaufman, 2004). These

authors proposed the name 'voraxin' for the native EF of ixodid ticks. When mated females were allowed to feed normally on a rabbit immunized against recombinant versions of both voraxin proteins, 74% of the females failed to feed beyond the CW, whereas those placed on a vehicle-injected control rabbit fed and engorged normally. These results suggested that antibodies against voraxin might be used to develop a novel anti-tick vaccine (Weiss & Kaufman, 2004).

In my study, recombinant *A. hebraeum* voraxin was produced in two distinct bacterial expression systems. Bacterial expression systems have several advantages over insect cells systems in that they are often easier to set-up and maintain, they allow for simpler scale-up of protein yields, and they are more cost-effective. Two bacterial expression constructs, originally prepared by Mr. Gary Ritzel (Department of Biological Sciences) were made available to our lab. The recombinant voraxinα construct was provided in the pRSETB expression vector (Invitrogen) with an amino terminal 6x His tag in *E. coli* BL21 (DE3), and the recombinant voraxinβ construct was provided in the pET26b+ expression vector (Novagen) with a carboxy terminal 6x His tag, in *E. coli* BL21 (DE3) pLysS. The inclusion of a 6x His tag on the recombinant protein allows for easy purification of the protein from the *E. coli* cells via a nickle column. Moreover, the small size of the 6x His tag makes it unlikely to interfere with (i) proper protein folding, (ii) the immunogenicity of the recombinant protein, or (iii) other properties of the recombinant protein. However His tagged-proteins can accumulate as two forms – biologically functional soluble proteins or insoluble aggregates of denatured protein, known as inclusion bodies, which must be

purified, bound to a column and refolded.

The recombinant proteins were also expressed using the Gateway expression system (Invitrogen) and archived in *E. coli* OmniMax2 T1^{R} cells with expression of the recombinant proteins done in L-Arabinose inducible *E. coli* BL21-A1 cells. Constructs were tagged with either an N-terminal glutathione S-transferase molecule (GST), or His tag, or a C-terminal GST or His tag. The large GST tag has an advantage over His tags in that it often produces large amounts of soluble protein. But due to the tag's large size it often must be cleaved from the target protein to prevent interference with the protein's biological activity. To this end, a Factor Xa cleavage site between the tag and the voraxin protein was included to allow for the eventual removal of the protein tags.

2.2 Materials and Methods

2.2.1 Tick rearing and feeding

Adult *A. hebraeum* used in these studies were from either an old laboratory colony (OC), established in 2003 from a population of ticks imported from a colony maintained at the *Université de Neuchâtel*, or from a new colony (NC) developed from ticks imported in May 2007 directly from the Onderstepoort Veterinary Institute, South African Rebublic, courtesy of Ms. Helena Steyn. All ticks were maintained in an incubator (under constant darkness, 26°C, and >95% RH). For feeding, ticks were confined to a cloth-covered foam arena attached with latex adhesive (Roberts 8502 Latex, Bramalea, Ontario, Canada) to the shaven back of a rabbit as described by Kaufman & Phillips (1973). For certain experiments, the arenas were divided into two compartments on selected rabbits to

allow for simultaneous feeding of two distinct experimental groups on the same rabbit. In order for the majority of females to attach to a host, feeding males must be present on the host to release an aggregation-attachment pheromone (Norval *et al.*, 1991). To obtain virgin females, males were either placed in a separate compartment in the arena or were confined to a cloth bag placed in the arena with virgin females, allowing the males to attach to the host and emit attachment pheromones, without mating.

Larvae and nymphs were fed in a similar manner to adults, but in a slightly modified arena that could be firmly sealed shut to prevent escape of the immature ticks. This was accomplished by means of a screw top lid adhered to the cloth of the arena that provided the only opening to the pack.

Adult *Dermacentor andersoni* used in this study were supplied by Dr. Tim Lysyk, Lethbridge Research Centre, Agriculture and Agri-Foods Canada. While off the host, adult *D. andersoni* were maintained at 10°C, held over water (RH ~100%) in darkness. A day or two prior to feeding, adults were moved to the colony incubator. The feeding conditions were the same as for the *A. hebraeum* ticks.

The use of rabbits for all research presented in this thesis was reviewed and approved by the Biosciences Animal Policy and Welfare Committee, University of Alberta.

2.2.2 Measurement of tick volume

In some experiments, tick body weight had to be estimated while the ticks were still attached to the host. To do this, the length, width and thickness of

partially fed and fully engorged female ticks were measured using electronic digital calipers, both on and off the host. Females at various stages of engorgement were weighed and measured. These measurements were used to calculate the volume of an ellipse of rotation, which approximates the shape and volume of a tick:

$$V= (1/6)\pi*XYZ$$

where X, Y and Z are body length, thickness and width, respectively. Because the weight of detached ticks is strongly correlated to the volume as calculated from the latter formula (Patriquin, 1991), the approximate weight of attached females could be calculated from a standard curve (Fig 2.1).

2.2.3 Collection and preparation of tissues

Ticks were immobilized on small plastic Petri dishes with a drop of cyanoacrylate glue (Loctite Corp., Rocky Hill, CT, USA), and chilled in a refrigerator for at least 30 min to anaesthetize the tick and inhibit smooth muscle contraction; the latter reduces the likelihood of puncturing the gut while removing the cuticle. The ticks were flooded with either a modified Hank's balanced saline (200 mM NaCl, 8.9 mM D-glucose, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 27 mM phenol red, pH 7.2) or with 1X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and the dorsal cuticle removed using a microscalpel. The male accessory gland (MAG) and testis/vas deferens (TVD) were excised, gently blotted to remove extra-glandular fluid, placed into microfuge tubes, flash frozen on dry ice, and stored at -80°C until required for

subsequent bioassays.

At the time of bioassay, the tissues were homogenized in microfuge tubes using teflon pestles after adding sufficient PBS to achieve a tissue concentration of one MAG, TVD, or whole gonad equivalent per 5 µl total volume. *A. hebraeum* tissues were sonicated for 3 min at 4°C and then passed through a 30 gauge needle to ensure a more complete disruption of the tissue, whereas *D. andersoni* tissues were homogenized using plastic pestles. For some experiments, following homogenization, the tissues were centrifuged at 8000 g for 5 min to pellet and discard the insoluble debris.

2.2.4 Construct design and preparation

The original bacterial expression constructs were designed and produced by Mr. Gary Ritzel of the Department of Biological Sciences. The N-terminally 6x His tagged recombinant voraxinα was produced using the pRSETB expression vector (Invitrogen, Grand Island, NY, USA) and the C-terminally tagged recombinant voraxinβ produced using the pET26b+ expression vector (Novagen).

N-terminally Glutathione-S-transferase (GST) tagged voraxinα and voraxinβ expression constructs were produced using the *E. coli* Expression System with Gateway technology (Invitrogen) according to the manufacturer's directions. The constructs were designed such that a Factor Xa cleavage site was located between the voraxin ORF and the tag sequence to facilitate tag removal following purification. In brief, voraxinα and voraxinβ were amplified in two rounds using sets of nested gene specific primers containing attB recombination sites (Table 2.1) to allow them to be later recombined into the appropriate vector

to produce the constructs. Each reaction contained 5 µl of PCR buffer, 50 uM dNTPs, 50 pmol of each primer, 2 µl of plasmid template containing either voraxinα or voraxinβ, 0.5 µl Taq polymerase and 0.1 µl Pfu polymerase in a total volume of 50 µl. Cycling conditions consisted of 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 2 min, followed by an additional 72°C extension step for 8 min. Following amplification, the PCR products were purified using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's directions.

Purified attB-flanked inserts were each combined with pDONR221 (Invitrogen) and BP Clonase II to produce initial pENTRY constructs via Gateway cloning according to the kit's directions. The recombination reactions were transformed into *E. coli* OmniMax 2 TI^{R} cells by electroporation, successfully transformed colonies identified by PCR (see colony PCR below), and the plasmids isolated using the QIAprep miniprep kit (Qiagen) according to the manufactures directions.

The final expression constructs were produced by combining the pENTRY plasmids with pDEST15 using LR recombinase, according to the kit directions, to obtain the final expression constructs with N-terminal GST tags. The recombination reactions were again transformed into *E. coli* OmniMax 2 TI^{R} cells by electroporation, successful transformants identified using colony PCR, and plasmids isolated using the QIAprep miniprep kit.

2.2.5 Colony PCR

Following transformation of various bacterial strains with a plasmid of

interest, colonies were screened by PCR to identify those containing the correct insert. In a total volume of 50 µl, 5 µl of 10x PCR buffer was combined with 0.2 mM dNTPs, 2 mM $MgSO_4$, 0.5 µM of each primer (M13, Table 2.1), and 0.25 µl of Taq DNA polymerase. One µl of a bacterial colony suspended in 50 µl of sterile H_2O was used as template for each reaction. PCR cycling conditions consisted of 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 2 min, followed by an additional 72°C extension step for 8 min. PCR products were run out on agarose gels and band sizes examined to determine if the correct product had been inserted.

2.2.6 Sequencing and sequence analysis

All plasmids were sequenced with BigDye® Terminator v3.1 Cycle Sequencing mix (Applied Biosystems, Foster City, CA, USA) using M13 primers (Table 2.1) to confirm sequence identity and that the constructs were in frame. Reactions consisted of approximately 200 ng of template DNA, 1.6 pmol of primer, 0.5x BigDye dilution buffer and 2 µl BigDye Sequencing premix in a 10 µl reaction volume. Cycling conditions consisted of 25 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 15 s and extension at 60°C for 60 s.

2.2.7 Production and detection of bacterially produced recombinant proteins

The original His-tagged recombinant voraxinα and voraxinβ proteins were expressed using *E. coli* BL21 (DE3) pLys S cells grown in LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) with antibiotic selection (100 µg/ml ampicillin and 100 µg/ml chloramphenicol) at 37°C in a shaking water bath, and

induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optical density (OD_{600}) of 0.5-0.8. Cells were grown overnight at 37°C following induction, then harvested by centrifugation and stored at -20°C.

Recombinant voraxin was partially purified by resuspending the cells in guanidinium lysis buffer (6M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl, filter sterilized) at 37°C, then lysed by passing the cells through a French press at 20 000 psi twice. Lysates were centrifuged to remove cellular debris and the supernatant retained for further purification using the ProBond Purification System (Invitrogen) according to the manufacturer's instructions for the purification of proteins using hybrid conditions (a combination of denaturing and native purification).

Partially purified protein samples were concentrated using Centriprep YM-3 columns (Millipore, Billerica, MA, USA), according to the manufacturer's directions. Following concentration, protein amounts were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's specifications, and samples were examined using SDS-PAGE and western blotting to determine the approximate amount and quality of purified proteins remaining. The approximate purity of each protein was determined by comparing the intensity of the bands at the appropriate size with the intensity of the all the other bands in the lane using Adobe Photoshop Imaging Software (Adobe Systems Incorporated, San Jose, CA, USA).

The N-terminally GST-tagged voraxinα and voraxinβ were freshly transformed into *E. coli* BL21 A1 cells (Invitrogen) by electroporation each time

the proteins were expressed. The cells were grown in LB medium containing 100 µg/ml ampicillin, 12.5 µg/ml tetracyline and 0.1% glucose at 37°C with shaking, until an OD_{600} of 1.2 – 1.5 was reached, then induced with 0.02% L-arabinose and allowed to grow for another 4 h at room temperature. Cells were harvested via centrifugation, resuspended in

50 mM Tris-Cl, pH 8.0, and cell lysates obtained using the French press as described above. Cell lysates were then passed through a 0.45 µm filter, and 100X protease inhibitor cocktail set I (EMD Chemicals, Billerica, MA, USA) was added to a final concentration of 1X. The cell lysates were examined using SDS-PAGE and western blotting to confirm the presence of the recombinant proteins and stored at 4°C until required for the voraxin bioassays.

2.2.8 SDS-PAGE and Western blotting

Recombinant proteins present in crude bacterial lysates or obtained following purification steps were separated by SDS-PAGE under reducing conditions (Laemmli, 1970), all gels consisting of a 4% stacking gel, and a 12% resolving gel. Following electrophoresis, gels were either stained with Colloidal Coomassie Brilliant Blue or transfered to Immobilon-PSQ polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) for immunoblotting. The approximate purities of the partially purified proteins were determined by analysis of band intensity using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

His-tagged proteins present on the PVDF membranes were detected either directly or indirectly. Direct detection was performed using the HisProbe-HRP

system (Pierce) and recombinant proteins visualized using the SuperSignal West Pico Chemiluminescent substrate kit (Pierce) followed by exposure of the membranes to X-ray film (Kodak, Rochester, NY, USA). Indirect detection was performed using a mouse anti-His primary antibody and a goat anti-mouse IgG secondary antibody conjugated to HRP and the locations of antibody binding examined using the SuperSignal West Pico Chemiluminescent substrate kit (Pierce) followed by exposure of the membranes to X-ray film.

Detection of the GST-tagged recombinant voraxin proteins was performed using a rabbit anti-GST primary antibody and a goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (AP). The locations of antibody binding were visualized using the colourimetric AP conjugate substrate kit (BioRad), according the manufacturer's directions.

2.2.9 Injection of virgin females

Partially fed virgin female ticks that were injected off the host were injected into the haemocoel through either the posterior ventral surface, or through the coxa of the fourth leg, using a microsyringe (Hamilton, Reno, Nevada, USA) fitted with a 30 gauge needle (BD, Franklin Lakes, NJ, USA). After injection, the females were held overnight in the colony incubator, or in a humidity chamber at room temperature, before being placed on rabbits the next day.

Partially fed female ticks, injected while they were attached and feeding on the host, were injected through the posterior body wall into the haemocoel. On occasions when the rabbit was overly active, the rabbit was first sedated with acepromazine 30 min before injection.

2.2.10 Voraxin Bioassays

Virgin female ticks were labelled by tying a strand of coloured cotton or silk thread to one or more legs and cementing it in place with a cyanoacrylate adhesive, and/or by painting the legs using coloured paint pens (Elmer's Products Inc., Westerville, OH, USA). Ticks were held overnight at room temperature at high RH and placed on the rabbit the following day. For off-host injections, virgin ticks were allowed to feed for 10 – 20 days, removed from the host, and weighed. Those weighing <300 mg were divided into equal groups and injected as described above, with 5 – 10 µl of partially purified recombinant protein, crude bacterial lysate, buffer control or one MAG, TVD, or one whole gonad equivalent. For on-host injections, individually marked ticks were allowed to feed for approximately two weeks; then their body volume and predicted weight were calculated. After division into approximately equal weighted groups, the ticks were then injected with 5 µl of N-terminally GST-tagged recombinant voraxin, or buffer control, or 1 – 2 MAG, TVD, or whole gonad equivalents. Some of these bioassays were performed by Mr. Andrew Hulleman, an undergraduate research assistant in the laboratory at the time.

Feeding progress was monitored daily for up to 3 weeks. During this time, all females that spontaneously detached were removed, weighed, measured (for those ticks injected while on the host) and stored in the colony incubator awaiting oviposition. Any ticks still remaining on the host at the end of the 3-week period were forcibly removed, weighed, measured and likewise stored in the colony incubator.

2.2.11 Immunization of rabbits against recombinant voraxin

Eight New Zealand White rabbits (*Oryctolagus cuniculus*) were inoculated with 150 μg of partially purified recombinant voraxin in Freund's complete adjuvant, followed by two booster inoculations in Freund's incomplete adjuvant, 4 weeks apart. Two rabbits were inoculated against voraxinα, two against voraxinβ, two against a mixture of both voraxinα and voraxinβ, and the final two against a 1.2% saline solution (control). All inoculations were performed by technicians at the University of Alberta Biological Sciences Animal Services Unit (BSAS).

One rabbit inoculated with voraxinα and one rabbit inoculated against voraxinβ were eventually sacrificed for serum collection; the remaining six rabbits were used for a feeding inhibition assay upon exposure to unfed male and female ticks. Twenty unfed females and 20 unfed male *A. hebraeum* were allowed to feed on each of the inoculated rabbits for up to 18 days, and the degree of female engorgement between treatments was compared. The ticks were then placed in the colony incubator and monitored for oviposition.

2.2.12 Bioassay of serum from immunized rabbits

Virgin female OC *A. hebraeum* were allowed to feed for 12 – 14 days then removed and weighed. All those weighing less than 181 mg (total = 60 females) were divided into nine groups and marked with coloured threads. The groups were injected with: 1.2% NaCl (control), serum from the rabbit injected with NaCl, pre-immune serum, anti-voraxinα serum, anti-voraxinβ serum, anti-voraxin, or a 3-fold dilution of the latter three sera. Feeding progress was monitored for up to 3 weeks. This work was performed by Ms. Kristy Myshaniuk, an undergraduate

research assistant in the laboratory.

2.2.13 Indirect ELISA of antibody titre and antibody content of tick haemolymph

Approximately 1 ml of serum was collected by the BSAS technicians from each of the latter rabbits; serum from each rabbit was taken before immunization (pre-immune serum), and 2 weeks following each inoculation with the experimental protein(s) or NaCl. Dr. Xiuyang Guo, a post doctoral fellow in the Kaufman laboratory at the time, used the KPL Protein Detector ELISA kit (KPL, Gaithersburg, MD, USA) to perform an indirect enzyme-linked immunosorbent assay (ELISA) to determine antibody titre in each rabbit injected with voraxinα, voraxinβ or both recombinant voraxin proteins, according to the manufacturer's protocols. Approximate antibody titre was estimated from the absorbance at 405 nm.

Haemolymph was collected 0 – 4 days post-engorgement from the 27 engorged females that had fed on the immunized rabbits, and subjected to an indirect ELISA. This allowed an estimate of the approximate amount of anti-voraxin antibodies taken up by the ticks during feeding. This assay was also performed by Dr. Guo using the KPL Protein Detector ELISA kit (KPL, Gaithersburg, MD, USA). The levels of voraxinα and voraxinβ absorbance were normalized with respect to the saline control for all analyses.

2.2.14 Statistical analysis

All statistical analyses were performed using Stata 10.0 software (StataCorp, College Station, TX, USA). Differences between experimental

treatments were analyzed by ANOVA; post hoc comparisons were achieved by the Sidak multiple comparison test. Differences between the mean initial and final weight of females were evaluated using unpaired two-sample mean comparison tests (t-tests). Mortality was evaluated using Fisher's exact test.

2.3 Results

2.3.1 Production and detection of bacterially produced recombinant proteins

Voraxinα and voraxinβ have no significant sequence homology as determined by NCBI BLAST alignments. The sequences of voraxinα and voraxinβ (Weiss & Kaufman, 2004) are shown in Fig. 2.2. Both His-tagged and GST-tagged recombinant voraxin constructs were produced. The sequences of the His-tagged constructs given in Fig. 2.3. Following expression and partial purification of the His-tagged recombinant proteins, the voraxinα and voraxinβ samples were separated using SDS-PAGE (Fig. 2.4) and the approximate purity of each was determined from band density analysis. The recombinant voraxinα was ~16 kDa and ~60% pure. The identity of the intense ~15 kDa band is unknown but is possibly a His-rich *E. coli* protein. The recombinant voraxinβ was ~10 kDa and ~88% pure, the intense smear below the band likely corresponding to voraxinβ degradation products. The concentration of proteins used for the initial bioactivity assay and immunization studies was 2325 ng/µl for voraxinα (of which 1395 ng was pure product) and 1900 ng/µl for voraxinβ (of which 1672 ng was pure product).

The ORFs and amino acid sequences of the recombinant voraxin constructs with N-terminal GST-tags are shown in Fig. 2.5. Analysis of crude

bacterial cell lysates of *E. coli* expressing these constructs indicated that moderate levels of the recombinant proteins were present, but western blot analysis indicated that a high proportion of the GST tags detected were not attached to the recombinant constructs (Fig. 2.6). In addition to expressing constructs with an N-terminal GST-tag, I also developed and expressed constructs with C-terminal GST-tags, N- and C-terminal His tags, and no tags; however none of these produced usable amounts of protein during trial expressions and no further work was done using these constructs.

2.3.2 Assessing the biological activity of voraxin

2.3.2.1 Off-host injections of partially-fed virgin females with recombinant voraxin and male gonad homogenates

Injection of partially purified His-tagged voraxin into partially fed OC females resulted in high mortality for all treatments (~41% overall); however, there were no significant differences among the groups as determined by Fisher's exact test ($p=0.121$; Fig. 2.7). The average weight of females following injection and 3 weeks of feeding did not increase significantly for any group ($p\geq0.24$), nor were there any significant differences among any of the treatment groups ($p=0.415$).

Following injection of OC MAG or TVD into partially fed OC virgin females, there was no significant increase in weight following feeding for any group ($p\geq0.43$), nor were there any significant differences in average weight among any of the groups ($p=0.650$; Fig. 2.8). Although there was high mortality for all treatment groups (58.3% overall), there were no significant differences in

the numbers of deaths among groups (p=1.000).

Injection of partially-fed NC virgin females with OC TVD, NC TVD, NC MAG or unpurified bacterial lysate containing N-terminally GST-tagged recombinant voraxin failed to stimulate any significant increase in fed weight for any group (p≥0.0593; Fig. 2.9). There were also no significant differences in fed weight among any of the treatment groups (p=0.180). The rabbit initially used to partially feed the females prior to injection fell ill, resulting in the death of a number of feeding female ticks, decreasing the number available for injection. A high mortality continued to be observed following injection, with ~66% of all female ticks dying over the course of the experiment and 100% of those injected with the bacterial lysate. These differences were not statistically significant, probably due to the low sample size.

2.3.2.2 On-host injection of feeding virgin *A. hebraeum* and *D. andersoni* females with male gonad homogenates or recombinant, GST-tagged voraxin

The approximate initial and final weights of all ticks injected on the host were calculated based on measurements of tick body volume as described in the Materials and Methods (Fig. 2.1). On-host injection of OC *A. hebreaum* with one gonad equivalent of OC TVD, NC TVD, NC MAG, non-purified bacterial lysate containing N-terminally GST-tagged recombinant voraxin, or a 50 mM Tris-Cl, pH 8.0 control (all in a volume of 5 µl) resulted in no significant increase in average tick weight (p=0.469) within each group, nor were there any significant differences in weight between any of the treatment groups (p≥0.177; Fig. 2.10). Approximately 19% of all injected females died over the course of the

experiment, but mortality did not vary significantly among the treatment groups ($p=0.357$).

Similarly, injections of NC MAG, TVD, MAG+TVD and a saline control into feeding virgin NC *A. hebraeum* females failed to elicit an engorgement response (Fig. 2.11). There were no significant differences among the final weights associated with each treatment group ($p=0.838$), nor was there a significant difference between the initial and final calculated weight for any of the treatment groups ($p\geq0.965$). A total of 35% of the injected ticks died over the course of the experiment, but there was no significant difference in mortality among the various groups ($p=0.578$).

Virgin *D. andersoni* were injected while feeding on the host with either one or two male gonad equivalents harvested from conspecific males, separated into MAG, TVD or MAG+TVD, as well as a saline control. Although females injected with one gonad-equivalent of both MAG+TVD increased significantly in weight (increasing from ~115 mg to ~351 mg, $p=0.027$), there were no significant differences observed among the various treatment groups (the remaining groups increasing from ~144 mg to ~288 mg on average, $p=0.672$; Fig. 2.12). Following injection of two gonad-equivalents, all groups, including the saline control, fed significantly beyond their initial weights (feeding on average from ~182 mg to ~338 mg, $p\leq0.0053$), but no significant differences were observed among the treatment groups ($p=0.389$). When the data for both trials were combined, there was a significant difference between the initial and final calculated weight for each group (feeding from ~162 mg to ~317 mg, $p\leq0.0072$). However, there were

no significant differences in calculated weight among any groups following feeding ($p=0.901$). There was no tick mortality following injection for either trial (with the exception of two ticks that died during the injection process).

2.3.3 Immunizations and indirect ELISA

The indirect ELISA performed by Dr. Guo indicated that levels of anti-voraxin antibodies increased significantly by the third inoculation in all rabbits immunized against these proteins ($p<0.000$, Fig. 2.13). After correcting for the absorbance of the pre-immune sera, both rabbits inoculated against voraxinα, had significantly more anti-voraxinα antibodies than did either rabbit immunized against both voraxinα and voraxinβ ($p<0.000$). However, the titre of anti-voraxinβ antibodies in the rabbits inoculated against voraxinβ was not significantly different from that of the double-immunized rabbits ($p=0.444$). The titre of anti-voraxinβ antibodies was significantly different between the two rabbits inoculated with both voraxinα and voraxinβ ($p=0.005$), but levels of voraxinα were not significantly different between those two rabbits ($p=0.894$). Levels of voraxinα or β were not significantly different between the two rabbits inoculated with voraxinα ($p=1.000$) or voraxinβ ($p=1.000$).

Following the third set of inoculations, ticks were allowed to feed on the immunized rabbits. There were no significant differences in engorged weight among females fed on rabbits immunized against voraxin or NaCl ($p=0.696$; Fig. 2.14). Similarly, there were no significant differences in the weight of oviposited eggs (expressed as % engorged body weight; $p=0.505$). Feeding success of females, was also compared among the six rabbits, to check for host-specific

differences in feeding success, but no significant differences were found either among treatment groups ($p=0.626$) or within those groups that were fed on multiple rabbits (the NaCl control, $p=0.891$, and the rabbits injected with both voraxinα and voraxinβ, $p=0.193$). A total of 7% of the females died over the course of feeding, but there were no significant differences among the treatment groups ($p=0.349$).

Ticks fed on rabbits immunized against voraxinα, voraxinβ, voraxinα+β or NaCl, were examined for the presence antibodies in the haemolymph. Ticks fed on the rabbit immunized against voraxinα had a significantly higher anti-voraxinα antibody titre than the NaCl immunized controls ($p<0.000$). However, the titres of those fed on the rabbit immunized against voraxinβ were not significantly different from those fed on the controls ($p=0.074$; Fig. 2.15). It should be noted that two of the five ticks in this group showed zero absorbance, with the other three exhibiting levels similar to those seen in the rabbit immunized against voraxinα. The low number of samples combined with the fact that two of the ticks did not appear to take up much antibody likely accounts for the lack of significance observed. Ticks fed on rabbits immunized against voraxinα plus voraxinβ, exhibited a level of voraxinα antibodies that was not significantly different from that seen in the controls ($p=0.845$), but the level of voraxinβ antibodies was significantly higher than those of the controls ($p=0.002$).

2.3.4 Bioassay of serum from immunized rabbits

Following injection of the anti-voraxin serum and allowing the ticks to feed, there were no significant differences among any of the groups ($p=0.491$).

Because of this, the data for the various treatments were pooled for all subsequent analyses: the three controls combined, and the full strength and diluted serum for each different immunization combined. As before, no significant differences among the groups were observed ($p=0.764$; Fig. 2.16), and the average weight of all four groups increased significantly compared to their uninjected weights ($p \leq 0.0004$).

2.4 Discussion

The major purpose of this study was to further investigate the biology of voraxin, and evaluate its suitability as a component of a novel anti-tick vaccine. In the majority of ixodid tick species, copulation occurs only on the host during feeding, and is a requirement for females to achieve full engorgement and subsequently oviposit (Kaufman, 2007). Weiss & Kaufman, (2004) demonstrated that the injection of homogenates of the TVD from fed males stimulated virgin females to greatly exceed the CW within 7 days of feeding compared to controls. They also showed that injection of recombinant voraxin was sufficient to trigger complete engorgement in virgin female *A. hebraeum*, followed by a significant degree of ovarian development (Weiss & Kaufman, 2004). Voraxin's importance as a stimulant for engorgement was also demonstrated by the marked inhibition of engorgement (a 72% reduction in weight compared to controls) in females fed on a rabbit immunized against the two voraxin proteins (Weiss & Kaufman, 2004).

Pappas & Oliver Jr. (1972), the first to demonstrate an engorgement factor in any tick, demonstrated in *D. variabilis* that it is produced in the male gonad and is passed to the female during copulation. Donohue *et al.* (2009) subsequently

identified a voraxinα transcript with 77% identity to the *A. hebraeum* voraxinα and BLAST homology searches have identified voraxinα transcripts from several other species including *Rhipicephalus appendiculatus*, *D. silvarum* and *Haemaphysalis longicornis*. A protein BLAST search has also revealed a hypothetical protein from *A. maculatum* showing 95% identity to the *A. hebraeum* voraxinα (e-value: 5e-73). Interestingly, this protein was identified from the salivary transcriptome of female *A. maculatum* (Karim *et al.*, 2011). It is unknown what function the voraxinα transcript plays in the salivary gland of female *A. maculatum*. Its presence in the sialome of *A. maculatum* notwithstanding, voraxinα has not been reported in transcriptomes of the synganglion, salivary gland and/or midgut from other tick species such as *A. americanum* (Aljamali *et al.*, 2009), *D. variabilis* (Anderson *et al.*, 2008; Donohue *et al.*, 2010), *D. andersoni* (Alarcon-Chaidez *et al.*, 2007), *R. sanguineus* (Lees *et al.*, 2010), *O. coriaceus* (Francischetti *et al.*, 2008), *I. scapularis* (Valenzuela, 2004), or *I. pacificus* (Francischetti *et al.*, 2005). It is also interesting to note that no genes or putative proteins with homology to the *A. hebraeum* voraxinβ have yet been identified, even though the male gonad transcriptome of *D. variabilis* has been examined (Sonenshine *et al.*, 2011).

There is substantial inter-specific variation in the extent to which a virgin female will feed. For example, in laboratory-reared ticks, the approximate maximum virgin-to-mated weight ratio is ~5% in *A. americanum*, ~10% in *A. hebraeum* (for the vast majority of virgins and ~20% for a small minority), ~17% in *R. sanguineus*, ~34% in *D. andersoni* and *D. variabilis*, and up to 39% in

Hyalomma anatolicum (Kaufman, 2007).

Harris & Kaufman, (1984) noted that physiological and behavioural changes occur when *A. hebraeum* females exceed ~10X the unfed weight, a degree of feeding they called the 'critical weight' (CW). If removed from the host while below the CW, females reattach to a host if given the opportunity, their salivary glands do not degenerate, vitellogenesis is curtailed and oviposition does not occur. If females above the CW are removed from the host, they do not reattach to a host if given the opportunity, the salivary glands undergo autolysis (within 4 days for mated females and 8 days for virgins), and vitellogenesis and oviposition occur (Kaufman & Lomas, 1996; Lomas & Kaufman, 1999). The physiological changes associated with the CW do not occur so long as the tick remains attached to the host. Thus, achieving the CW is only the prerequisite for the changes; it is the act of detachment that provides the trigger (Friesen & Kaufman, 2009). After exceeding the CW, virgins allowed to remain attached to the host for a further 8 days did not undergo SG degeneration nor did they complete egg maturation. Rather, these changes occurred (within 8 days) only following detachment from the host (Friesen & Kaufman, 2009). The location and nature of the sensory receptors that monitor attachment/detachment remain unknown, although they are assumed to be somewhere in the mouthparts or oral cavity.

Weiss & Kaufman (2001) determined that the precise value of the CW in *A. hebraeum* depends on how it is measured. When measured by reluctance to reattach to a host following detachment, it was 9X the unfed weight. When

measured by the onset of salivary gland degeneration and increase in haemolymph ecdysteroid titre it was 10X. When measured by ovary weight, oocyte length and oocyte vitellin content, the CW was 12X, 12X and 13X, respectively (Weiss & Kaufman, 2001). The underlying cause of these differences is still unknown, but it may relate to a differential sensitivity to haemolymph ecdysteroid titre (Kaufman, 2007).

Although virgin female *A. hebraeum* rarely exceed the minimum weight required for oviposition, this is not the case for other species. Virgin *D. variabilis* and *D. andersoni* regularly feed to ~35X the unfed weight, or even to repletion, and will lay (infertile) eggs at that size (Donohue *et al.*, 2009; personal communications from Dr. Daniel Sonenshine, Old Dominion University, Norfolk Virginia, USA for *D. variabilis* and Dr. Tim Lysyk, Agriculture and Agri-Food Canada, Lethbridge Alberta, for *D. andersoni*). The question as to whether the CW in these species is higher than that of *A. hebraeum* remains, although recent work on *D. andersoni* indicates that the CW in this species is similar to that of *A. hebraeum* in spite of differences in the extent to which virgins will feed (Ullah & Kaufman, in preparation).

This work detailed several attempts to replicate the results obtained by Weiss & Kaufman (2004), but using bacterially-produced rather than insect cell-produced recombinant voraxin. Initial attempts showed that off host injections of the original construct, at concentrations comparable to those used by Weiss and Kaufman, had no significant effect on female feeding (Fig. 2.7). It is possible that the bacterially-produced proteins lacked certain post-translational modifications

necessary to confer biological activity to the proteins, or that the protein were misfolded due to a lack of the appropriate chaperone proteins. However, size comparisons between the bacterially produced proteins and the reported sizes of those produced by Weiss & Kaufman (2004) in the insect cell expression system, showed minimal differences, implying that whatever post-translation modifications normally take place *in vivo*, they do not result in an obvious increase in molecular size. Despite the lack of chaperones in the *E. coli* expression system, the small size alone of the proteins implies that they should be able to fold into the appropriate energetically stable conformation spontaneously. So the inactivity of the bacterially-produced voraxin remains a puzzle.

Weiss & Kaufman (2004) immunized a single rabbit against the insect cell-line produced recombinant voraxin proteins, and obtained an impressive 72% reduction in the weight of the females fed on the rabbit. Similarly, Yamada *et al.* (2009) were able to obtain a 40% reduction in engorged weight of female *R. appendiculatus* fed on rabbits immunized against voraxinα, followed by a 50% reduction in average egg masses. In spite of the lack of biological activity of the original bacterially-produced recombinant voraxin, I used it to immunize rabbits against voraxin. However, there was no decrease in female weight or in the egg masses produced (Fig. 2.14). Analysis of tick haemolymph for IgG and IgM by ELISA revealed the presence of antibodies in the majority of the ticks, although the antibody titre against voraxinα was low in ticks fed on the doubly-immunized rabbits (Fig. 2.15). It is unclear why this was so because the rabbit serum had a high titre of anti-voraxinα antibodies, albeit at a lower level than the anti-voraxinβ

antibody (Fig. 2.13). It is possible that immunization against a single component of voraxin was insufficient to inhibit feeding. Due to the low level of anti-voraxinα antibodies present in the doubly-immunized rabbit, it is possible that it was effectively immunized against only voraxinβ and thus was unable to inhibit feeding. However, I would have expected that ticks, injected directly with serum from the immunized rabbits, should have exhibited at least some decline in blood intake compared to the controls, but this was not the case (Fig. 2.16). All this said, my inability to inhibit tick feeding seems unlikely to be due to only immunizing against a single component of voraxin. Yamada *et al.* (2009) were able to obtain a substantial decrease in mean female body weight and egg production after feeding *R. appendiculatus* on rabbits immunized solely against voraxinα. Although they did not obtain the same level of inhibition of feeding as described by Weiss & Kaufman (2004), a fact they attributed to the lack of immunization against voraxinβ, they still managed to obtain a much greater degree of inhibition than I was able to. This implies that immunization against just voraxinα should have been enough to inhibit tick feeding to some degree in *A. hebraeum*. However, other possibilities exist that may potentially explain the immunizations' lack of effect.

The proteins used for both the original bioassays and immunization experiments were only partially purified, and contained numerous contaminating *E. coli* proteins. It is conceivable that the bacterial proteins had a much higher immunogenicity, and if so, the majority of the antibodies produced would have been against these proteins as opposed to voraxin. This would result in the ELISA

falsely indicating the presence of anti-voraxin antibodies present in the rabbits, instead detecting the presence of antibodies against the *E. coli* protein contaminants. The anti-*E. coli* antibodies would also not have any effect on tick feeding, possibly explaining the lack of feeding inhibition in both the ticks fed on the immunized rabbits as well as those directly injected with the immune sera.

The absence of biological activity and the failure to protect the rabbits against tick infestations by the recombinant voraxin may be further explained by the fact that the His-tagged voraxin constructs had undergone multiple recombination events in the expression cells. The constructs were originally provided in the bacterial expression strain BL21 DE3 pLys S, which possess functional copies of the genes allowing DNA recombination and endonuclease activity (*recA* and *endA* respectively). Propagation and archival of the expression constructs in this cell line allowed substantial amounts of DNA cleavage and recombination to occur, which may have altered the sequences of the proteins being expressed. Although the sequence of the expression constructs was verified before beginning the initial expression experiments, the sequences were not continuously re-evaluated to ensure that no changes had occurred over time. If the sequences of the expression constructs was altered, this could have led to the production of non-functional proteins that did not contain epitopes resembling those of native voraxin.

Recall that injection of the N-terminally GST-tagged proteins off the host resulted in a very low survival rate for all ticks, likely due to the development of a severe illness in the rabbit initially used to pre-feed the ticks (Fig. 2.9). I also

considered the possibility that on-host injection would be a protocol superior to off-host injection. However, this was not the case: mortality was lower, but the injections failed to stimulate engorgement (Fig. 2.10, 2.11).

Another disturbing result was the failure of on-host or off-host injection of male gonad homogenates to trigger full engorgement (Fig. 2.8 – 2.11). In the experiment performed by Weiss & Kaufman (2004), the ticks were allowed to feed for only 7 days following injection; they assumed that this would be more than enough time to engorge, because the ticks had already fed for a week or so before injection. Although most of the ticks did not achieve full engorgement, they did feed to at least double the weight of the controls. However, this was not observed in any of my experiments, none of the ticks feeding significantly more than the controls, regardless of injection method or time spent on the host (Fig. 2.8 – 2.11). In several experiments, ticks injected with TVD or MAG appeared to feed more than the controls (Fig. 2.8 – 2.10); however, in each case, the increase in weight was not significantly more than that of the control. Although the small sample size likely played a role in the lack of significance, it should be noted that even if significance had been attained, the majority of ticks in each of these groups fed only slightly above the CW, with very few reaching engorged weights.

Virgin *D. andersoni* females, on-host injected with homogenates of whole fed male gonads, MAG or TVD, fed only to the same extent as the controls (Fig. 2.12). This lack of response was not entirely surprising, considering that *D. andersoni* virgins will often feed to ~ 35X their unfed weight and may then lay (infertile) eggs (Dr. Tim Lysyk, personal communication; Ullah & Kaufman, in

preparation). Further work to determine the CW in *D. andersoni* is currently underway (Ullah & Kaufman, in preparation).

Interestingly, although Sonenshine *et al.* (2011) were able to identify voraxinα in the male gonad transcriptome of *D. variabilis*, they found no evidence of the voraxinα protein in either the fed male TVD, MAG or the spermatophore by LC MS/MS. On the other hand, Donohue *et al.* (2009) stimulated a significant weight increase in virgin female *D. variabilis* by injecting both the TVD and MAG from fed males. Unlike Weiss & Kaufman (2004), however, they were unable to trigger engorgement by injecting TVD alone. However, the injection of MAG homogenate directly into the female gonopore simulated 27% of females to feed to repletion, compared to (a) 17% of untreated virgins or TVD injected virgins, (b) 0% of PBS injected controls and (c) 0% of females whose gonopore was mechanically stimulated by inserting a sephadex bead (Donohue *et al.*, 2009). These data, along with the fact that knockdown of the *D. varabilis* voraxinα gene had no effect on the ability of females to engorge (Donohue *et al.*, 2009), implies that the stimulus required for engorgement in *D. variabilis* may differ substantially from that of *A. hebraeum*, and that voraxinα in *D. variabilis* may play a different role in the reproductive process.

Donohue *et al.* (2009) obtained a partial *D. variabilis* voraxinα sequence at 2704 bp, of a predicted 7 kb as determined by northern blot, but they couldn't find voraxinβ by either degenerate PCR or examination of the male gonad transcriptome (Sonenshine *et al.*, 2011). Extensive BLAST searching of public sequence databases by both Donohue *et al.* (2009) and I have failed to reveal a

voraxinβ orthologue in any other tick species for which sequence data are available. Alignments of the reported *A. hebraeum* voraxinα to those from *D. variabilis*, *I. scapularis* and *R. microplus* indicate that the *A. hebraeum* voraxinα is a 5' partial sequence (Donohue *et al.*, 2009). The conservation of voraxinα suggests that it plays a role in engorgement in multiple tick species; but it remains a puzzle why voraxinβ has not been found in other species, considering that in *A. hebraeum* voraxinβ is essential for EF bioactivity.

2.5 Conclusion

In spite of much effort, I was unable to replicate the results of Weiss & Kaufman (2004). Likewise, the injection of bacterially-produced recombinant voraxin with either His or GST tags failed to provoke an engorgement response in the feeding ticks. Ticks fed on rabbits immunized against the recombinant voraxin proteins fed and engorged normally, likewise displaying none of the inhibition reported by Weiss & Kaufman (2004). It is possible that some of the difficulties I encountered with this project were due to the nature of the recombinant proteins produced. Weiss & Kaufman (2004) produced their recombinant voraxin proteins in an insect cell expression system, whereas I produced my proteins in a bacterial expression system. This may have resulted in a lack of proper folding and/or post-translational modification of the proteins. Although I did not attempt to produce recombinant voraxin in an insect cell expression system, this was the primary project of the post-doctoral fellow, Dr. Xiuyang Guo, who was in the laboratory at the time. Dr. Guo spent a year attempting to produce recombinant voraxin via transient transfection using CellFectin (Invitrogen) of both sf9 and sf21 insect cell

lines. He also attempted stable transfection of both cell lines using CellFectin and Blasticidin-S selection. However, he was unable to express either voraxinα or voraxinβ in any quantity, despite being able to produce large amounts of his positive control protein, β-galactosidase. Dr. Guo's difficulties in producing the recombinant voraxin proteins in an insect cell line, suggested that my time would be better spent on other projects as opposed to me attempting further production of recombinant voraxin in an insect cell system in an attempt to better replicate the work of Weiss and Kaufman (2004).

I was also unsuccessful in stimulating engorgement in *D. andersoni* by the on-host injection of *D. andersoni* male gonad homogenates (Fig. 2.12). Injections of fed male gonads, both on and off the host into virgin *A. hebraeum* elicited no significant increase in body weight above that observed in the controls (Fig. 2.10, 2.11), indicating that a major change in the physiology of *A. hebraeum* has occurred in our tick colonies. It raises the question of how closely the inbred lab colonies used in both my experiments, as well as in those of Weiss & Kaufman (2004) actually resemble the wild populations. I myself observed numerous morphological abnormalities in our lab colonies as well as the brief appearance of a parthenogenic line of *A. hebraeum* (see Chapter 4). These sorts of anomalies are only rarely observed in wild populations of ticks and had not been previously encountered in significant numbers in the lab before I began my studies.

Table 2.1 Primers used for cloning and sequencing

Target	Primer ID	Direction	Sequence (5'-3')	Purpose
Voraxinα ORF	pRSETa-F	Forward	AAGGATCCGATGTTGATCACCAAGGAC	Original construct generation
	PRSETa-R	Reverse	TTCTCGAGCTATCGACCAGTGTCAAG	
Voraxinβ ORF	pET26b-F	Forward	CTCTCTCATATGGCGAAACAGGGACTTCTG	Original construct generation
	pET26b-R	Reverse	CTCTCTAAGCTTCCGCAGGCTCCCCAGGACCCT	
M13 (-20)	ADS73	Forward	GTAAAACGACGGCCAG	Sequencing
M13	ADS74	Reverse	CAGGAAACAGCTATGAC	
Voraxinα N-terminal GST tagged construct	ADS55	Forward	TTCATTGACGGGCGGATGTTGATCACCAAGGACCTGATGCAG	Construct generation set 1
	ADS56	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATTGACGGGCGG	Construct generation set 2
Voraxinβ N-terminal GST construct	ADS63	Forward	GGCTTCATTGACGGGCGGATGGCGAAACAGGGACTTCTG	Construct generation set 1
	ADS64	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATTGACGGGCGG	Construct generation set 2
Voraxin N-terminal GST tagged constructs	ADS59	Reverse	GAAAGCTGGGTCCTATCGACCAGTGTCAAGCTCGGCGAG	Construct generation set 1
	ADS60	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATCGACC	Construct generation set 2

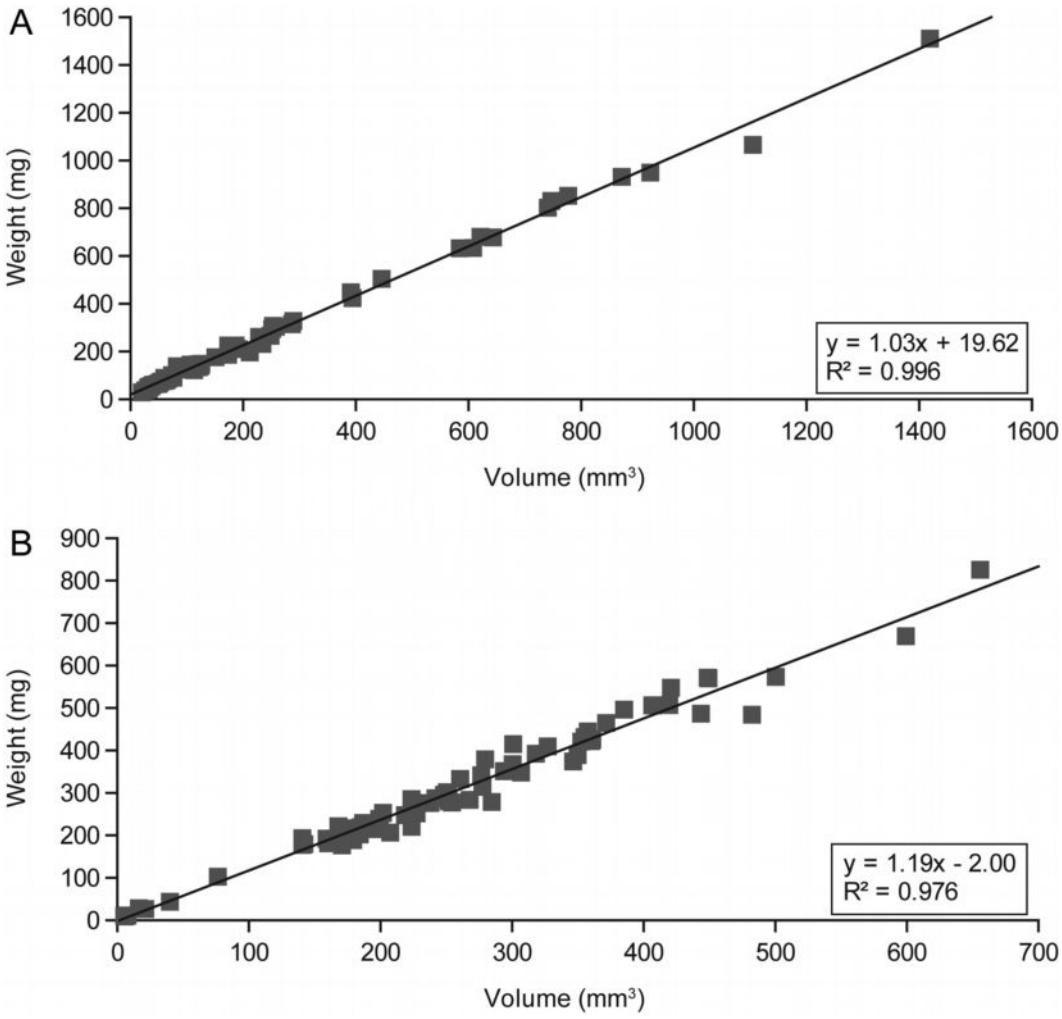

Figure 2.1. Tick weight plotted as a function of body volume for *A. hebraeum* and *D. andersoni* ticks. Female ticks were weighed and measured before and after feeding and their body volume calculated as described in Materials and Methods. This plot was used to calculate the approximate weights of feeding ticks from volume measurements taken while (A) *A. hebraeum*, and (B) *D. andersoni* females fed on the host.

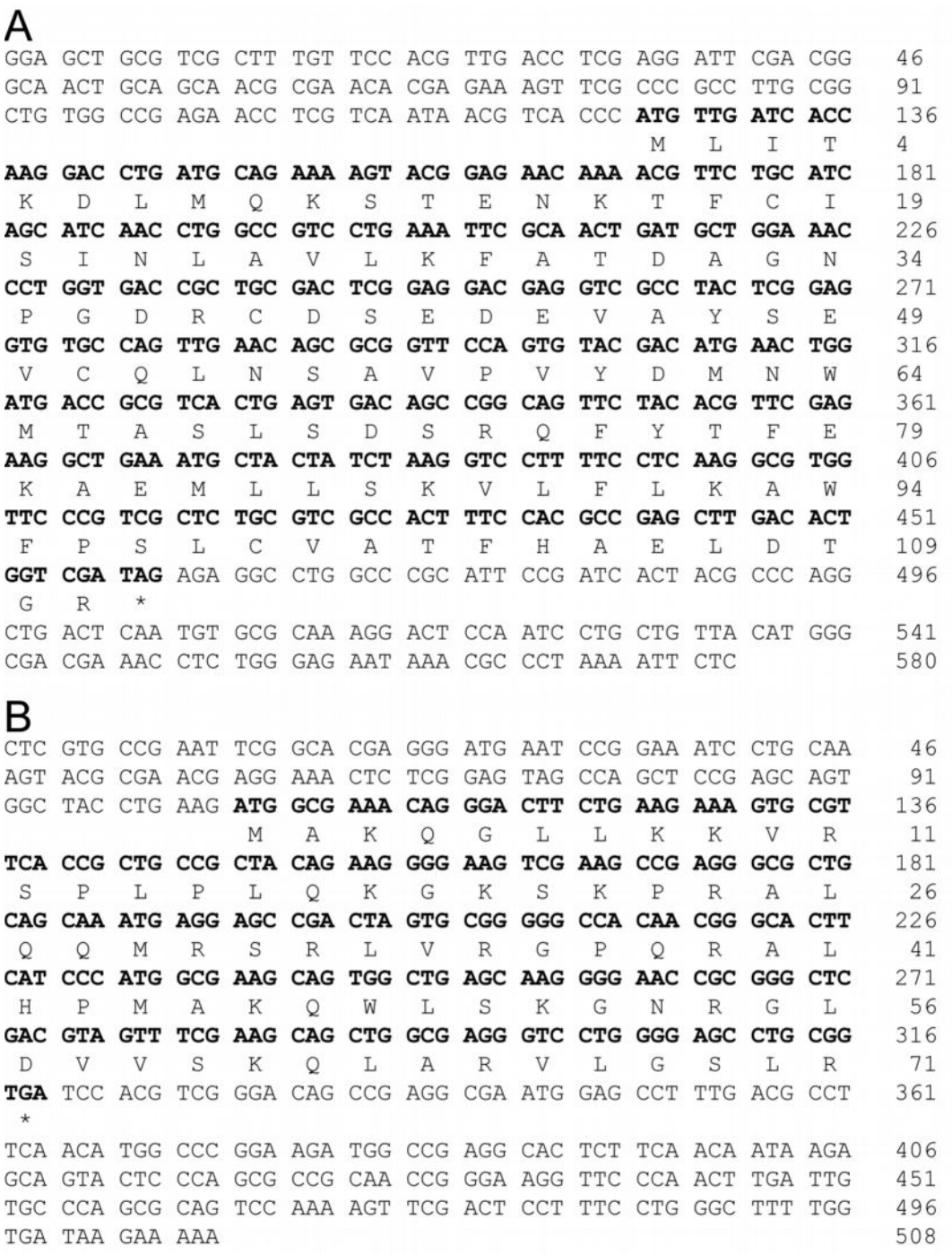

Figure 2.2. The nucleotide and amino acid sequences (single-letter code) of both (A) voraxinα and (B) voraxinβ as determined by Weiss & Kaufman (2004). The ORF of each gene is indicated in bold.

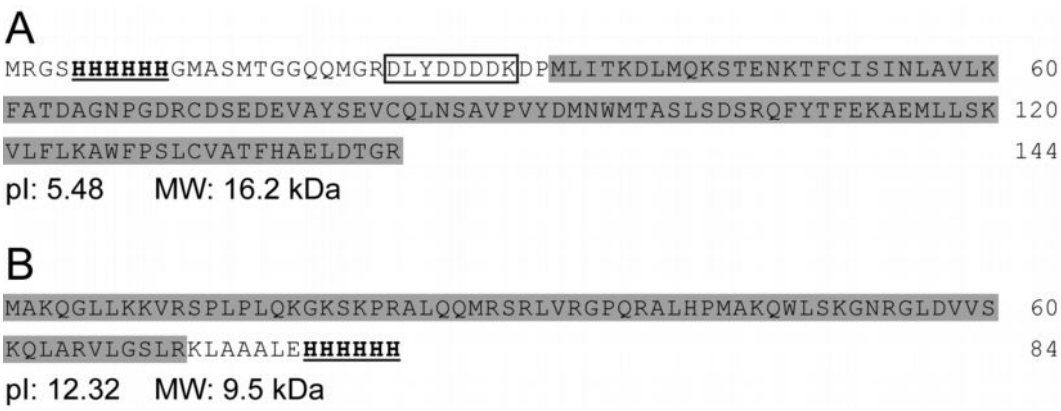

Figure 2.3. The amino acid sequences of the voraxinα (A) and voraxinβ (B) His-tagged constructs designed and produced by Mr. Gary Ritzel of the Department of Biological Sciences. The N-terminally 6x His-tagged recombinant voraxinα was produced using the pRSETB expression vector (Invitrogen) and the C-terminally tagged recombinant voraxinβ produced using the pET26b+ expression vector (Novagen). The voraxin ORFs are indicated by grey shading, the His-tags are in boldface and underlined, and the voraxinα construct enterokinase recognition site marked by an open box. Non-marked sequence corresponds to translated regions of the vector polylinker. The predicted isoelectric point and molecular weight of each protein are indicated.

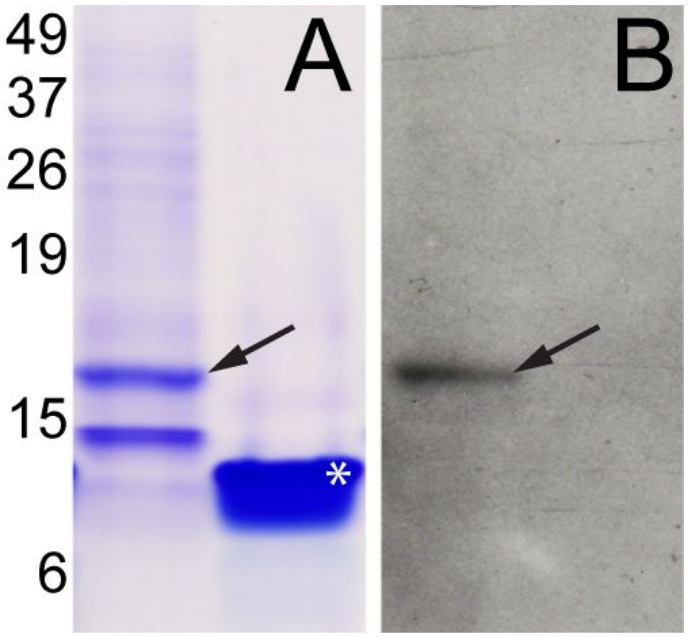

Figure 2.4. Partially purified His-tagged recombinant voraxinα and voraxinβ samples separated via SDS-PAGE and analyzed by western blot. Approximate purity of each sample was determined by analysis of band intensity using Adobe Photoshop. Voraxinα, ~16 kDa was ~60% pure and voraxinβ, at ~10 kDa was ~88% pure. (A) The partially purified His-tagged voraxinα and voraxinβ separated by SDS-PAGE with the band corresponding to voraxinα indicated by an arrow and the band corresponding to voraxinβ marked with an asterisk. The identity of the second strong band at ~15 kDa in the voraxinα lysate is unknown, and the low molecular weight smear observed in the voraxinβ containing lysate may be degradation products of voraxinβ. (B) Radiograph of the partially purified proteins analyzed by western blot, with recombinant proteins indicated as before. Voraxinβ is not present on this blot due to initial difficulties in transferring the highly positively charged protein to the PVDF membrane.

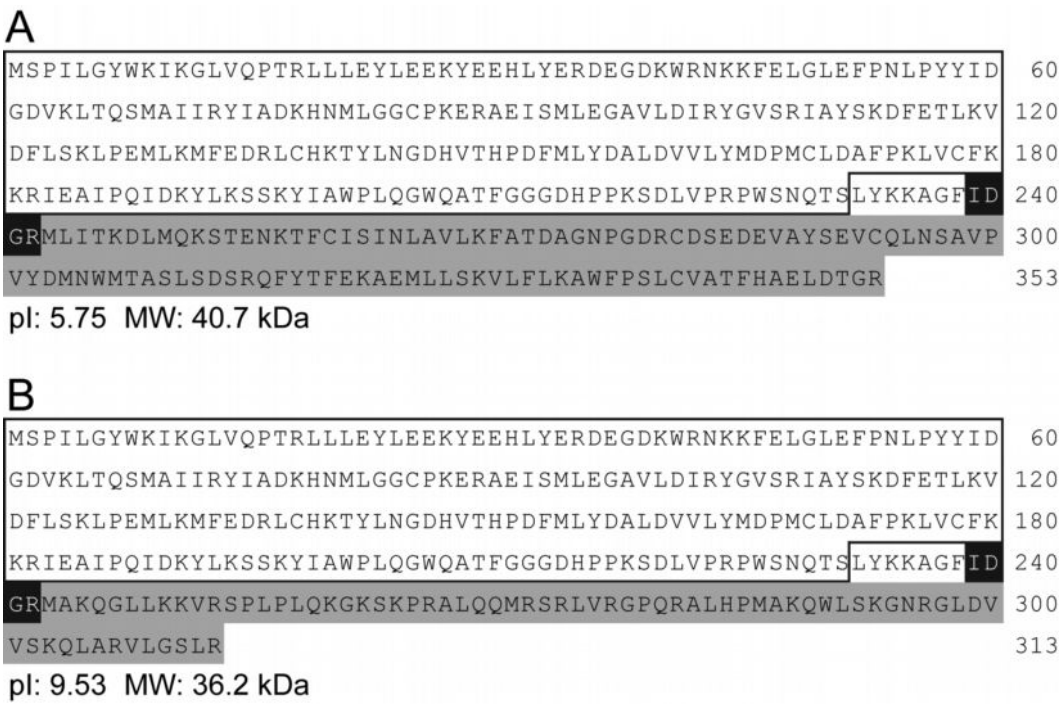

Figure 2.5. The one-letter amino acid sequences of the N-terminal GST-tagged voraxinα (A) and voraxinβ (B) expression constructs are shown. The regions of the protein corresponding to the GST-tag are framed in open boxes, the factor Xa cut sequences are indicated by black shaded boxes, the voraxin ORFs marked by grey shading, and non-marked regions indicate expression vector linker sequence. The predicted isoelectric point and molecular weight of each protein are indicated.

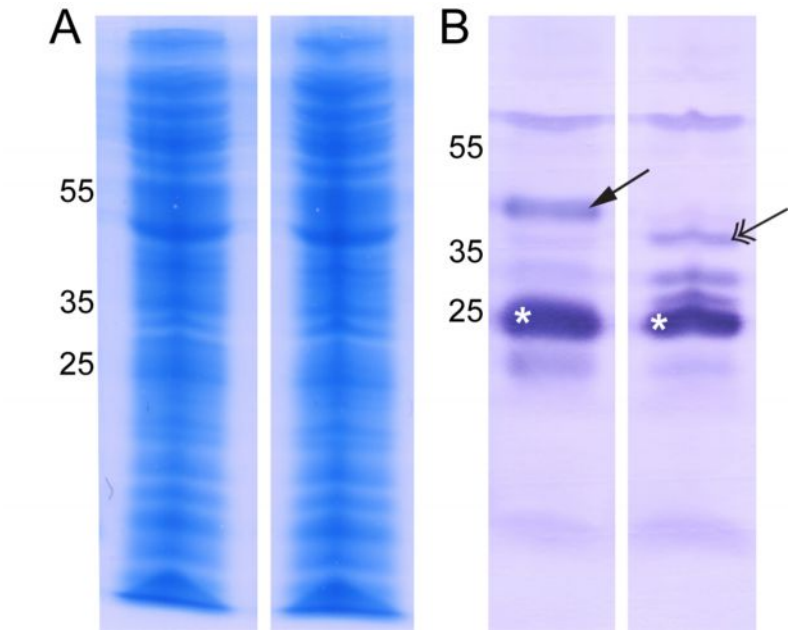

Figure 2.6. SDS-PAGE and western blot analysis of crude bacterial cell lysates containing recombinant N-terminal GST-tagged voraxinα and voraxinβ. (A) Lysates containing GST-tagged recombinant voraxin separated by SDS-PAGE. (B) Colourimetric detection of the GST tagged proteins following western blot analysis. Labelled are bands likely corresponding to decoupled GST (asterisk), the tagged voraxinα (arrow) and the tagged voraxinβ (double arrow). Additional voraxinβ bands located between tagged protein and the decoupled GST are possibly voraxinβ degradation products The band >55 kDa in both samples is due to non-specific binding of the anti-GST antibody.

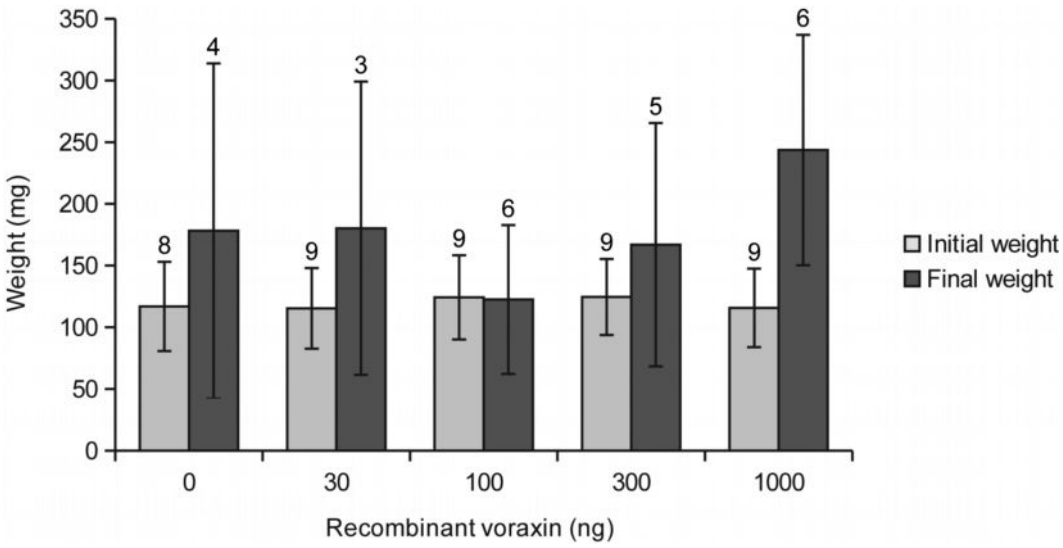

Figure 2.7. The effect of off-host injection of recombinant His-tagged voraxin on partially fed OC virgin female *A. hebraeum*. Females were injected with the indicated dose of each of the partially purified His-tagged recombinant voraxin proteins (voraxinα and voraxinβ), or the saline control (0 ng). After 3 weeks of feeding, all females that had not yet spontaneously detached were removed and weighed. There were no significant differences as a function of injected dose after feeding ($p=0.415$), nor were there any significant differences between the initial and final weights for each group ($p\geq0.24$). For this and all subsequent figures, the differences between experimental treatments were analyzed by ANOVA with post hoc comparisons achieved by the Sidak multiple comparison test.

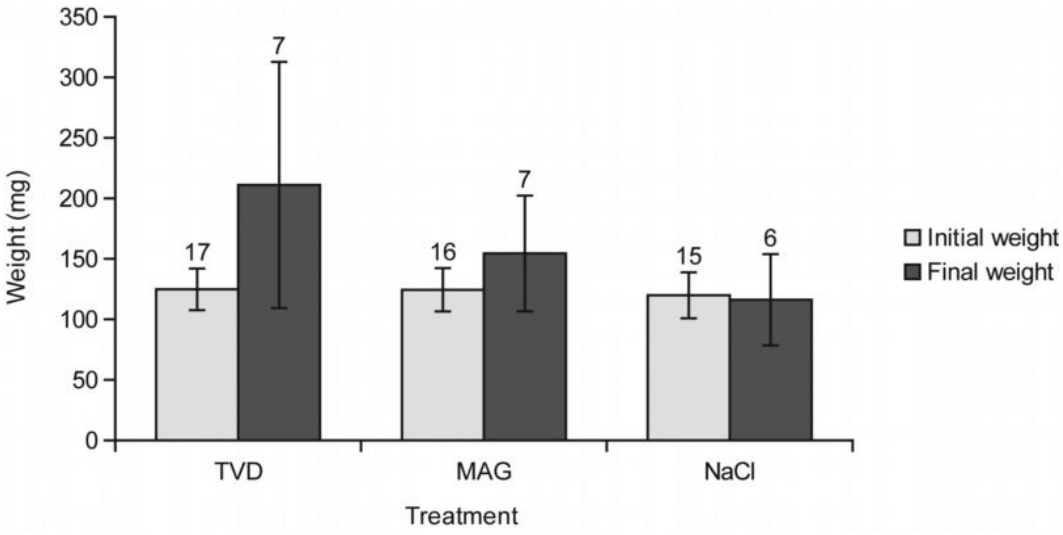

Figure 2.8. The effect of off-host injection of one gonad equivalent of homogenized OC TVD, MAG or a saline control on partially fed virgin OC female *A. hebraeum*. No significant differences were observed between the initial and final weights of each group ($p \geq 0.43$), nor among the treatment groups following feeding ($p = 0.650$).

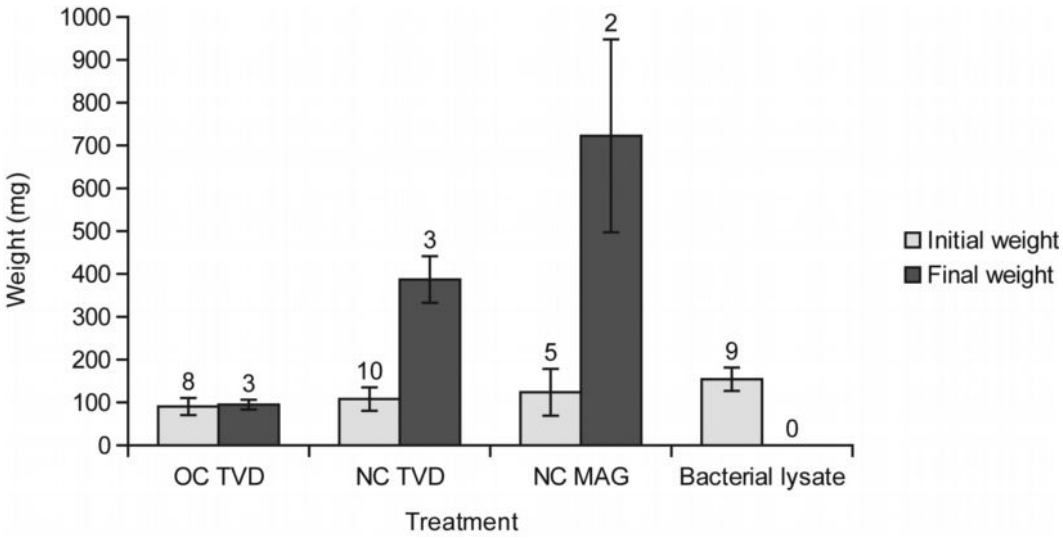

Figure 2.9. The effect of off-host injection of one gonad equivalent of homogenized OC TVD, NC TVD, NC MAG or non-purified bacterial lysate containing N-terminally GST-tagged recombinant voraxin on partially fed virgin NC female *A. hebraeum*. The approximate initial and final weights for each tick were calculated from volume measurements taken before and after injection and feeding as described in the Materials and Methods. Possibly due in part to the low numbers of surviving ticks, there were no significant differences among the groups following injection and feeding ($p \geq 0.18$). Similarly there was no significant increase from the initial weight of any of the groups following injection and feeding ($p \geq 0.20$). None of the females injected with the bacterial lysates survived.

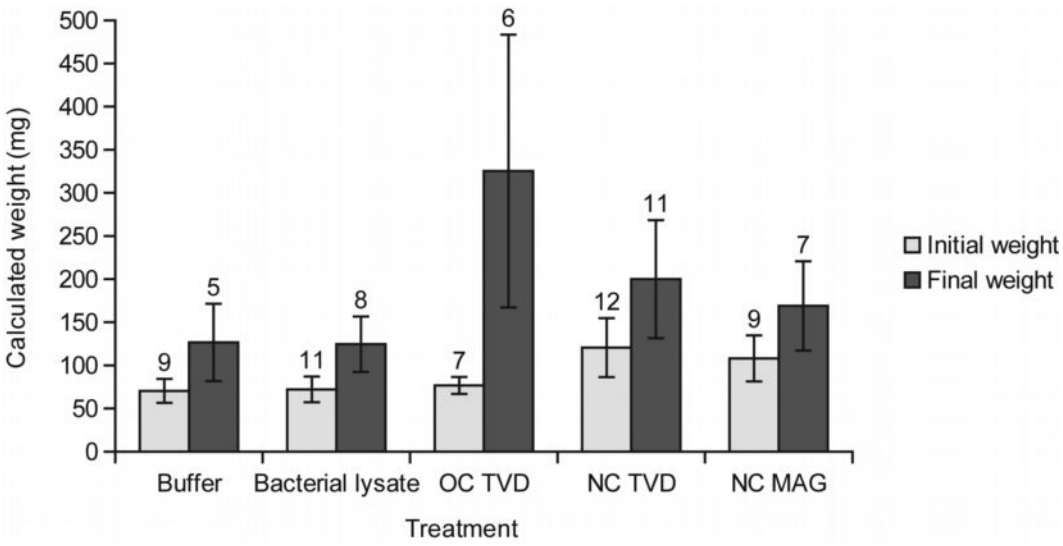

Figure 2.10. The effect of on-host injection of one gonad equivalent of homogenized OC TVD, NC TVD, NC MAG, non-purified bacterial lysate containing N-terminal GST-tagged recombinant voraxin, or a 50 mM Tris-Cl, pH 8.0 control on feeding virgin OC female *A. hebraeum*. Following injection and feeding there were no significant differences among the treatment groups ($p=0.469$), nor was there a significant increase from the initial average weight for any of the groups ($p \geq 0.177$). The large SEMs observed are likely due in part to the low sample size, and in the case of the OC TVD, the fact that the majority of the ticks did not feed past the CW and only a single tick fed to repletion.

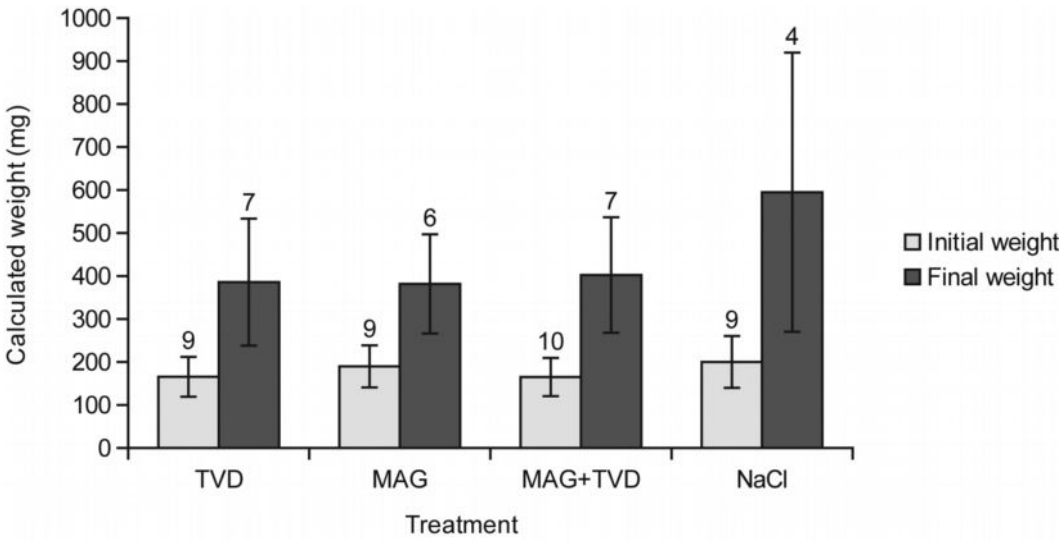

Figure 2.11. The effect of on-host injection of one gonad equivalent of homogenized NC male gonads on feeding virgin NC female *A. hebraeum*. Following injection, females exhibited no significant differences in the calculated mean weights among treatment groups ($p=0.838$), nor was there any significant increase from the initial average calculated weight within any group following injection and feeding ($p \geq 0.965$). Particularly high SEM in the NaCl group likely arises from small sample size and the single tick from this group that fed to repletion.

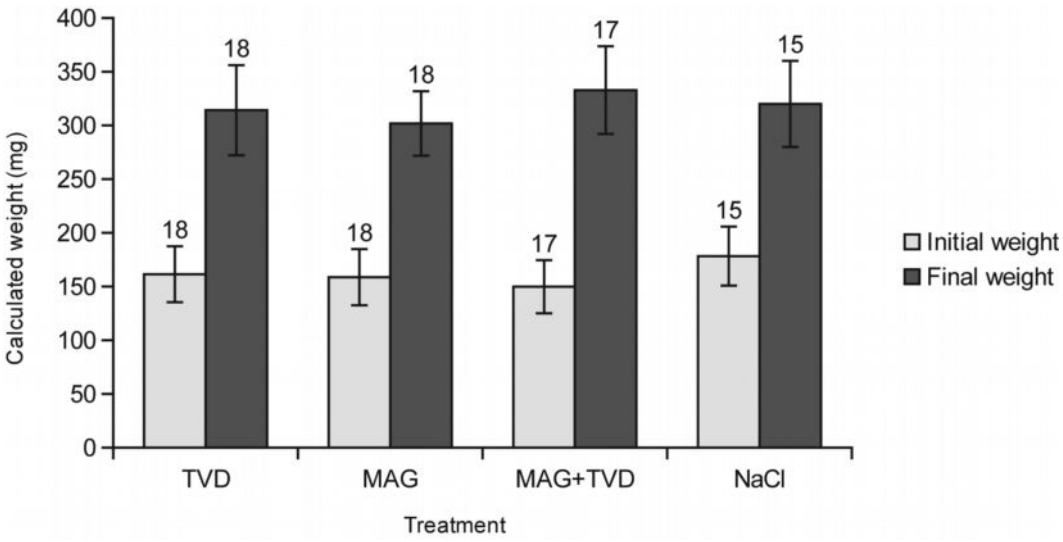

Figure 2.12. The effect of on-host injection of one or two gonad equivalents of homogenized *D. andersoni* TVD, MAG, TVD+MAG or a saline control on feeding virgin *D. andersoni* females. There were no significant differences among the calculated weights of any of the treatment groups after feeding ($p=0.951$), but there was a significant increase in fed weight within each group following injection and feeding ($p \leq 0.0072$).

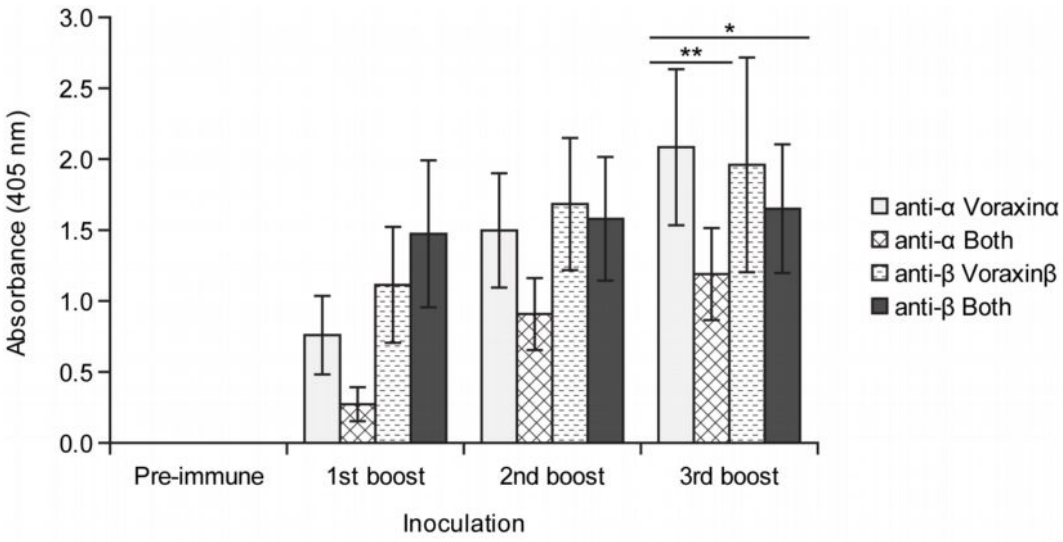

Figure 2.13. Anti-voraxin antibody titre in rabbits following immunization against voraxinα, voraxinβ, both voraxinα+β, or a saline control. An indirect ELISA indicated that the levels of anti-voraxin antibodies increased significantly by the third inoculation in all rabbits (*, $p<0.000$). Levels of anti-voraxinα antibodies were examined in the rabbits inoculated against voraxinα and rabbits immunized against both voraxinα+β. Levels of anti-voraxinβ antibodies were examined likewise in the rabbits immunized against voraxinβ and voraxinα+β. Significantly higher levels of voraxinα were seen in the rabbits inoculated solely against voraxinα compared to the doubly immunized rabbits (**, $p<0.000$). Absorbance of the samples in rabbits inoculated against voraxinβ alone were not significantly different from those in the doubly immunized rabbits ($p=0.444$).

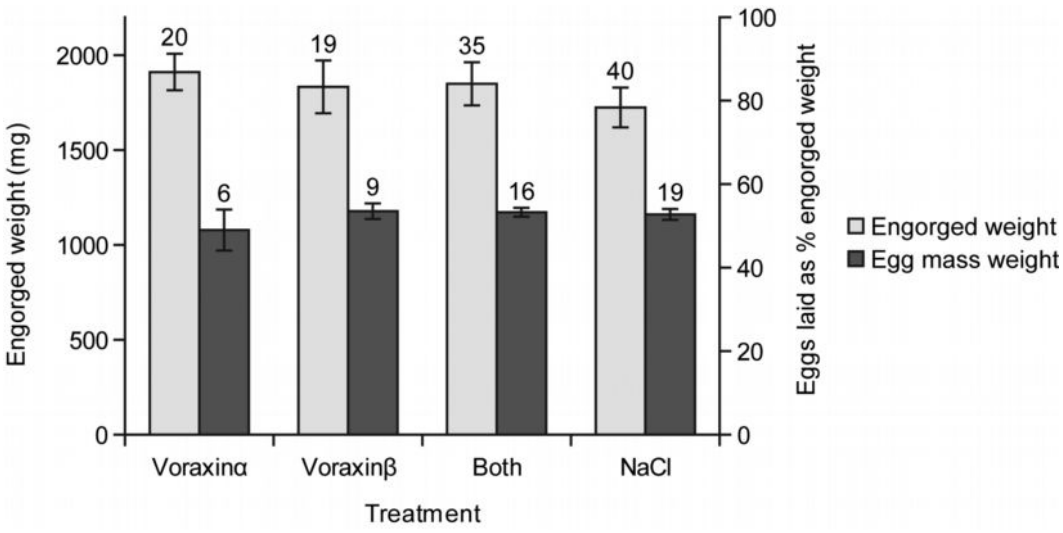

Figure 2.14. The effect of feeding on rabbits immunized against voraxinα, voraxinβ, both voraxinα+β, or a saline control on mated *A. hebraeum* females. Following feeding and engorgement, females were weighed and monitored for egg production. There were no significant differences in engorged body weight ($p=0.696$) or egg mass laid ($p=0.505$) among the treatment groups.

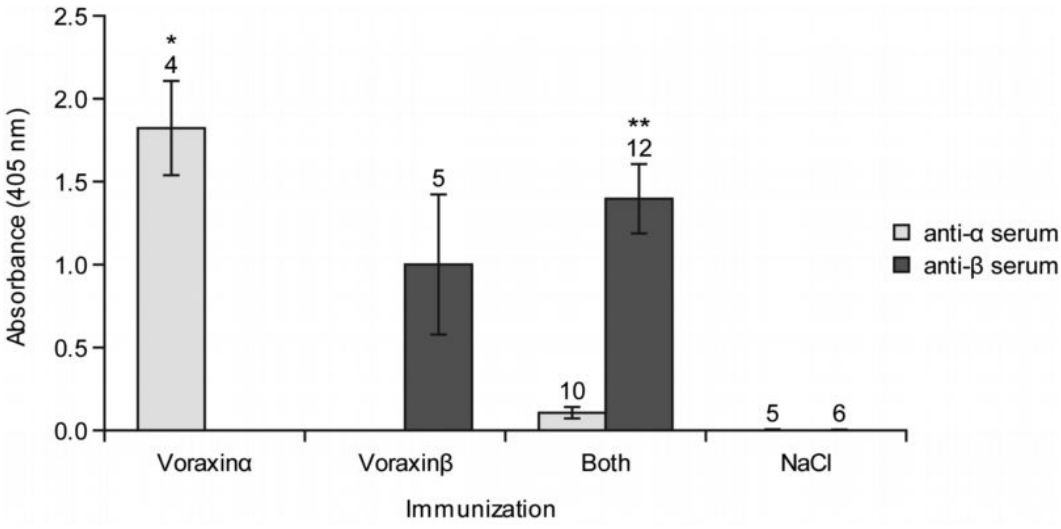

Figure 2.15. Anti-voraxin antibody titre in the haemolymph of engorged females allowed to feed on rabbits immunized against voraxinα, voraxinβ, both voraxinα+β, or a saline control. Haemolymph extracted from these females was assayed for the presence of anti-voraxin antibodies in an indirect ELISA assay. Ticks fed on the rabbits immunized against voraxinα had significantly higher levels of anti-voraxinα antibodies in their haemolymph compared to the saline-injected controls (*, $p<0.000$). However, ticks fed on the rabbits immunized against voraxinβ had only marginally higher levels than the control ($p=0.074$). Ticks fed on the doubly immunized rabbits, exhibited an anti-voraxinα titre that was not significantly different from the control ($p=0.845$), but the level of anti-voraxinβ antibodies in these ticks was significantly higher than the control (**, $p=0.002$).

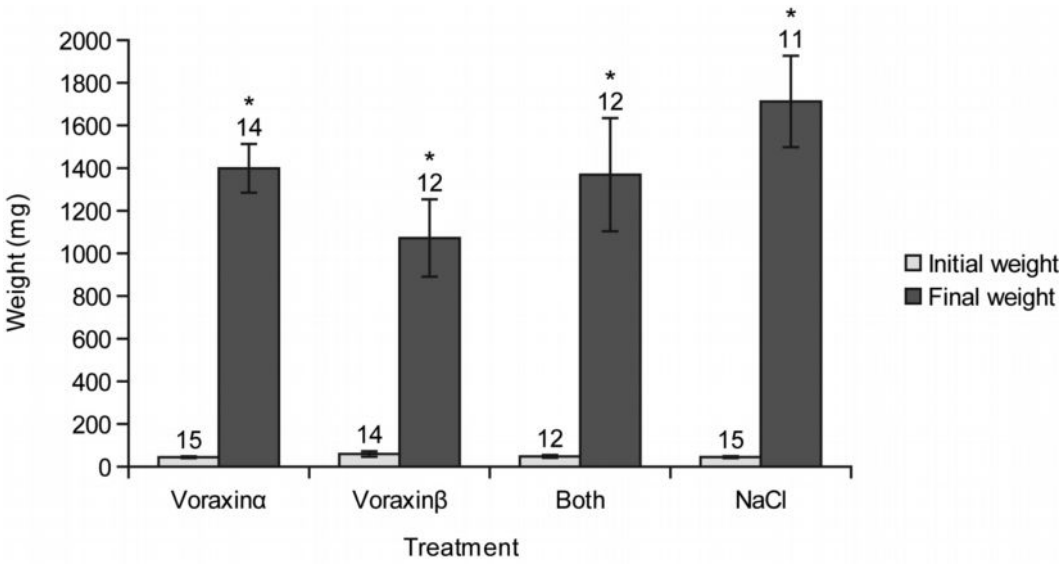

Figure 2.16. The effect of off-host injection of serum obtained from rabbits immunized against voraxinα, voraxinβ, both voraxinα+β, or a saline control on OC *A. hebraeum* females. Following injection, the females were allowed to mate and feed to engorgement on a fresh host. There were no significant differences between ticks injected with the anti-voraxin sera vs the 3-fold diluted sera ($p=1.000$); consequently, data for the two doses were pooled for this figure and statistical analysis. Regardless of the injected serum, no significant differences in average tick weight were observed ($p=0.491$), and the four treatment groups increased their weight significantly compared to the pre-injection weight (*, $p<0.000$).

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Chapter 3. The impact of RNA interference of the subolesin and voraxin genes in male *Amblyomma hebraeum* (Acari: Ixodidae) on female engorgement and oviposition[1]

3.1 Introduction

Ticks are major vectors of disease to humans and domestic animals (Parola & Raoult, 2001; Peter *et al.*, 2005). Notably, ticks and tick-borne pathogens have a negative impact on cattle production, with annual economic loss worldwide estimated at hundreds of millions of dollars due to direct effects of the tick infestations as well as the diseases caused by tick-borne pathogens (Peter *et al.*, 2005). Along with the impact of tick feeding itself (reduced weight gains and milk production), cattle also suffer from diseases caused by the pathogens transmitted by *A. hebraeum* including *Ehrlichia* (formerly *Cowdria) ruminantium* (heartwater disease; (Norval *et al.*, 1989; Norval, 1990). *A. hebraeum* is also the principal vector of *Rickettsia africae*, the agent of African tick bite fever (Norval *et al.*, 1989; Kelly & Mason, 1991).

Presently, acaricides constitute a major component of integrated tick control programs. However, use of acaricides is often accompanied by selection of acaricide-resistant ticks, environmental contamination, and contamination of milk and meat products with drug residues (Graf *et al.*, 2004). Novel cost-effective strategies are therefore urgently needed for control of ticks that avoid the drawbacks of acaricides.

1 A version of this chapter has been published as Smith, *et al* (2009) Experimental and applied acarology 47: 71 – 86.

Use of vaccines has proven to be effective for controlling selected tick species on cattle (de la Fuente & Kocan, 2003; Willadsen, 2004). A tick vaccine, based on the midgut antigen, Bm86, has been used for over ten years in selected integrated programs for control of the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, a species prevalent in Africa, Asia, Australia, the Caribbean and South America. Use of this vaccine has resulted in markedly reduced severity of tick infestations, a reduction in the incidence of some tick-borne diseases, and has reduced the numbers of required acaricide applications (reviewed by de la Fuente *et al.*, 2007a). Because these commercial vaccines, based on Bm86 (TickGard™ in Australia and GAVAC™ in the Caribbean and South America), are labelled for control of *R. (Boophilus) spp.* only (reviewed by de la Fuente *et al.*, 2007a), there is an ongoing search for vaccine antigens useful for other tick species.

The complex feeding cycle of female *Amblyomma hebraeum* Koch requires up to 14 days of on-host feeding for repletion, and thus allows for considerable exposure of ticks to vaccine-derived antibodies during the blood meal. A feeding lesion is established during the preparatory phase (24 to 36 h), which is followed by a slow feeding phase (up to 10 d), during which the females undergo an approximate 10-fold weight gain. Mating occurs on the host and is required for full engorgement. The final, rapid feeding phase (up to 24 h) results in a further 10-fold increase in weight gain. Initiation of the rapid phase of feeding requires an ‘engorgement factor’ produced in the male gonad and transferred to the female during copulation (Pappas & Oliver Jr., 1972; Kaufman, 2007). This engorgement factor, called 'voraxin', comprises two proteins, voraxinα and

voraxinβ (Weiss & Kaufman, 2004).

Immunization of a rabbit with recombinant voraxin caused a 74% reduction in the number of females that fed to engorgement and that ultimately oviposited (Weiss & Kaufman, 2004). Feeding success of female ticks on the immunized rabbit fell into two categories: eight ticks achieved normal engorged weights and oviposited viable eggs. Twenty-three ticks, however, achieved only 4 – 5% of the normal engorged weight, and so were too small to oviposit.

The tick-protective antigen, subolesin (initially named '4D8'), was recently discovered in *Ixodes scapularis* by use of expression library immunization and sequence analysis of expressed sequence tags in a mouse model of tick infestations (Almazán *et al.*, 2003*a*, 2003*b*, 2005*a*, 2005*b*). Vaccine trials in mice, rabbits and sheep using recombinant subolesin resulted in significant reductions in larval, nymphal and adult *I. scapularis* infestations (Almazán *et al.*, 2005*a*). While the subolesin gene is highly conserved among diverse ixodid tick species, the function of its expressed product is not known. Silencing of subolesin by RNA interference (RNAi) in *I. scapularis*, *A. americanum*, *Rhipicephalus sanguineus*, *Dermacentor variabilis* and *D. marginatus* resulted in markedly reduced feeding success, subsequent oviposition, and survival after feeding (de la Fuente *et al.*, 2006*a*). Male ticks in which the subolesin gene was silenced were unable to successfully mate with females, thus preventing the rapid stage of engorgement and oviposition (de la Fuente *et al.*, 2006*b*).

Collectively, the foregoing suggest that subolesin and/or voraxin might show promise as candidate antigens for use in development of tick vaccines. In

this study, we used RNAi to test the effect of silencing the expression of voraxin, subolesin or both together in male *A. hebraeum*.

3.2 Materials and methods

3.2.1 Ticks and hosts

Adult OC *A. hebraeum* were used for these studies, and were fed and reared as described in Chapter 2.

3.2.2 Cloning of A. hebraeum subolesin and dsRNA synthesis for subolesin and voraxin

The *A. hebraeum* subolesin cDNA was amplified by RT-PCR[2], according to procedures reported previously, using oligonucleotide primers 4D8R5 and 4D833 (Table 3.1, de la Fuente *et al.*, 2006a). Oligonucleotide primers containing T7 promoter sequences for *in vitro* transcription and synthesis of dsRNA were synthesized for amplification of the genes encoding *A. hebraeum* subolesin, voraxinα and voraxinβ (Table 3.1). RT-PCR and dsRNA synthesis reactions were performed according to procedures described previously for other tick species (de la Fuente *et al.*, 2005) using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA)[3]. The dsRNA was purified and quantified by spectrophotometry (260 nm) using a Nanodrop ND-1000 spectrophotometer. The Genbank accession number for *A. hebraeum* subolesin is EU262598. Genbank accession numbers for *A. hebraeum* voraxinα and voraxinβ are AY442319 and AY442320, respectively.

2 The cloning of *A. hebraeum* subolesin cDNA was performed by Dr. Xiuyang Guo, a postdoctoral researcher in our lab at the time.
3 Both I and Dr. Guo prepared various dsRNA constructs.

3.2.3 RNAi in unfed male ticks

In the first round of tick feeding, four treatment groups of 40 – 48 males ticks each were used: (1) subolesin dsRNA, (2) voraxinα & voraxinβ (hereafter referred to simply as 'voraxin') dsRNA, (3) subolesin + voraxin dsRNA and (4) control ticks injected with injection buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone. The ticks from each treatment group were fed on two rabbits (half of the ticks on each), and each rabbit served as a host for two treatment groups, each group feeding in separate halves of the chamber.

For RNAi, unfed male *A. hebraeum* were injected with approximately 1 µl of dsRNA ($2.4 – 3.2 \times 10^{11}$ molecules/µl), an amount that has proved successful in our earlier RNAi studies[4] using subolesin dsRNA (de la Fuente *et al.*, 2006*a*, 2006*b*). Injection of ticks was done in the lower right quadrant of the ventral surface. The exoskeleton was first pierced with the tip of a 30g needle to create an opening, and then the dsRNA was injected through this opening into the haemocoel using a Hamilton® syringe fitted with a 33g needle. After injection, the males were held overnight in the colony incubator before being placed on rabbits in the appropriate feeding chamber. One day after the males had attached to the rabbits, an equal number of unfed, virgin females was added to each feeding chamber. Feeding progress in the females was monitored for up to 20 days, after which all remaining females were removed, weighed and stored in individual gauze-covered plastic vials in the colony incubator for oviposition. In this study we define ‘engorgement’ as females that detached from the host spontaneously

4 Injection of dsRNA was performed by Dr. Katherine Kocan of Oklahoma State University, Dr. Xiuyang Guo and me.

within the 20-day feeding period; ticks that were forcibly removed from the host on the 20th day are defined as 'partially fed'. At 42 – 51 days after engorgement or removing ticks from the rabbits, the egg mass weight produced by each female was recorded, because by this time, under these holding conditions, oviposition in *A. hebraeum* is known to have stopped (Friesen & Kaufman, 2002). Hatching success for individual egg masses was recorded 36 days after oviposition. Five to ten male ticks from each group were used for histological studies, and an additional five ticks from each group were used to determine gene silencing by semi-quantitative RT-PCR (see below).

3.2.4 Second feeding of previously fed males

Some of the surviving dsRNA-injected male ticks (nine for the subolesin group and 15 for each of the other groups) were placed on a second group of tick-naïve rabbits and allowed to feed again for 23 days with an equal number of unfed females, in order to see whether gene silencing in the males was prolonged beyond a single feeding cycle. Engorgement success, oviposition, hatching and gene silencing in the males were evaluated in the same manner as during the first round of tick feeding.

3.2.5 Second feeding of females

To assess the duration of the inhibitory effect of dsRNA-injected males on the females, a group of female ticks that had been previously fed with males injected with subolesin or subolesin+voraxin dsRNAs, and did not enter the rapid phase of feeding, were placed on a fresh, tick naive rabbit and allowed to feed

with normal males. Feeding success and fecundity of these females were monitored as described above.

3.2.6 RNAi in fed male ticks

For reasons outlined in Results (*Ticks pre-fed before injection of dsRNA*) we also attempted RNAi by injecting dsRNA into fed males as follows: Three groups of 15 male ticks were allowed to feed for one, two, or three days on tick-naïve rabbits, then injected with 3 µl (2.0×10^{12} molecules/µl) of voraxin dsRNA or injection buffer alone as described above, held overnight in the colony incubator, and then returned to the rabbits on which they had fed previously. One day after each group of males had reattached, an equal number of females was added to each feeding chamber on the rabbit. Feeding progress was monitored for up to 17 days, after which all remaining females were removed and weighed. Females were stored in individual gauze-covered plastic vials in the colony incubator, and total egg mass of each female was weighed 48 – 56 days post removal from the host. Subsequent hatching success was recorded for each female 66 – 71 days later. Five males from each group were used to determine voraxin silencing by semi-quantitative RT-PCR (see below). Ten unfed male ticks were injected with 3 µl (1.2×10^{12} molecules/µl) of subolesin-, voraxin-, or both-dsRNAs, or injection buffer, according to the protocol above. These injected males were allowed to feed on a tick-naïve rabbit, half of each group were allowed to feed for 5 days and the other half were allowed to feed for 10 days, before being forcibly removed and assayed for gene expression levels by semi-quantitative RT-PCR (see below).

3.2.7 Determination of subolesin and voraxin mRNA levels after RNAi[5]

Subolesin and voraxin mRNA levels after RNAi in male ticks was measured by semi-quantitative RT-PCR as follows: Total RNA was extracted from testes and midguts of male ticks after 5, 10, and 18 days of feeding and reverse-transcribed according to the protocols outlined previously. Subolesin, voraxinα, voraxinβ and 16S rRNA levels were analyzed using using gene-specific primers (Table 3.1) in a 50 µl reaction mixture (1 µl cDNA, 1.5 mM $MgCl_2$, 25 mM KCl, 5 nM EDTA, 10mM Tris-HCl, pH 9.2, 50 nM each deoxynucleoside triphosphate [dNTP]), 0.5 U Hot-Start Taq DNA polymerase (Fermentas, Burlington, ON, Canada, 0.5 µM each primer). All reactions were incubated at 94°C for 5 min, then 35 cycles of (94°C, 30 s; 60°C, 60 s; 72°C, 2 min for denaturation, annealing and extension conditions, respectively), followed by 72°C, 10 min. Control reactions were performed using the same procedures but without reverse transcriptase to test for DNA contamination in the RNA preparations and without DNA to detect contamination of the PCR. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (GeneRuler 1kb DNA Ladder Plus, Fermentas).

3.2.8 Light microscopy[6]

Selected male ticks collected after the first and second rounds of tick feeding (2 – 5 ticks per treatment group) were fixed and processed for light

5 Both Dr. Xiuyang Guo and I were responsible for dissecting all the ticks, and both extracted RNA from the excised tissues. I performed the majority of RT-PCR testing for levels of subolesin and voraxin mRNA.

6 I was responsible for the preparation and fixation of the tick samples. All sectioning and microscopy work was performed by Dr. Katherine Kocan.

microscopy studies of resin-embedded sections. Tick fixation and processing were done according to the procedures of Kocan *et al.* (1980). Briefly, ticks were cut in half at the midline using a razor blade, separating the right and left halves. The two tick halves were fixed immediately in individual vials containing 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) and held at 4^0C until further processing. The tick halves were post-fixed in 2% cacodylate-buffered osmium tetroxide (pH 7.4), dehydrated in a graded series of ethanol (70% – 100%), and embedded in epoxy resin. Sections (1 µm) were cut with an ultramicrotome and stained with Mallory's stain (Richardson *et al.*, 1960). Photomicrographs were recorded with a light microscope equipped with a 3-chip digital camera.

3.2.9 Statistical analysis

The weight of females after feeding and the weight of egg masses were compared using the Kruskal-Wallis test, the nonparametric analogue of the one-way ANOVA, followed by pairwise comparisons. Tick mortality and engorgement rates were recorded as the percentage of dead and engorged ticks, respectively, to the total number of ticks fed after 20 days (first round of feeding) or 23 days (second round of feeding). Tick mortality, engorgement and hatching success rates were compared by using Fisher's Exact test. All statistical analyses were performed using Stata statistical software (College Station, TX: StataCorp LP).

3.3 Results

3.3.1 The first round of tick feeding

The mortality rate of males injected with subolesin dsRNA (53%) was significantly higher compared to all other treatments ($p < 0.001$), whereas significant differences in tick mortality rates were not observed between the voraxin (0%), subolesin+voraxin (7%) and control groups (2%; $p = 0.105$ and $p = 0.355$, respectively; Fisher's exact test; Table 3.2).

Representative samples of the ticks in the four treatment groups that engorged or were removed after 20 days of feeding are shown in Fig. 3.1. In this figure it is visually apparent that females co-fed with subolesin dsRNA-treated or subolesin+voraxin dsRNA-treated males fed substantially less successfully than females co-fed with either the voraxin dsRNA-treated or the control males, and this impression was verified statistically in Table 3.2. All but one of the females that were fed with voraxin dsRNA-injected males or with control males fed to engorgement (98% and 100%, respectively; Table 3.2). On the other hand, very few of the females that fed with subolesin dsRNA-injected or subolesin+voraxin dsRNA-injected males engorged fully (10% and 0%, respectively; $p <0.001$; Table 3.2). Females co-feeding with either the subolesin or the subolesin+voraxin group had significantly lower body weights after feeding (309 mg and 187 mg, respectively) than either the voraxin or the control group (1813 mg and 1945 mg, respectively; $p <0.001$). There was no significant difference, however, between the mean weights of females feeding with subolesin dsRNA-treated males or with subolesin+voraxin dsRNA-treated males (309 mg and 187 mg, respectively ($p =$

0.340; Table 3.2).

The average weight of the egg masses produced by females from the subolesin and subolesin+voraxin groups (33% and 16% of female body weight (bw), respectively) was significantly lower than that of the control or voraxin group (54% and 48% bw, respectively; $p < 0.001$). The average weight of the egg masses produced by females from the voraxin group (48% bw) was marginally lower than that of the control group (54%; $p = 0.0422$). Viability of the eggs (i.e., hatching success) produced by females that fed with subolesin or subolesin+voraxin dsRNA injected males (50% and 0%, respectively) was significantly lower than that of eggs produced by females fed with buffer-injected or voraxin dsRNA-injected ticks (100% and 98%, respectively; $p < 0.001$). The proportion of eggs that hatched from egg masses produced by females in the subolesin+voraxin group (0%) was significantly lower than that of the subolesin group (50%; $p = 0.033$; Table 3.2).

3.3.2 The second round of tick feeding

Males available after the first round of feeding were removed, held overnight in the colony incubator and then allowed to feed on rabbits for 24 days with a fresh batch of unfed virgin females, in order to determine the duration of RNAi in the males. Males that had been injected previously with subolesin or subolesin+voraxin dsRNAs, and allowed to feed a second time with unfed untreated females, had significantly higher mortality rates than did the buffer-injected controls (78%, 53% and 7%, respectively; $p = 0.001$ and $p = 0.014$, respectively; Table 3.2). Males from the subolesin+voraxin group also had a

significantly higher mortality rate during the second round of feeding compared to the first (53% vs 7%, $p < 0.001$). Only 29% of females fed with males injected previously with subolesin dsRNA, and none of females fed with subolesin+voraxin group ticks, fed to repletion. Both of these groups fed significantly less successfully than either the voraxin dsRNA injected ($p = 0.017$ and $p < 0.001$, respectively) or control groups (86% and 80% respectively; $p = 0.052$ and $p <0.001$ for subolesin and subolesin+voraxin, respectively; Table 3.2).

Females from the subolesin and the subolesin+voraxin groups (701 mg and 369 mg, respectively) weighed significantly less than those from either the voraxin (1967 mg, $p = 0.007$ and $p <0.001$, respectively) or control group (1844 mg, $p = 0.020$ and $p <0.001$, respectively). Weights of the females from the subolesin and the subolesin+voraxin groups (701 mg and 369 mg, respectively) were not significantly different from each other ($p = 0.193$; Table 3.2).

The mean egg mass weights from both the subolesin and subolesin+voraxin groups (17% bw and 9% bw, respectively) were significantly lower than that of the control (34% bw, $p = 0.045$ and $p = 0.0045$, respectively). The difference between the subolesin and subolesin+voraxin groups was not statistically significant (17% and 9%, respectively; $p = 0.248$; Table 3.2).

Hatching success in the subolesin and subolesin+voraxin groups (20% and 0%, respectively) was significantly lower than that of the voraxin (100%, $p = 0.002$ and $p <0.001$, respectively) or control groups (92%, $p = 0.010$ and $p < 0.001$, respectively; Table 3.2), but were not significantly different from each other ($p = 0.455$). In brief, the effect of dsRNA treatment of males on female

feeding success and fecundity was maintained over at least two feeding cycles by the males.

3.3.3 Determination of subolesin and voraxin mRNA levels after RNAi

Semi-quantitative RT-PCR analysis of gene expression levels showed a reduction of the subolesin transcript compared to the voraxin dsRNA-injected or buffer- injected control males after 5 or 10 days of feeding (Fig. 3.2). Levels of both voraxinα and voraxinβ in the voraxin dsRNA injected males were not reduced relative to the control ticks. Levels of voraxinβ were low for all samples and could not be amplified from the subolesin-silenced males, but could be amplified from the males injected with subolesin+voraxin dsRNAs (Fig. 3.2).

3.3.4 Second round of feeding of the females that had not engorged after the first round of feeding

We next tested whether females, failing to engorge when fed with gene-silenced males (subolesin or subolesin+voraxin) during the first round of feeding, were subsequently able to engorge if co-fed with normal males on a tick-naïve host. The data are found in Table 3.2, "2nd feeding of females". Mortality of these females during the course of feeding was 0%. Engorgement occurred in 80% and 89% of these females, vs 10% and 0% during their first opportunity to engorge. Similarly, these females achieved engorged weights within the normal range (1353 mg and 1652 mg, respectively). Their egg masses (35% bw and 38% bw, respectively) were within the normal range, and hatching success (100% and 96%, respectively) was much higher than that of similarly treated females during the

first round of feeding (50% and 0%, respectively; Table 3.2).

3.3.5 Ticks pre-fed before injection of dsRNA

Injection of voraxin dsRNA into unfed males failed to inhibit engorgement in co-feeding females (Table 3.2). One possibility for this negative result relates to the fact that voraxin is up-regulated during feeding (Weiss & Kaufman, 2004), and so voraxin mRNA was unlikely to have been present in unfed males for the dsRNA to act on. We thus repeated these experiments with fed male ticks. We injected voraxin dsRNA in males fed for 1, 2 or 3 days, and then allowing them to feed with females. Most of these females engorged and produced normal egg masses (Table 3.2). Semi-quantitative RT-PCR analysis of gene expression levels did not show substantial decreases in levels of the voraxin transcripts in the males injected with voraxin dsRNA (Fig. 3.2).

3.3.6 Light microscopic changes in tick salivary gland, midgut and testis after RNAi

Noticeable light microscopic differences were observed in salivary gland, midgut, and testis following RNAi of subolesin or subolesin+voraxin, as compared to the injection-buffer controls, and these changes were similar after the first and second tick rounds of feeding (Fig. 3.3). Injection of ticks with voraxin dsRNA alone did not appear to affect these tissues, which were histologically similar to those of the controls. However, male ticks injected with subolesin or subolesin+voraxin dsRNA had salivary gland acini that were crenated and appeared smaller in diameter (Fig. 3.3B) compared to the injection-buffer controls

(Fig. 3.3A). Tick midgut appeared to be profoundly affected in both the subolesin and subolesin+voraxin groups, and showed signs of advanced degeneration (Fig. 3.3D) compared to the control (Fig. 3.3C). Few gut epithelial cells were attached to the basement membrane, and sloughed cells and cellular debris were seen within the gut lumen (Fig. 3.3D). In the testis of males injected with subolesin or subolesin+voraxin dsRNA, few prospermia (mature spermatids) were seen (Fig. 3.3F), and these prospermia appeared deformed relative to those from controls (Fig. 3.3E). Cellular debris and clear spaces surrounded the scattered prospermia (Fig. 3.3F).

3.4 Discussion

The focus of this study was attempting to silence targeted genes in male ticks, and then assessing the ability of these males to mate successfully with normal females, leading the latter to engorge and oviposit. In most ixodid tick species, copulation occurs only on the host during feeding; copulation is necessary for females to achieve full engorgement and subsequently oviposit (reviewed by Kaufman, 2007). Transfer of voraxin via the spermatophore is required for female feeding and oviposition in *A. hebraeum* (Weiss & Kaufman, 2004). The importance of voraxin as an engorgement stimulus in *A. hebraeum* was demonstrated by marked inhibition of engorgement in females feeding on a rabbit that had been immunized against the two voraxin proteins (Weiss & Kaufman, 2004).

Whereas homologues of voraxin are suspected in other ixodid tick species, they have not yet been widely reported, the exceptions being three entries in

GenBank: a peptide from *D. variabilis* showing 83% identity to voraxinα of *A. hebraeum* and two EST entries from the BmGI dataset for *Rhipicephalus (Boophilus) microplus,* showing 77% and 76% similarity to voraxinα of *A. hebraeum.* To date, no homologues of voraxinβ have been reported. However, among tick species in which partially fed virgin ticks do not engorge fully, there is substantial inter-specific variability in the extent to which they feed. For example, the approximate maximum virgin-to-mated weight ratio has been reported for laboratory-reared ticks as follows: *A. americanum* (~5%), *A. hebraeum* (~10% for the vast majority of virgins and ~20% for a small minority), *R. sanguineus* (~17%), *D. andersoni* (~34%), *D. variabilis* (~35%), and *Hyalomma anatolicum* (up to 39%) (reviewed by Kaufman, 2007). In *A. hebraeum*, physiological and behavioural changes were observed to occur when females exceed ~10X the unfed weight, a transition that has been called the critical weight (CW; Harris & Kaufman, 1984). Below the CW, (1) females will reattach to a host if given the opportunity, (2) the salivary glands will not undergo autolysis within 4 days (mated) or 8 days (virgin), and (3) vitellogenesis is curtailed and oviposition does not occur. Females above the CW (1) will not reattach to a host if given the opportunity, (2) the salivary glands undergo degeneration (4 days for mated and 8 days for virgins) and (3) vitellogenesis and oviposition occurs (Kaufman & Lomas, 1996; Lomas & Kaufman, 1999). The exact value of the CW depends on which parameter is used to measure it. Thus, the CW as measured by reluctance to reattach to the host was 9X the unfed weight, while for haemolymph ecdysteroid titer, salivary gland degeneration, ovary weight, oocyte length and oocyte vitellin

content the CW was 10X, 10X, 12X, 12X and 13X, respectively (Weiss & Kaufman, 2001). The biological significance of these small but distinct differences is not known. Although in *A. hebraeum*, virgin females rarely feed above the minimum weight for laying eggs, this is not the case for at least some other species. Virgin *D. variabilis* and *D. andersoni*, which normally feed to ~35 X the unfed weight, do lay (infertile) eggs at that size (personal communications from: Dr. Dan Sonenshine, Old Dominion University, Norfolk Virginia, USA for *D. variabilis* and Dr. Tim Lysyk, Agriculture and Agri-Food Canada, Lethbridge Alberta, for *D. andersoni*).

The effects of silencing the subolesin gene has been tested in *I. scapularis, A. americanum*, *R. sanguineus*, *D. variabilis*, *D. marginatus* and *R. microplus* (Almazán *et al.*, 2003*a*, 2003*b*, 2005*a*, 2005*b*; de la Fuente *et al.*, 2006*a*, 2006*b*; Kocan *et al.*, 2007; Nijhof *et al.*, 2007). The silencing of subolesin by RNAi resulted in production of sterile males that apparently did not mate successfully with females because these females failed to engorge, complete the rapid stage of engorgement or oviposit (de la Fuente *et al.*, 2006*b*). Immunization of sheep with recombinant *I. scapularis* subolesin reduced larval, nymphal and adult tick infestations, further demonstrating the usefulness of this protein as a candidate vaccine antigen (Almazán *et al.*, 2005*a*, 2005*b*).

In this study, most females that fed together with males, in which subolesin or subolesin+voraxin were silenced by RNAi, failed to engorge (Table 3.2). The females that failed to engorge had unlimited opportunity to pair and copulate, but neither the frequency of pairing nor confirmation of spermatophore

transfer from the male was confirmed. Therefore, the extent to which failure of the females to engorge was due a failure to copulate was not determined. However, a previous study in *D. variabilis* demonstrated that subolesin dsRNA-treated males paired with females at a frequency similar to controls, but spermatophore transfer apparently still did not occur because the females did not engorge and produce egg masses (de la Fuente *et al.*, 2006*b*). Injection of males with subolesin dsRNA in this study caused noticeable histological degeneration of the testis (Fig. 3.3). So even if pairing had occurred, inhibition of sperm production and spermatophore transfer were most likely the main reasons for the deleterious effects we observed here. Males injected with subolesin dsRNA alone suffered significantly higher mortality compared to that suffered by males injected with subolesin+voraxin dsRNA (Table 3.2); we do not know the reason for this difference. One possible explanation is that the simultaneous injection of voraxin dsRNA offered some protection against the deleterious effects of subolesin dsRNA; if so, there is no obvious explanation for the mechanism of the hypothetical protection. The following data are not consistent with this hypothesis, however. Whereas males injected with subolesin or the subolesin+voraxin dsRNA suffered significantly different mortality, the females mated to the males of each group exhibited similar failures to feed to engorgement, lay normal egg masses, and produce viable larvae (Table 3.2). Clearly, more work is required to investigate this matter.

During the second round of male feeding on a tick-naïve rabbit, most females were still unable to feed successfully and oviposit (Table 3.2). One

possibility for this failure could have been the high mortality of the males. Thus there were only two of the nine injected males alive and available for the seven females in the subolesin group, and seven males alive and available for the 14 females in the subolesin+voraxin group. However, the fact that both groups of females fared poorly to a similar degree, even though there were significantly more males available to the latter group of females, leads us to interpret these results as continued efficacy of gene silencing through a second round of feeding.

We expected that silencing of voraxin alone in male *A. hebraeum* would result in an inhibition of engorgement and subsequent oviposition in co-feeding females. However, the mean engorged weight of the females and the mean weight of the egg masses they laid were similar to those of the controls (Table 3.2). In other words, the voraxin genes were still being expressed, notwithstanding treatment with dsRNA on day 1, 2 or 3 of feeding (Table 3.2). Our semi-quantitative RT-PCR results confirmed that the voraxin genes were not silenced (Fig. 3.2), the reason(s) for which are not known. In contrast, expression of subolesin was reduced following semi-quantitative RT-PCR (Fig. 3.2), and subolesin dsRNA-treatment of *A. hebraeum* led to the types of pathological effect shown to be associated with gene silencing in other tick species (de la Fuente *et al.*, 2007).

This study demonstrated the utility of RNAi to study the role of tick genes in tick biology. Although RNAi is not a direct predictor of the success of using gene products in a vaccine formulation, this technique does allow for study of the direct impacts of gene silencing.

Table 3.1 List of oligonucleotide primers used in this study

Primer	Sequence (5'-3')	Purpose	Amplicon Size (bp)
4D8R5	GCTTGCGCAACATTAAAGCGAAC	subolesin cloning primer	466
4D833	TTTGGTCGTACGTAAACTTGACAAATGTG	subolesin cloning primer	
D8AAT75	TAATACGACTCACTATAGGGTACTGACTGGGATCCCCTGCACAGT	subolesin dsRNA synthesis	466
D8AAT73	TAATACGACTCACTATAGGGTACTCAAGCTTGGTGGAGAGCACG	subolesin dsRNA synthesis	
EFa5'	TAATACGACTCACTATAGGGTACTATGTTGATCACCAAGGACCTG	voraxinα dsRNA synthesis	384
EFa3'	TAATACGACTCACTATAGGGTACTCTATCGACCAGTGTCAAGCTC	voraxinα dsRNA synthesis	
EFb5'	TAATACGACTCACTATAGGGTACTATGGCGAAACAGGGACTTCTG	voraxinβ dsRNA synthesis	264
EFb3'	TAATACGACTCACTATAGGGTACTTCACCGCAGGCTCCCCAGG	voraxinβ dsRNA synthesis	
Sub93F	GACTGGGATCCCCTGCACAGTC	measure subolesin transcript levels	419
Sub94R	CAAGCTTGGTGGAGAGCACG	measure subolesin transcript levels	
VorA43R	CCACGCCTTGAGGAAAAGG	measure voraxinα transcript levels	311
VorA44F	GGCCGAGAACCTCGTCAA	measure voraxinα transcript levels	
VorB51R	TGCGGCGCTGGGAGTA	measure voraxinβ transcript levels	323
VorB87F	ATGGCGAAACAGGGACTTCTGAAG	measure voraxinβ transcript levels	
16S89F	CTGCTCAATGATTTTTTAAATTGCTGTGG	measure 16S rRNA transcript levels	456
16S90R	CCGGTCTGAACTCAGATCAAGT	measure 16S rRNA transcript levels	

Table 3.2 Effect of subolesin and voraxin silencing on *A. hebraeum* survival, feeding, and fecundity

dsRNA-treatment of males (and initial n)	Number of males dead/total (and %) after feeding	Number of females fed to repletion/total (and %)	Average weight of females after feeding (mg/tick) ± SEM (n)	Average weight (mg) of egg mass ± SEM (n)	Egg mass (% of female body weight) ± SEM (n)	Number of ticks producing viable larvae/total fed (and %)
1st round of feeding						
Subolesin (40)	21/40 (53%)**	4/39 (10%)**	309 ± 82 (39)**	401 ± 142 (10)**	33 ± 5 (10)**	5/10 (50%)**
Voraxin (48)	0/48 (0%)	47/48 (98%)	1813 ± 100 (48)	885 ± 66 (48)*	48 ± 2 (48)*	47/48 (98%)
Subolesin+Voraxin (44)	3/44 (7%)	0/44 (0%)**	187 ± 24 (44)**	71 ± 10 (11)**	16 ± 2 (11)**	0/10 (0%)**
Control (46)	1/46 (2%)	46/46 (100%)	1945 ± 81 (46)	1069 ± 55 (46)	54 ± 1 (46)	46/46 (100%)
2nd round of feeding						
Subolesin (9)	7/9 (78%)**	2/7 (29%)	701 ± 257 (7)*	248 ± 194 (6)*	17 ± 8 (6)*	1/5 (20%)*
Voraxin (15)	1/15 (7%)	12/14 (86%)	1967 ± 270 (14)	950 ± 174 (14)	40 ± 5 (14)	13/13 (100%)
Subolesin+Voraxin (15)	8/15 (53%)*	0/14 (0%)**	369 ± 69 (14)**	60 ± 16 (7)**	9 ± 2 (7)**	0/6 (0%)**
Control (15)	1/15 (7%)	12/15 (80%)	1844 ± 273 (15)	746 ± 135 (13)	34 ± 6 (13)	11/12 (92%)
2nd feeding of females[#]						
Subolesin (25)	0/25 (0%)	20/25 (80%)	1353 ± 176 (25)	633 ± 107 (22)	35 ± 4 (22)	21/21 (100%)
Subolesin+voraxin (28)	0/28 (0%)	25/28 (89%)	1652 ± 98 (28)	679 ± 77 (28)	38 ± 3 (28)	27/28 (96%)
Pre-fed before injection						
Voraxin - 1 day (15)	1/15 (7%)	15/15 (100%)	1541 ± 88 (15)	759 ± 56 (15)	49 ± 1 (15)	15/15 (100%)
Voraxin - 2 days (15)	0/15 (0%)	15/15 (100%)	1575 ± 180 (15)	837 ± 117 (15)	50 ± 3 (15)	15/15 (100%)
Voraxin - 3 days (14)	0/14 (0%)	14/14 (100%)	1992 ± 194 (14)	1111 ± 116 (14)	54 ± 2 (14)	14/14 (100%)
Control (13)	0/13 (0%)	13/13 (100%)	1952 ± 131 (13)	1045 ± 86 (13)	53 ± 2 (13)	13/13 (100%)

[#]25-28 female ticks that had been previously fed with males injected with subolesin or subolesin+voraxin dsRNAs, and did not enter the rapid phase of feeding, were placed on a fresh, tick naive rabbit and allowed to feed with uninjected males.

* and **: Level of significance with respect to the respective control is indicated: *$0.01 < p < 0.05$, **$p < 0.01$

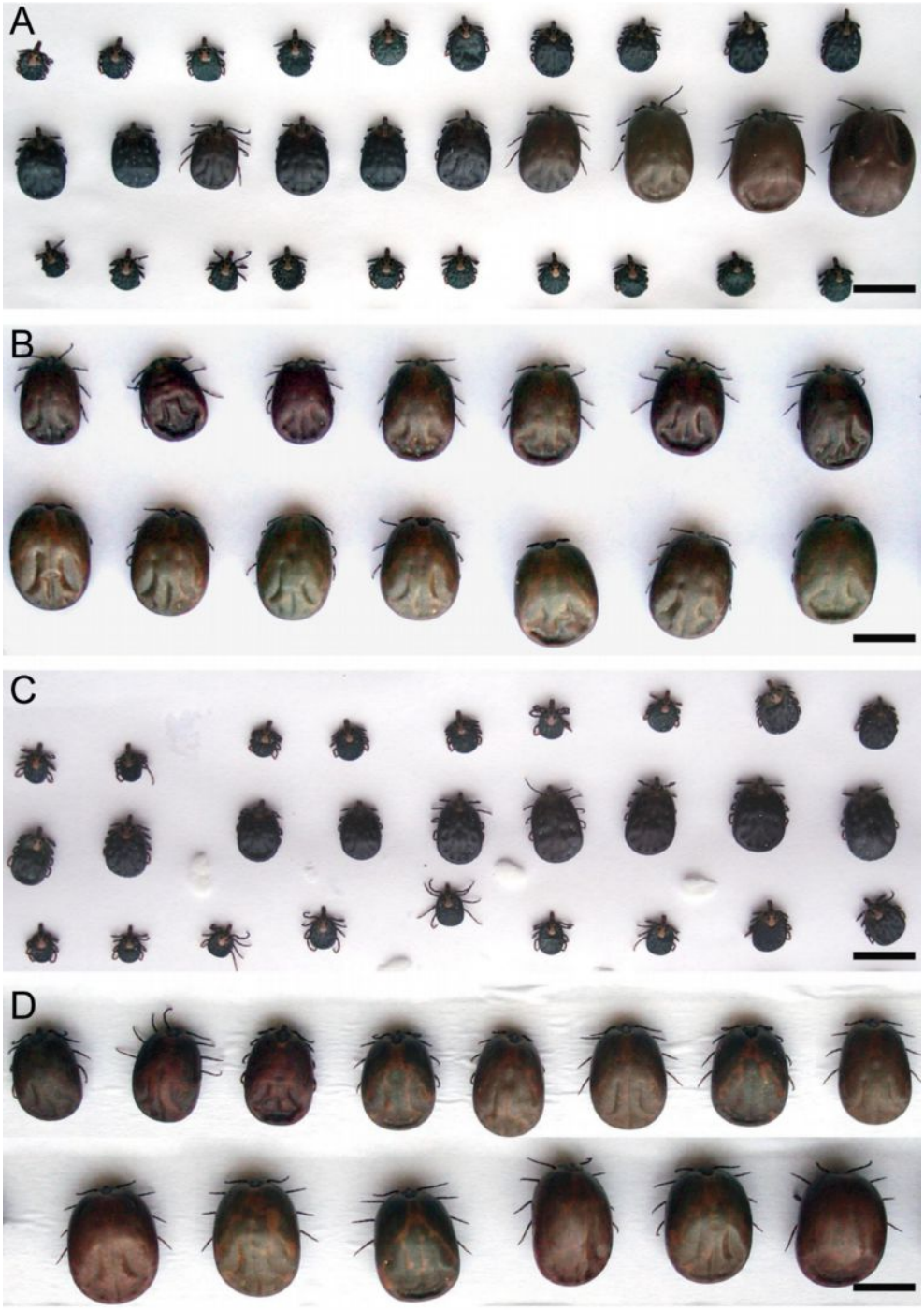

Figure 3.1. Appearance of a representative sample of engorged ticks and of those removed after 20 days of feeding for the four treatment groups: (A) subolesin dsRNA, (B) voraxin dsRNA, (C) subolesin plus voraxin dsRNA, (D) injection-buffer control. Bars in all panels, 1 cm.

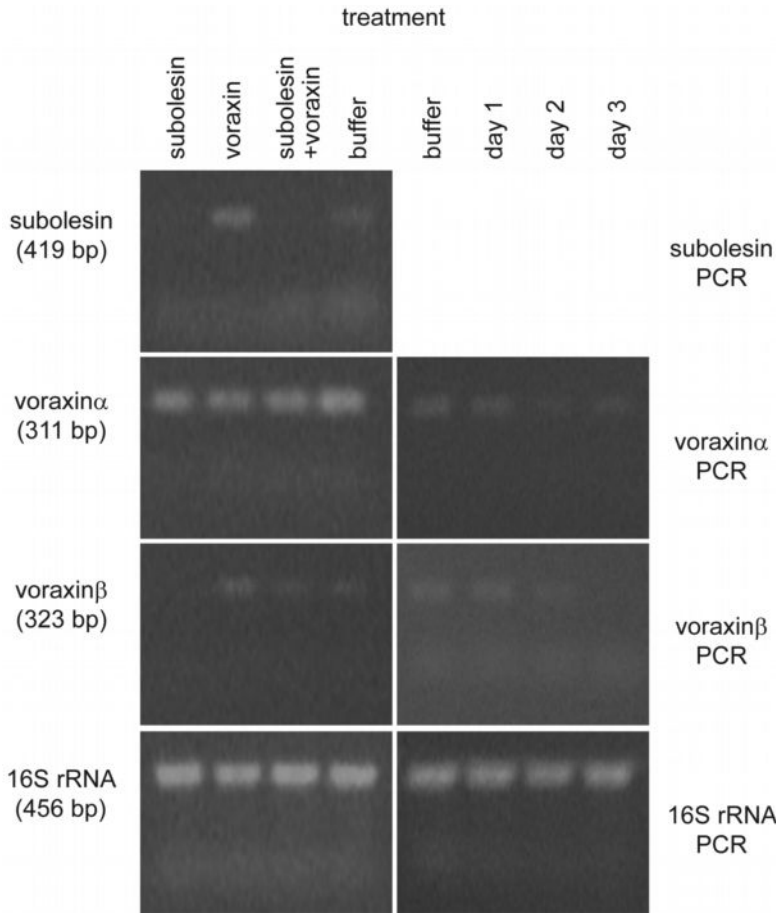

Figure 3.2. Semi-quantitative RT-PCR to determine gene expression levels of subolesin, voraxin and 16S rRNA transcripts. The left column (numbers in parentheses indicate size of gene product amplified by PCR reaction) shows levels of subolesin, voraxinα, voraxinβ or 16S rRNA present in male ticks injected with (top row) subolesin, voraxin, subolesin+voraxin dsRNAs or buffer and allowed to feed for 5 or 10 days. No differences in gene expression levels were observed between males fed for 5 or 10 days after injection (data not shown). The right column shows levels of voraxinα, voraxinβ and 16S rRNA present in male ticks that were injected with voraxin dsRNA or buffer after 1, 2 or 3 days of feeding, then allowed to feed a further 18 days before being sacrificed for gene level analysis.

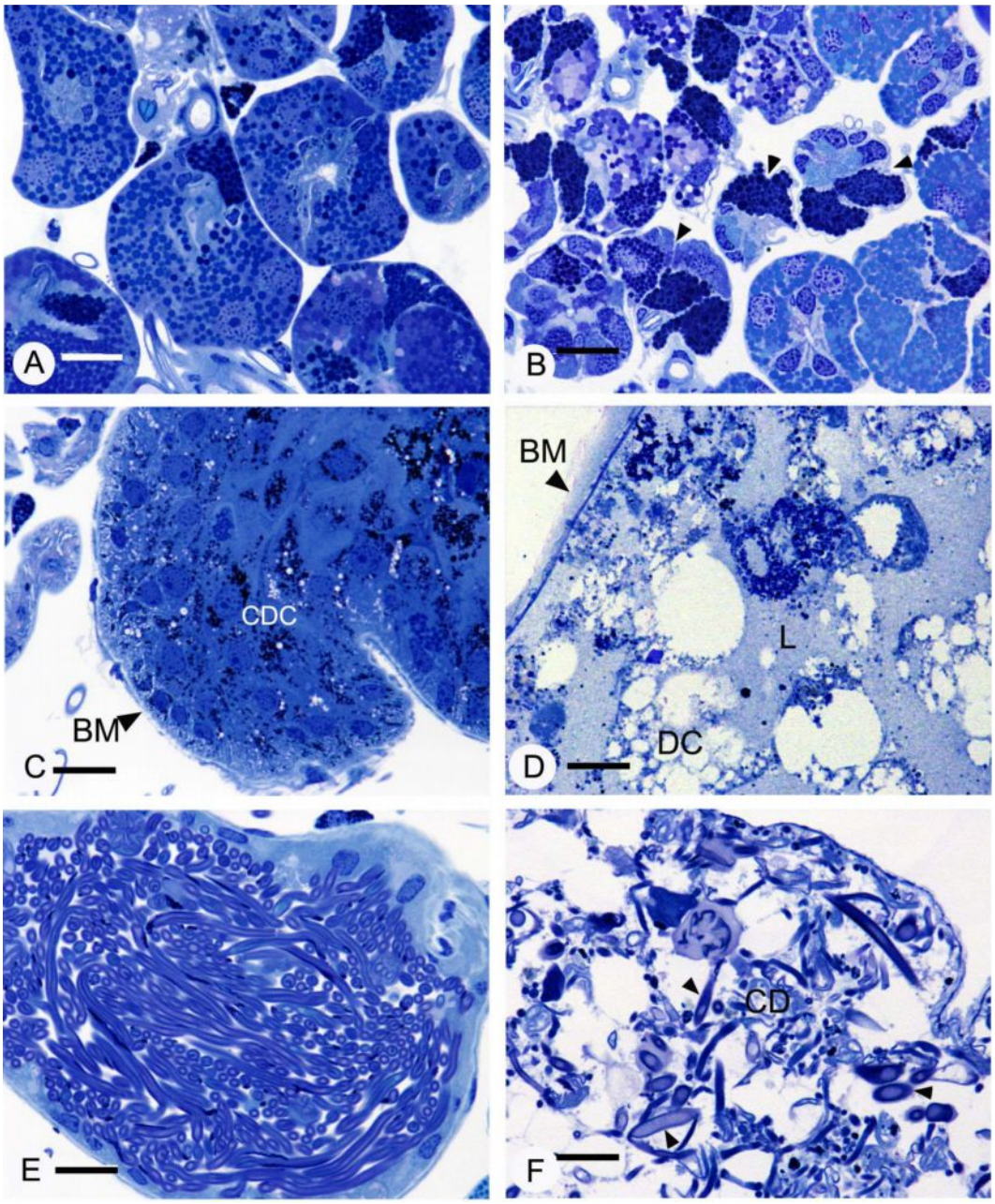

Figure 3.3. Light photomicrographs of 1 µm plastic sections of salivary gland, midgut, and testis from male *A. hebraeum*. Photomicrographs are from representative ticks injected with subolesin dsRNA (n = 6), subolesin+voraxin dsRNAs (n = 7) or injection buffer alone (n = 10). In contrast to salivary glands from the control (injection buffer) ticks (A), the salivary gland acini from subolesin dsRNA treated ticks (B) were noticeably smaller, and many acini were

crenated as evidenced by the smaller size and scalloped edges (small arrows). Differences in granule structure were not apparent between the granular cells of control and treated ticks. In the control midgut (C) large columnar digestive cells (CDC) were situated along the midgut basement membrane (BM), and contained numerous dark hematin granules. Midguts from subolesin or subolesin+voraxin silenced ticks (D) were largely filled with sloughed and degenerating cells (DC), many of which were not attached to the basement membrane (BM). These cells filled the midgut lumen (L) along with debris. The control testis (E) was packed with large, mature prospermia. In contrast (F) testis from subolesin and subolesin+voraxin silenced males contained only scattered prospermia (small arrows) which appeared to be deformed. Cellular debris (CD) was seen surrounding the prospermia. All bars, 20 μm.

3.5 Literature cited

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Chapter 4. Developmental abnormalities and parthenogenesis in the South African bont tick, *Amblyomma hebraeum*

4.1 Introduction

Since Neumann's (1899) initial description, there have been numerous reports of morphological abnormalities in ticks, both naturally occurring and experimentally induced. Campana-Rouget (1947, 1959a, 1959b) compiled an extensive list of teratologies, including the malformation of the legs, capitulum, scutum, spiracular and adanal plates, as well as gross morphological changes such as twinning. To date, the most severe malformations have been reported from laboratory colonies; only few abnormalities have been reported in field-collected ticks (Estrada-Peña, 2001; Dergousoff & Chilton, 2007). The reason for this disparity is probably due to the high degree of inbreeding found in laboratory colonies, with the use of non-natural hosts and environmental conditions also possibly playing a role. In addition to the latter abnormalities, more than 60 cases of gynandromorphism have been reported in various species of *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus* (Gothe, 1967; Rechav, 1977; Clarke & Rechav, 1992; Guglielmone *et al.*, 1999; Labruna *et al.*, 2000, 2002).

With the exception of some *Ixodes* spp., bisexual ixodid ticks copulate only during the feeding cycle, both sexes requiring several days of feeding in order to complete gonad maturation (Khalil, 1970). Virgin females, on the other hand, will remain attached to the host indefinitely, and in the case of *Amblyomma hebraeum*, rarely feed beyond 10X their unfed weight. The 5 – 15% minority that

do feed beyond 10X, rarely exceed 20X their unfed weight (Kaufman & Lomas, 1996). However, there also exist several parthenogenic species. *A. rotundatum* is an obligatory parthenogenetic species, with only three male specimens having ever been collected (Labruna *et al.*, 2005). *Haemaphysalis longicornis* consists of three 'races' – a diploid bisexual race, a triploid obligatory parthenogenetic race, and an aneuploid race capable of reproducing both bisexually and parthenogenetically (Oliver Jr. *et al.*, 1973). Parthenogenic reproduction also occurs sporadically and at low frequency in a number of argasid ticks such as *Ornithodoros moubata*, *O. tholozani*, and *O. verrucosus*, as well as in a number of ixodid ticks, such as *D. variabilis*, *R. bursa*, *R. microplus*, *Hyaloma anatolicum*, and *A. dissimile* (Davis, 1951; Oliver Jr., 1971, 1989; Balashov, 1972; Homsher *et al.*, 1984). However, the few larvae produced are generally weak, unable to feed and soon die (Oliver Jr., 1989).

Although a wide range of gross morphological defects and gynandromorphs have been reported in *A. hebraeum*, to this time parthenogenesis has not. The purpose of this study was to characterize and record the increasingly frequent appearance of ticks possessing a range of morphological abnormalities in our laboratory colony of *A. hebraeum*, as well as the appearance of a short-lived parthenogenic strain. Although there have been multiple previous reports of gynandromorphism in this species (see above), and one case was discovered in our lab a few years ago (Kaufman, unpublished), no obvious gynandromorphs were observed in the 1181 ticks examined in this study.

4.2 Materials and methods

4.2.1 Tick rearing and feeding

Both adult and immature ticks were reared as outlined in Chapter 2. Within several weeks of the moult to the adult stage, ticks were examined to identify morphological abnormalities, sorted by sex, and weighed. Tick morphology was examined and photographed using a stereomicroscope and digital camera. Much of this work, as well as a substantial amount of the photography of malformed ticks were performed by, or with the assistance of, Mr. Andrew Hulleman, an undergraduate student who was conducting a research project in our laboratory at the time.

4.2.2 Statistical analysis

All statistical analyses were performed using Stata 10.0 software (StataCorp, College Station, TX, USA). Differences between groups were analyzed by oneway ANOVA; post hoc comparisons were achieved using the Bonferroni multiple comparison test. Differences in sex ratios were determined using the chi-squared goodness of fit test. The various malformations were examined using pairwise correlations and the Bonferroni adjustment to calculate significance levels.

4.3 Results

4.3.1 Gross morphological abnormalities

Of the 1181 OC adult ticks examined for morphological abnormalities shortly after moulting, 513 ticks (43%) displayed easily distinguishable

morphological defects. Approximately 49% of female ticks exhibited some degree of morphological abnormality, whereas significantly fewer males (36%, $p=0.011$) exhibited any detectable morphological defects. The nymphs had been fed on one of two rabbits, K1-4 and K1-6, with a total of three vials of nymphs placed on K1-4 and four vials placed on K1-6, each vial containing approximately 200 – 500 nymphs. There were significantly more deformed ticks obtained from the nymphs fed on rabbit K1-6 than K1-4 ($p<0.000$). Of the 415 adults derived from the nymphs fed on K1-4, 121 (29%) displayed one or more morphological abnormalities, whereas 345 (45%) of the 766 adults obtained from K1-6 exhibited some degree of deformation. The ticks fed on K1-6 also had significantly more defects per tick on average (2.2 deformities/tick) compared to those fed on K1-4 (1.8 deformities/tick; $p<0.000$).

On average, females weighed significantly more than males (26.8 ± 0.3 mg vs 22.2 ± 0.3 mg, respectively, $p<0.000$), regardless of the particular host used for nymphal feeding. Males with morphological defects weighed significantly less than did normal males (21.0 ± 0.4 mg vs 22.8 ± 0.4 mg, respectively, $p=0.002$), but this was not the case for females (27.3 ± 0.4 mg vs 26.3 ± 0.4 mg for normal and deformed females, respectively). OC unfed females weighed significantly more than the NC females (26.8 ± 0.3 mg vs 23.4 ± 1.1 mg, respectively, $p= 0.021$), but the average weight of unfed males was not significantly different between the OC and NC ticks (22.2 ± 0.3 mg vs 20.9 ± 0.9 mg, respectively, $p= 0.302$). It has been recently noticed that a large number of our NC ticks bear one or more morphological abnormalities; however, the degree to which this colony is

malformed has not been quantified.

The most common class of morphological defect was malformation of the legs, comprising ~89% of all defects (morphologically normal adults can be seen in Fig. 4.1, with those exhibiting deformations of the legs displayed in Fig. 4.2). The observed defects ranged in severity from the complete absence of one or more legs, including the coxa (Fig. 4.2 A & D), to missing one or more of a given leg's articles, but retaining the coxa (Fig. 4.2 A, B & F). Of these, the most common malformation was the loss of a portion of one or more legs (~68% of limb defects), with the complete absence of the limb being the second most common limb defect (~29% of limb defects). The third most common type of limb malformation were joint defects, comprising ~3% of all limb malformations, which generally resulted in one or more articles appearing as though they were attached backwards to the leg (Fig. 4.2 E). Other malformations such as dwarfism of one or more limbs, making it appear as though one or more nymphal limbs had been retained after moulting (Fig. 4.2 C); gigantism of a portion of a limb (Fig. 4.2 E); and one instance of limb bifurcation (photograph lost) were also observed, but much less common, making up <1% of all observed limb defects. Also included in this category were instances in which the cuticle comprising the distal portion of a limb was discoloured, extremely dry, and fragile (Fig 4.2 F). In these instances, the discoloured sections would frequently break off, causing the tick to appear to have lost the distal articles of the leg. After this section would break off, it was not always possible to tell that the limb previously possessed this fragile region. As such, it is possible that a number of ticks counted as missing a portion

of a limb, actually belonged to this category. There was a correlation between defects of right legs III and IV ($p=0.0018$); if one was deformed, the other was also likely to be deformed, but there were no other significant correlations among the defects of the other legs.

The second most common class of morphological abnormality, comprising ~10% of all malformations, affected the capitulum (Fig. 4.3). Many instances of ticks with shrivelled and/or shortened palps, missing one or more articles, were observed (Fig. 4.3 A – D), as were deformities of the hypostome, chelicerae and basis capitulum (Fig. 4.3 D). Defects in a particular palp, were positively correlated with defects, in leg I on the same side of the body ($p<0.000$).

The least commonly observed class of defects, at ~2% of all malformations were abnormalities of the cuticle and scutum (Fig. 4.4). *A. hebreaum* is an ornate tick species. Both the males and females display a characteristic patterns of coloured pigment, known as enamel, on the scutum. This is in contrast to many other tick species (including all members of the genus *Ixodes*) whose integument is usually a solid brown colour, lacking any patterns of pigmentation. Normal adult *A. hebraeum* females generally show a conserved pattern of black and gold pigment (Fig. 4.1 C, Fig. 4.4 D). This pattern was somewhat correlated to the size of the female (Fig 4.4 B – D). In particularly small unfed adult females (7 mg), the scutum displayed minimal patterning, showing only a single gold dot (Fig 4.4 B), and was similar in appearance to that of a nymph (Fig 4.4 A). Somewhat larger females (15 mg), showed partial pattern formation (Fig. 4.4 C), but this was not as complex as that seen in fully formed

adults (25 mg, Fig. 4.4 D). Some variation in patterning was also observed in adult females of approximately average size (Fig. 4.4 E). One of the most striking malformations was the presence of scapular spikes – long protrusions of cuticle from the scapular area (Fig. 4.3 C, Fig. 4.4 F & I). These spikes were often associated with the complete absence of leg I and were positively correlated with defects in right leg I ($p=0.0007$) and the right palp ($p=0.0002$), but not left leg I or the left palp ($p=0.3363$ and $p=0.0807$, respectively). Other scutal abnormalities such as discoloured, cracked cuticle (Fig. 4.4 H), or soft translucent areas of cuticle on the dorsal surface of some males (Fig 4.4 I & J) were also observed. When compared to normal males (Fig 4.4 G), some males also had aberrant scutal patterning (Fig 4.4 K & L) and unusually enamelled festoons[7] (Fig. 4.4 I), as well as other cuticular defects, such as wrinkling of the intercervical[8] and central[9] fields, a character not normally present in this species. It should be noted that the 'normal male' also exhibits a slight defect in cuticular patterning, as the two festoons to the right of the midline are partially fused on the dorsal surface, but appear completely separate on the ventral surface (Fig. 4.1 A & B).

I observed one instance of extreme "gluttony" (Fig. 4.5). Generally, when fed to repletion, female *A. hebraeum* will reach approximately 1800 mg to 3000 mg, with ~3500 mg being the approximate maximum. One mated female, however, appeared to reach a very large size by the 16th day of feeding, but did not detach from the host for an additional 6 days, increasing in size each day. After it

7 Small, rectangularly shaped areas, separated by grooves, present along the posterior body margin in the majority of metastriate ticks.

8 A raised region on the central anterior surface of the scutum bounded on either side by the cervical grooves.

9 The region on the central anterior surface of the scutum and conscutum directly posterior to the intercervical field.

spontaneously detached, it was 5306 mg — over twice the weight of an average engorged female (Fig. 4.5 A), and over 200X the weight of a typical unfed female (Fig. 4.5 C); unfortunately, I don't know what the actual fed/unfed ratio was for this tick. In addition to its extreme size, this tick was missing left leg II, with only a small sclerotized point marking the location where the leg should have been (Fig. 4.5 B). The tick was monitored for egg production, but did not lay any eggs, dying approximately 28 days post engorgement. Upon dissection, the body was filled with a large amount of fluid, and the various organs small and discoloured, though still intact and, in some cases, bulging with fluids. This tick's large size and the excessive amount of fluids present suggest that it may have had a non-obviously visible defect in its salivary glands, preventing it from removing excess fluids taken in with the blood meal during feeding.

In one instance, a fed male was completely missing left leg III, but upon dissection, a hard sclerotized mass was found inside the tick, at the site corresponding to the location of the missing leg (Fig. 4.6 A). This phenomenon was not limited to *A. hebraeum*; a hard sclerotized mass was also found within a *D. andersoni* male missing right leg IV at that leg's corresponding internal attachment position (Fig. 4.6 B). The sclerotized mass was loosely attached to the body wall by muscle and fat body and was easily removed, revealing smooth cuticle below (Fig 4.6 C). Generally, only exterior morphological abnormalities were noticed, although occasionally I observed some internal defects even in ticks appearing outwardly normal. On a few occasions in both *A. hebraeum* and *D. andersoni*, the tick possessed only a single salivary gland; the space where the

second should have been was occupied by fat body.

4.3.2 Parthenogenesis

Although virgin female *A. hebraeum* do not normally feed above the critical weight (CW), three of them engorged (2116 mg, 1689 mg, and 1307 mg) and laid viable eggs (5.2%, 51%, and 45% of female body weight (bw), respectively) that hatched into apparently healthy larvae. Neither the engorged weight (p=0.989) nor the weight of the egg mass as %bw (p=0.454) were significantly different from those of normally mated females[10]. Larvae from one of the females were placed on a tick-naïve rabbit and subsequently fed, engorged and moulted into healthy nymphs. These nymphs were placed on another tick-naïve rabbit, fed and in due course moulted into 125 apparently morphologically normal females. Only 125 female adults were ultimately obtained from a large number of larvae, because many of those larvae died before being offered a chance to feed. No immediate physiological or behavioural differences were apparent when compared to adults derived from eggs of normally mated females. These F1 parthenogenetic females weighed significantly less than normal bisexual females (24.5 ± 0.62 mg vs 27.3 ± 0.40 mg, respectively; p=0.001). Of 30 females allowed to feed on a rabbit in the absence of males, four engorged (1186 ± 334 mg) and laid eggs (14.2 ± 4.5% bw), none of which hatched. Fig. 4.7 shows a diagram of the observed life cycle. The females remaining on the host did not significantly increase in weight from their unfed states (p=1.000). After placing 30

10 The "normal engorged ticks" referred to in this section is a single population of 66 females to which all others are compared. Eggs produced by 20 of these females were used to obtain the mean egg mass for this group. Average female weight for this group was 1504 ± 66 mg and the average egg mass as %bw was 47 ± 2.3%.

females on a rabbit with males present, but enclosed in a cloth bag to prevent mating, only 2 fed above the CW (1005 mg and 423 mg), and of which only one produced any eggs, none of which hatched. When 10 parthenogenic females were given the opportunity to co-feed and mate with males, four fed above the critical weight (631 ± 69.2 mg) and three produced eggs (21.8 ± 3.0 % bw), none of which hatched. Unlike their parent generation, the average engorged weight of the F1 females was significantly less than that of normal mated females ($p=0.004$), as was the average egg mass size as a proportion of female body weight ($p<0.000$).

A separate incidence of spontaneous virgin female engorgement was noted on a different rabbit, 1 year after the first engorged virgin females were discovered. Two virgin females engorged (2168 mg and 1225 mg) and laid eggs (57.8% and 21.2% bw) that successfully hatched to larvae. When the seminal receptacles of the females were examined approximately 35 days following engorgement, there was no evidence of a spent spermatophore. Similar to the initial instance of parthenogenesis observed in this colony, neither the engorged weight nor the egg mass produced as a proportion of body weight were significantly different from those of normally mated females ($p=0.997$ and $p=0.959$ respectively). No attempt was made to feed the larvae produced from these virgin eggs.

4.4 Discussion

Although various morphological abnormalities have been reported for many tick species, the shear number of ticks reported here with some manner of defect(s) is unusual. Although a greater number of ticks with morphological

abnormalities were recovered from the rabbit used to feed the larger number of nymphs, no definitive conclusion can be made with regard to the effects of tick load on the development of morphological defects, because all of the data in this study come from only two rabbits. Latif *et al.* (1988) noted that immature ticks fed on a host that had acquired resistance to tick infestation exhibited an altered scutal morphology in later developmental stages, in addition to generally decreased feeding and reproductive success. Following from this, it is possible that the use of a non-native host such as *Oryctolagus cuniculus*, which may be more sensitive to tick infestation than a native host-species would be, could have contributed to the appearance of the morphological defects observed. However, this seems unlikely due to the fact that our lab has always used *O. cuniculus* for tick feeding and the extensive morphological abnormalities reported here have not been previously observed in our lab.

Buczek (2000) examined the eggs and larvae of *Hyalomma marginatum* ssp. (a tick generally found in dry savannah regions) for morphological abnormalities, after maintaining the developing embryos at their preferred RH of ~75% or at RH 90%. Approximately 13% of embryos maintained at 90% RH died during embryonic development, and a further 7% of the embryos didn't hatch, the process halting following the initial cracking of the eggshell or during the emergence of various body parts. The 90% RH embryos had significantly decreased hatching success (78%) compared to the embryos reared at 75% RH (88%); and those 90% RH embryos that did hatch, 0.2% displayed various morphological abnormalities, most commonly affecting the legs (Buczek, 2000).

Buczek suggested that low RH adversely affects formation of the blastoderm and metamerization of the germ band, which causes morphological anomalies in various appendages. In other ixodid species, which prefer higher RH, such as *I. rubicundus* (Van Der Lingen *et al.*, 1999), *R. microplus* (Sutherst & Bourne, 2006), *I. pacificus* (Peavey & Lane, 1996), *H. longicornis*, *I. holocyclus*, and *R. sanguineus* (Heath, 1979), both low RH and temperatures affect hatching success. Embryogenesis in many tick species is similar, differing primarily in the duration of the pre-hatch period and the mechanisms of germinal band contraction (Buczek, 2000). It is thus conceivable that such factors could affect morphological development. Immature and adult *A. hebraeum* require high RH for long-term survival (Norval, 1977). However, all of our ticks were maintained at a RH >95%, so the malformations I saw could not have been caused by low RH.

Larvae and nymphs were not examined for morphological abnormalities, making it difficult to determine whether the defects were due to genetic mutations, external conditions, or some combination of the two. Young *et al.* (1995) reported that in a laboratory population of *R. appendiculatus*, inbreeding resulted in reduced feeding capacity and various leg defects. It is likely that at least some proportion of the abnormalities I observed are due to extensive inbreeding in our lab colony. Inbreeding resulted from the small number of ticks used to generate the colony, and the difficulty and expense involved in importing wild ticks. Moreover, at one point an incubator malfunction caused the majority of our colony to die, perhaps further magnifying the genetic bottleneck.

Whereas sporadic instances of parthenogenesis have been reported in a

number of normally bisexual species (Oliver Jr., 1971, 1989; Balashov, 1972; Oliver Jr. & Pound, 1985), this is apparently the first reported incidence of parthenogenesis in a population of *A. hebraeum*. A total of five virgin females engorged and produced viable egg masses, some of which were successfully reared to adulthood, with all the resulting adults being female. These females were significantly smaller than other, non-parthenogenically derived females, similar to thelytokus (a type of parthenogenesis that produces only females) *D. variabilis* (Homsher *et al.*, 1984). The few that engorged and produced eggs, attained a smaller fed weight and produced significantly smaller egg masses, even when normalized to engorged bw, than did normally mated females. It is particularly interesting that whereas the five P1 females laid only small clutches of eggs, they all had a very high (>90%) hatch rate. In contrast, in both *A. cajennense* (Gunn & Hilburn, 1991) and *D. variabilis* (Homsher *et al.*, 1984), only a very small proportion of the eggs produced by virgin females gave rise to larvae. These were generally weak, with only a small number surviving and feeding; this phenomenon also occurs in a number of other species such as *Ornithodoros moubata*, *O. tholozani*, *O. verrucosus*, *R. microplus*, *R. bursa*, *H. anatolicum*, and *A. dissimile* (Oliver Jr., 1989). However, the percentage of eggs that hatch from a particular parthenogenic strain can be quite variable. In *D. variabilis*, hatching percentages from virgin eggs ranged from 1.5% to 50% (Nagar, 1967; Gladney & Dawkins, 1971; Homsher *et al.*, 1984). In this study, I observed a high hatching percentage among the eggs produced by the P1 females; however, none of ticks from the F1 generation reared from the eggs of one of these females produced

viable eggs.

The mechanism used to produce parthenogenic offspring is unknown. The obligate thelytokous race of *H. longicornis* is known to be triploid, containing ~30 – 35 chromosomes whereas an aneuploid race of this species contains ~22 – 28 chromosomes and is able to reproduce both bisexually and parthenogenetically (Oliver Jr. *et al.*, 1973). There is also a bisexual species that is diploid with an XO-XX sex determining system, possessing 21 and 22 chromosomes in the males and females respectively (Oliver Jr., 1977). It is uncertain whether the triploid thelytokous *H. longicornis* reproduce via apomictic parthenogenesis, in which the mature eggs are produced directly through mitotic divisions without entry into meiosis, or are automictic, a process in which meiosis is entered and ploidy restored by fusion of the products of anaphase I or II (Oliver Jr. *et al.*, 1973). However, it is thought that the aneuploid race is automictic, as hybridization experiments between aneuploid females and bisexual males resulted in offspring that were generally diploid (Oliver Jr. *et al.*, 1973). Homsher *et al.* (1984) speculated that the high number of morphological abnormalities in the F1 generation of their thelytokus *D. variabilis* indicated that they likely possessed genetic differences from their progenitors, and as such were probably automictic. This is different from what I observed in *A. hebraeum*, where all the F1 thelytokus females appeared morphologically normal, suggesting that they may have been the result of an apomictic process.

It has been suggested that the genes for thelytoky may occur at low frequency in some *Ambylomma* spp. (Gladney & Dawkins, 1973) and that the

distribution of these genes may be highly variable within a population (Oliver Jr., 1981). This would lead to a relatively higher (or lower) incidence of parthenogenesis in an inbred line compared to a larger population(Gunn & Hilburn, 1991).

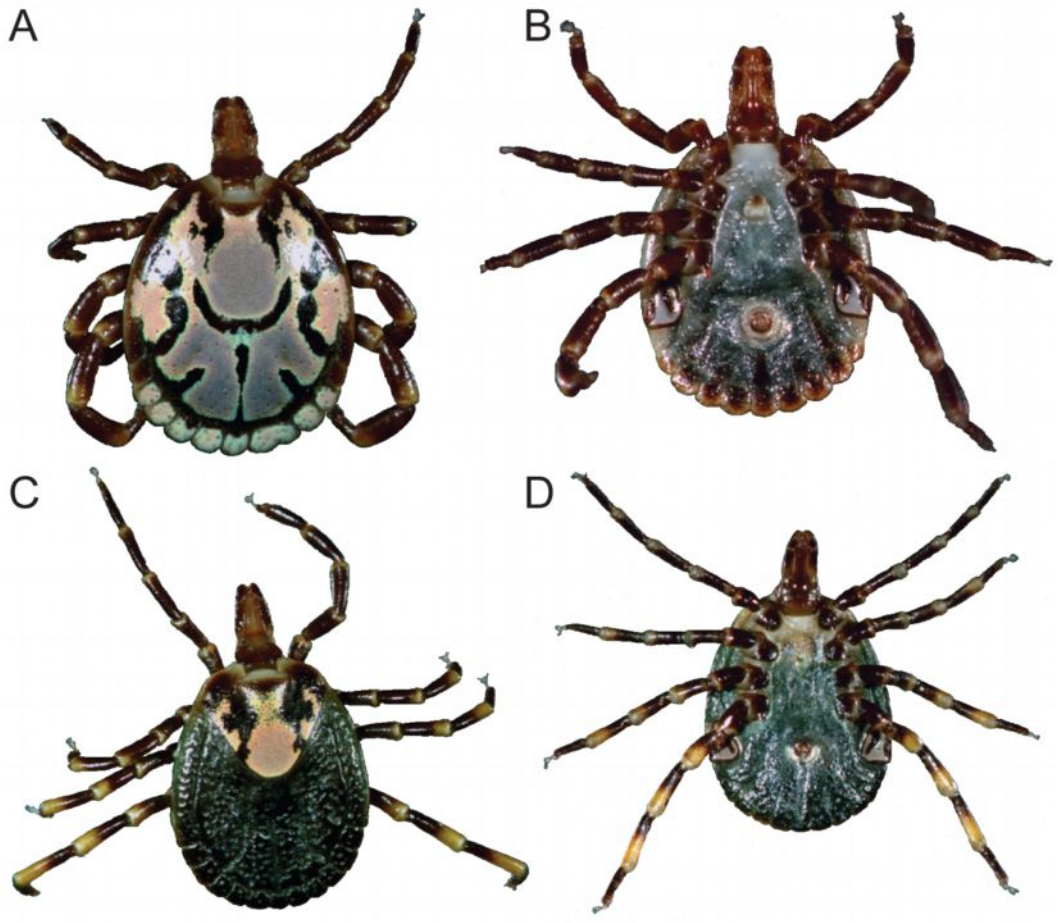

Figure 4.1. Normal unfed adult *A. hebraeum.* (A) Male dorsal aspect, (B) male ventral aspect, (C) female dorsal aspect, and (D) female ventral aspect. In all subsequent images, “right” and “left” refer to the tick's perspective.

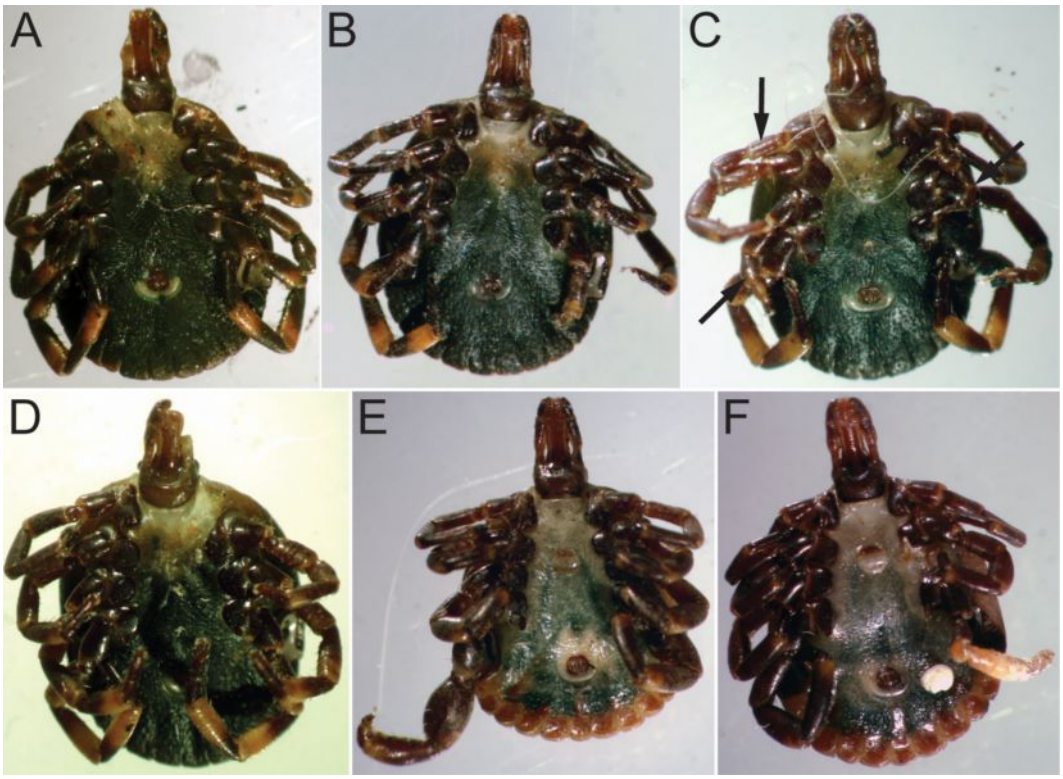

Figure 4.2. Some examples of leg abnormalities in unfed *A. hebraeum*. (A) Female exhibiting multiple abnormalities including a shortened right palp, the complete absence of right leg I, and the loss of all but the coxa of left leg I; (B) female missing parts of the left leg IV distal from the femur; (C) female possessing three abnormally small, nymph-like legs: left legs I and III, and right leg II, marked with arrows; (D) female missing left leg I, and possessing a shortened right palp; (E) male showing gigantism of right leg IV; (F) male with a complete absence of left leg III, a malformed joint between the femur and patella of left leg IV, and a high degree of whitened, brittle cuticle from the patella onwards, on the same leg.

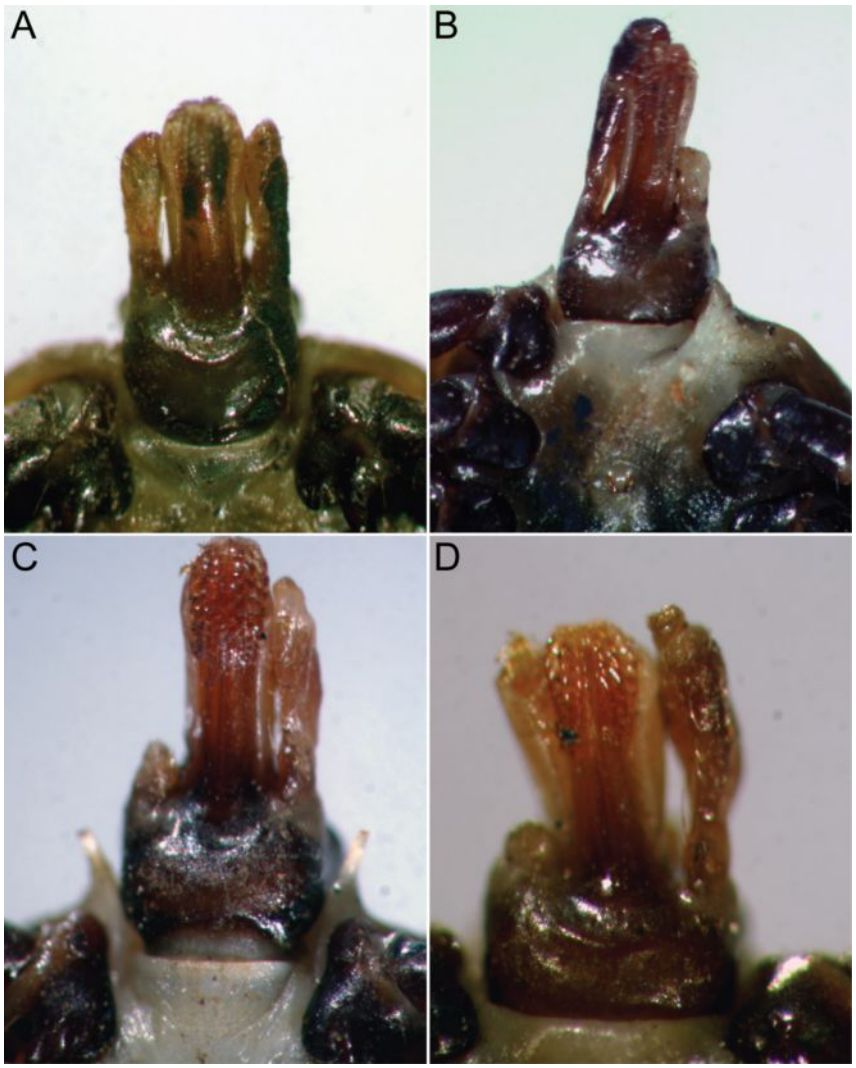

Figure 4.3. Ventral aspect of the capitulum of several abnormal unfed adults. (A) Female with abnormal shortening of both palps, missing articles three and four from each palp; (B) female missing all but a portion of the second article of the left palp, as well as missing left leg I in its entirety; (C) male missing the second through fourth articles of the right palp, portions of the third and all of the fourth article of the left palp in addition to exhibiting small scapular spikes; (D) female displaying a malformation of the basis capitulum, the absence of the second through fourth articles of the right palp, as well as a malformed right cheliceral sheath that extends to the right of the hypostome.

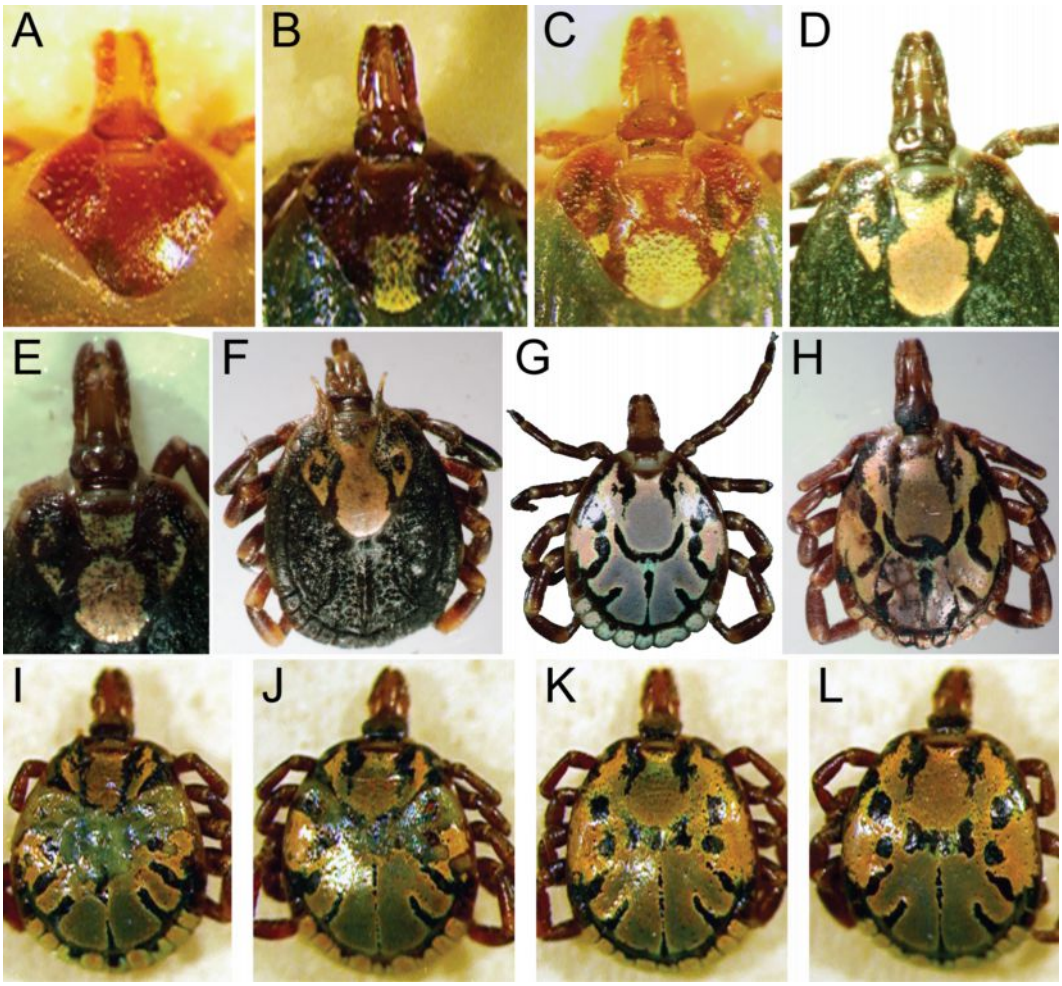

Figure 4.4. Abnormalities and variation in scutal patterning. (A-E) A comparison of the scuta of (A) a normal partially fed nymph, (B) a small 7 mg adult female, (C) a 12 mg adult female, (D) a normal 25 mg adult female; (E) an unfed adult female with unusual scutal patterning; (F) an adult female with malformed, shortened palps and chelicerae displaying prominent spikes extending from the scapulae as well as discoloured cuticle at the anterior edge of the scutum; (G) Dorsal aspect of morphologically normal male; (H) male displaying a region of cracked and discoloured cuticle near the posterior of the scutum; (I) male with a region of abnormally soft, wrinkled, and translucent cuticle, as well as wrinkling of the intercervical and central fields, unusual black markings on three festoons,

and a small spike protruding from the left scapula; (J) male showing an abnormally soft and translucent area of the scutum, as well as a wrinkled intercervical and central fields and unusual posterior ornamentation; (K) male with abnormal scutal ornamentation, distinct wrinkles in the central field and depressions on the left side; (L) male showing abnormal scutal ornamentation.

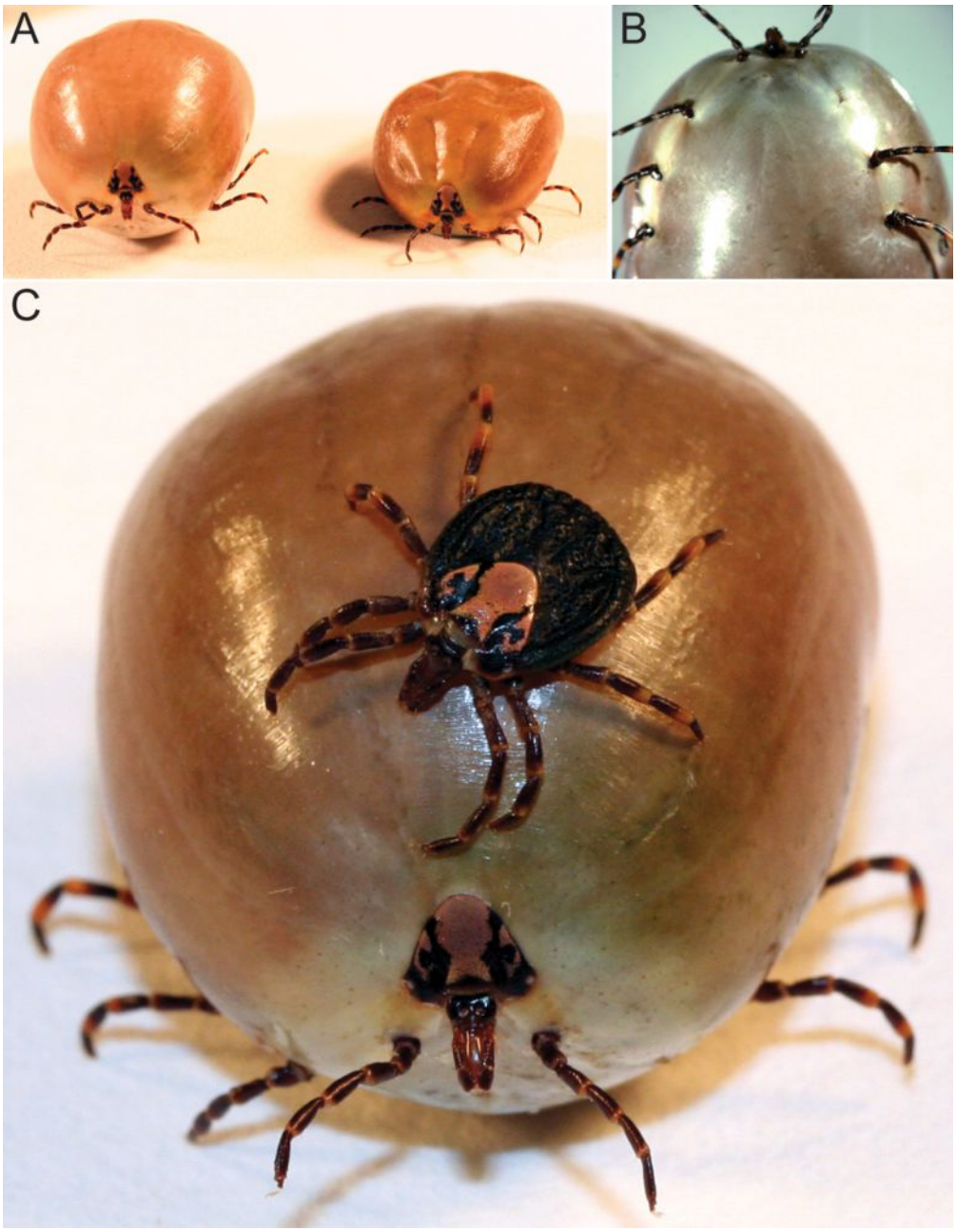

Figure 4.5. (A) An extremely large female (5306 mg) placed beside a very large, ~3500 mg, engorged female. The cuticle of the larger female is noticeably strained, with the sites of muscle attachment nearly invisible, compared to the furrowed sites seen on the smaller female. (B) A view of the ventral surface of the 5306 mg female showing distension of the ventral surface in addition to the missing left leg II. (C) The 5306 mg female with a normal ~ 30 mg unfed female.

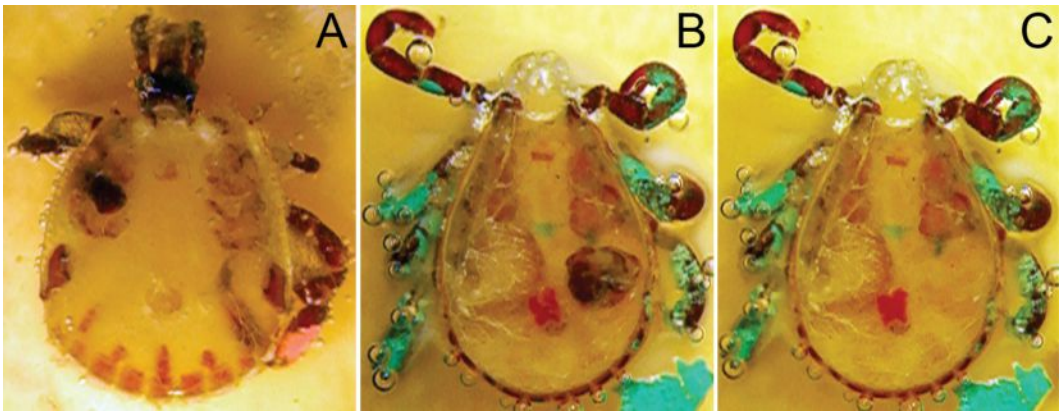

Figure 4.6. Dissected fed male ticks possessing a mass of sclerotized cuticle at a typical site of leg attachment. (A) Male *A. hebraeum* that was missing left leg III contained a hard sclerotized mass internally, at a location corresponding to that of the missing leg. This tick was labelled with pink paint prior to feeding, explaining the spot on its right leg IV. (B) A male *D. andersoni* missing right leg IV possessed a hard sclerotized mass inside the body, at the site of attachment for the absent leg; (C) removal of the cuticular mass revealed that it was attached to they body wall by only muscle and fat body. The mass was not contiguous with the cuticle, which was completely intact, showing no signs of a typical leg attachment point. In both (B) and (C), the green splotches are the remnants of the paint label used to identify the tick during feeding.

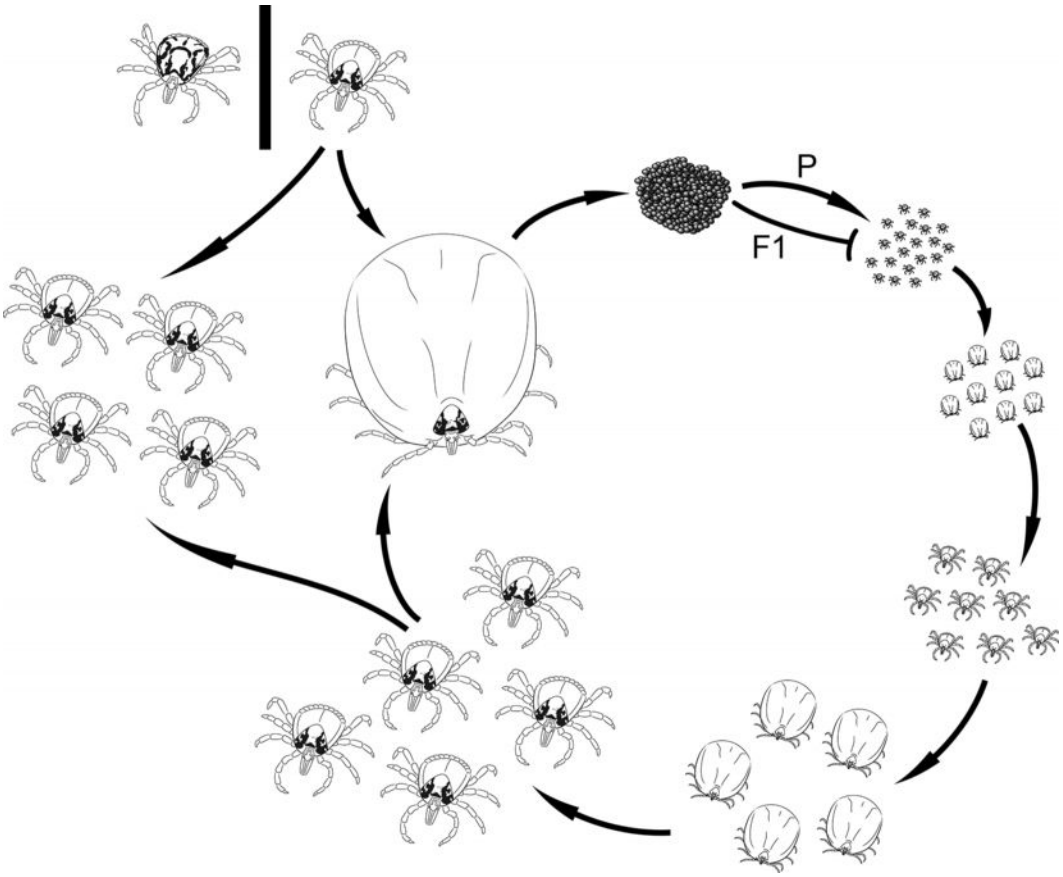

Figure 4.7. Diagram of the life cycle of the short-lived parthenogenic population of *A. hebraeum*. A small number of virgin females of the parental generation (P), fed on tick-naive rabbits, engorged and laid eggs, nearly all of which hatched into larvae. These larvae successfully fed and moulted into nymphs, which were also able to feed to engorgement then moult into adults. All resulting adults were female. Only 125 female adults were ultimately obtained from a large number of larvae, because many of those larvae died before being offered a chance to feed. Mortality during feeding was otherwise similar to that obtained during the feeding of normal bisexually derived ticks. When these females, the F1 generation, were allowed to feed, either with or without males present, only a few engorged and produced eggs, none of which hatched.

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Chapter 5. Identification and localization of a *Coxiella*-like symbiont in the southern African bont tick *Amblyomma hebraeum*

5.1 Introduction

As outlined in Chapter 1, ticks and their pathogens are the cause of numerous medical, veterinary and economic losses around the world. However, ticks are also known to harbour a number of non-pathogenic *Rickettsia*-like or *Wolbachia*-like proteobacteria, some of which may be mutualistic endosymbionts (Noda *et al.*, 1997; Zhong *et al.*, 2007). Most of these studies depended on ultrastructural observations (Suitor Jr. & Weiss, 1961; Hayes & Burgdorfer, 1979; Lewis, 1979; Šuťáková & Řeháček, 1991). However, the increased use of molecular techniques such as PCR amplification and DNA sequencing has expanded our knowledge of the relationships among these microbes (Forsman *et al.*, 1994; Niebylski *et al.*, 1997*b*, 1997*a*; Noda *et al.*, 1997; Beninati *et al.*, 2004; Lo *et al.*, 2006; Jasinskas *et al.*, 2007). The symbiont found in *Argas arboreus* (formerly *A. persicus*), had been classified as *Wolbachia persicus* on the basis of its ultrastructure. But molecular phylogenetic analyses have suggested that this bacterium is related to the genus *Francisella*, a group of γ-proteobacteria, and not other *Wolbachia* spp., which are α-proteobacteria (Suitor Jr. & Weiss, 1961; Forsman *et al.*, 1994; Niebylski *et al.*, 1997*a*; Noda *et al.*, 1997; Sun *et al.*, 2000; Scoles, 2004). Several other tick species, such as *D. andersoni*, *Ornithodoros moubata*, *O. porcinus*, and *A. maculatum* also harbour *Francisella*-like endosymbionts. These symbionts are not passed on to their vertebrate hosts, unlike the pathogenic *F. tularensis*, which some of these ticks do transmit

(Niebylski *et al.*, 1997*a*; Kugeler *et al.*, 2005).

Whereas several symbionts belonging to the α-proteobacteria have been identified, such as the *Rickettsia*-like symbiont in *Ixodes scapularis* (Weller *et al.*, 1998; Lo *et al.*, 2006), and *Midichloria mitochondrii*, a novel α-proteobacterium that resides within the mitochondria of some ixodid ticks (Beninati *et al.*, 2004; Epis *et al.*, 2008), reports remain rare of true *Wolbachia* spp. in ticks. *Wolbachia pipientis* has been reported in several *Ixodes* and *Rhipicephalus* spp. as well as *A. americanum*. However, in each of these cases, the tick population was also heavily parasitized by either a hymenopteran parasitoid (*Ixodiphagus hookeri*, in the case of the *Ixodes* and *Rhipicephalus* spp.) or a filarial nematode (*Acanthocheilonema* sp., in the case of *A. americanum*), so questions remain regarding the pervasiveness and stability of the infection (Tijsse-Klasen *et al.*, 2011; Zhang *et al.*, 2011; Plantard *et al.*, 2012).

The obligate intracellular bacterium, *Coxiella burnetii*, is the causative agent of Query (Q) fever, a zoonotic disease with a worldwide distribution, and is transmitted by numerous tick species (Maurin & Raoult, 1999; Voth & Heinzen, 2007). Human infection with *C. burnetii* is generally asymptomatic, although it may present as a flu-like illness; however, it may also develop into an acute form that manifests as mild to severe atypical pneumonia, or hepatitis (Maurin & Raoult, 1999). Up to 9% of patients may also develop a chronic, debilitating form of the disease after a case of acute Q fever, most commonly manifesting as endocarditis (Fournier *et al.*, 1998*a*; Maurin & Raoult, 1999; Waag, 2007). *C. burnetii* is found in many organisms, with wild rodents, farm animals and pets

demonstrated to be reservoirs and potential sources of outbreaks (Fournier *et al.*, 1998*a*). More than 40 species of ticks also contain *C. burnetii* or other *Coxiella* spp., and ticks have been hypothesized to play a major role in the transmission of coxiellosis among wild vertebrates, especially rodents, lagomorphs and birds (Maurin & Raoult, 1999; Lee *et al.*, 2004; Jasinskas *et al.*, 2007).

This study was prompted by the sudden and unexpected deaths of several rabbits used to feed nymphs from the OC. Histopathological examinations of the rabbit tissues, performed by Dr. Nick Nation of Animal Pathology Services, Ltd. were inconclusive, and I was unable to amplify any bacterial sequences from genomic DNA isolated from blood samples taken from the rabbits post-mortem. As a result of this, this study was undertaken to determine if either our 'old lab colony' (OC) of *Amblyomma hebraeum* (established in 2003 from a colony maintained at the *Université de Neuchâtel* in Switzerland), or a 'new colony' (NC) of the same species (established in 2007 from specimens obtained directly from South Africa) contained any putative pathogens. A combination of general and specific primer pairs were used to detect microorganisms in whole ticks as well as various tissues. The small subunit (16S) rRNA gene sequence was sequenced from multiple specimens and used to identify the bacterial species present, as well as to perform phylogenetic analyses. These analyses revealed the presence of a *Rickettsia* sp., closely related to *R. conorii*, the causative agent of Boutonneuse fever in NC ticks, and a *Coxiella* sp., closely related to both the *A. americanum* and *A. cajennense Coxiella* symbionts, in both our OC and NC ticks. *Coxiella* symbionts have not been previously reported in *A. hebraeum,* However no

putative pathogens that could have been responsible for the observed rabbit deaths were identified.

5.2 Materials and methods

5.2.1 Tick rearing and feeding

Tick rearing and feeding was performed as described in Chapter 2.

5.2.2 Isolation of genomic DNA

Tick genomic DNA was isolated using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's directions from both OC and NC whole unfed male and female ticks as well as eggs, and unfed nymphs. To examine the distribution of the bacterial symbionts in various tissues over time, individual ticks at different feeding stages (unfed, partially fed, day zero post-engorgement and day 10 post-engorgement) were dissected and the midgut, Malpighian tubules, salivary glands, fat body, and ovary or testis/vas deferens harvested for genomic DNA extraction as above.

5.2.3 PCR amplification of bacterial sequences

Genomic DNA isolated from either whole ticks or dissected tissues was used as template for PCR to amplify an ~1200 bp region of eubacterial 16S rDNA (45F: 5'-GCTTAACACATGCAAG-3'; 1242R: 5'-CCATTGTAGCACGTGT-3') or a 456 bp region of *A. hebraeum* 16S rDNA (16S+1F: 5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3'; 16S-1R: 5'-CCGGTCTGAACTCAGATCAAGT-3') as a template control. Cycling conditions for these primers consisted of an initial 5 min denaturation at 94°C followed by 35

cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 1 min, ending with a final extension at 72°C for 10 min.

5.2.4 Cloning and Sequencing of PCR products

The amplified PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and either directly sequenced or sequenced following subcloning into a pGEM-T vector (Promega, Madison, WI, USA). The resulting plasmids were transformed into *E. coli* OmniMAX 2 T1^{R} cells (Invitrogen) by electroporation and the plasmids purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). All PCR amplicons and plasmids were sequenced using BigDye® Terminator v3.1 Cycle Sequencing mix (Applied Biosystems, Foster City, CA, USA) and either gene specific primers (above) or primers corresponding to the flanking regions of the plasmid (T7F: 5'-TAATACGACTCACTATAGGG-3'; SP6R: 5'-TATTTAGGTGACACTATAG-3'). Reactions consisted of approximately 70 ng of template PCR DNA or approximately 200 ng of plasmid DNA, 1.6 pmol of primer, 0.875x BigDye dilution buffer and 1 µl BigDye Sequencing premix in a 10 µl reaction volume. Cycling conditions consisted of a 60 s denaturation at 96°C followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min.

5.2.5 Electron microscopy studies

Various organs (midgut, Malpighian tubule, salivary gland, synganglion, trachea, ovary, accessory gland and testis) were collected from fed males and

partially fed virgin females. Tissues were fixed overnight in 2.5% glutaraldehyde, and post-fixed in 2% osmic acid for 2 h. The samples were dehydrated through a series of gradated ethanol washes, incubated in a propylene oxide and Spurr's resin mixture overnight and embedded in Spurr's resin at 60°C for 3 days. Sections were cut with an ultramicrotome (Reichert-Jung), stained with uranyl acetate and Reynold's lead citrate and imaged using a Morgagni 268 transmission electron microscope.

5.2.6 Sequence and phylogenetic analyses

Sequences were assembled using GeneTool (Biotools, Inc. Edmonton, Canada) after the removal of all primer and vector sequences from the raw sequence data, then compared against the non-redundant GenBank nucleotide database using BLASTN (www.ncbi.nlm.nih.gov) searches (Altschul *et al.*, 1997) to determine the closest related species. Homology of the eubacterial 16S sequences were compared to the 16S sequences of other closely related eubacterial species. Sequences were aligned using the multiple sequence alignment program MUSCLE (Edgar, 2004), and phylogenetic relationships inferred using the Molecular Evolutionary Genetics Analysis (MEGA) software package, version 5 (Tamura *et al.*, 2011). Trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) from distance matrices based off of the nucleotide sequences according to the Kimura 2-parameter method (Kimura, 1980). The reliability of the branching-order was assessed using 1000 bootstrapped replicates (Felsenstein, 1985). To construct the trees, all positions containing gaps or missing data were deleted, resulting in final datasets of 690

positions, 757 positions and 1067 positions for the large 52-taxa tree, the 19-taxa *Coxiella* tree and the 13-taxa *Rickettsia* tree respectively. The 16S rDNA gene sequences of various members of the alpha-, beta- and gamma-proteobacteria classes were used for phylogenetic analysis and the gram-positive *Bacillus thuringienis* (D16281) from the phylum Firmicutes, selected as an outgroup (Noda *et al.*, 1997). A full list of the organisms and their accession numbers can be found in Table 5.1.

5.2.7 Nucleotide sequence accession numbers

The nucleotide sequence data reported in this work will be deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers JX846589 (NC *A. hebraeum Coxiella*-like endosymbiont), JX846590 (OC *A. hebraeum Coxiella*-like endosymbiont), and JX846591 (NC *A. hebraeum Rickettsia*-like endosymbiont).

5.3 Results

5.3.1 Life stage and tissue specific PCR amplification of eubacterial 16S rDNA

PCR analysis of tick genomic DNA (gDNA), indicated the presence of a previously unrecognized bacterial symbiont in all examined ticks and tissue samples. Following PCR amplification of the eubacterial 16S rDNA gene, ~1200 bp bands were observed (Fig. 5.1). The amount of amplification appeared lower in both the egg and unfed male samples, compared to both the nymphs and females, as well as compared to the *A. hebraeum* 16S control reactions. Bacterial prevalence was also examined in the midgut, Malpighian tubules, salivary glands,

fat body and reproductive organs of male and female ticks at various stages of feeding (Fig. 5.1). In both unfed males and females, high levels of eubacterial 16S rDNA were observed in the Malpighian tubules, but only negligible levels in all other tissues (Fig. 5.1). Following feeding, males showed low levels of bacteria in all examined tissues, whereas the partially fed females had high levels of bacterial 16S amplification in the midgut and ovary, but lower levels in the fat body or salivary glands. A similar pattern was observed in the females zero and 10 days post-engorgement, with high levels of amplification seen in the ovary, but comparatively lower amounts in the Malpighian tubules, salivary glands and fat body. Interestingly, all the bands amplified from the fed males, the female salivary glands and female fat body were smaller (~1150 bp) than the ~1200 bp bands amplified from the Malpighian tubules or ovaries. These smaller bands were only amplified from NC ticks and were also seen in one NC egg gDNA sample. OC gDNA isolated from the salivary glands, midgut or fat body did not show detectable levels of eubacterial 16S rDNA following PCR amplification, with the exception of one unfed female who exhibited high amounts of eubacterial 16S rDNA in the salivary glands and ovary (~1200 bp bands), but not in other tissues.

5.3.2 Sequencing and phylogenetic analysis of A. hebraeum *symbionts*

Following the sequencing of all eubacterial 16S rDNA bands (from a total of 16 OC and 19 NC individuals), the sequences were aligned and compared to the non-redundant nucleotide database in GenBank by BLASTN homology searches. This revealed the presence of two distinct bacterial species. The 16S rDNA amplified from OC ticks as well as from the Malpighian tubules of unfed

NC males, and the Malpighian tubules and ovaries of unfed, partially fed and engorged NC females, showed 94% identity to *Coxiella burnetii* (D89800), 92% identity to the *Coxiella* symbiont of *A. americanum* (AY939824) and 95% identity to both the Brazilian and US *A. cajennense Coxiella* symbionts (HM133589 and HM133590 respectively). Additionally, the 16S *Coxiella* sequences isolated from OC and NC ticks, though not identical, showed a 99% identity with each other. In all samples examined, there were two sites where their sequences differed (Fig. 5.2). Sequencing of the smaller ~1150 bp 16S rDNA amplified from fed males, the salivary glands and fat bodies of fed females, and one egg sample, revealed the presence of a second bacterial species in NC *A. hebraeum*. BLASTN searches indicated that over the 1070 bp of sequence obtained, the bacteria present in these tissues had 100% identity with *Rickettsia conorii* (L36107), the etiological agent of Mediterranean spotted fever, as well as Strain S, a spotted fever group (SFG) *Rickettsia* isolated from *Rhipicephalus sanguineus* ticks in Armenia (U25042; Eremeeva et al., 1995).

Phylogenetic analysis supports the initial identifications obtained through homology searching, indicating that the *Coxiella*-like symbionts identified in both the old and new colonies form a cluster with other *Coxiella*-like symbionts within the gamma-proteobacteria. In contrast, the *Rickettsia*-like symbiont found in the NC, clusters with other *Rickettsia* spp., which are alpha-proteobacteria (Fig. 5.3A). The two *Coxiella*-like symbiont sequences obtained from the OC and NC form a well-supported clade with the other *Coxiella* symbionts isolated from *A. americanum* and *A. cajennense* (Fig. 5.3B). This clade forms a polytomy with two

other clades of *Coxiella* symbionts isolated from hard ticks: one consisting of the three *H. longicornis Coxiella* symbionts and the *H. shimoga Coxiella* symbiont, and the other comprising the *Coxiella* symbionts of *R. sanguineus* and *H. conncinae*. The *Coxiella* symbionts of these hard ticks form a distinct cluster, separate from the single clade containing both *Coxiella burnetii* and the various *Coxiella* symbionts isolated from four species of soft ticks.

The *Coxiella* symbiont found in the crayfish *Cherax quadricarinatus* is completely separated from the various tick-borne *Coxiella* symbionts and *Coxiella burnetii*. The second, *Rickettsia*-like symbiont found in the NC forms a moderately-supported polytomy with *Rickettsia africae*, *R. conorii*, and the Strain S SFG *Rickettsia* isolated from *Rhipicephalus sanguineus*. This polytomy is separated from *R. rickettsia* and the various *Rickettsia*-like symbionts found in *D. hunteri*, *D. andersoni* and *D. variabilis*, with good support (Fig. 5.3C).

5.3.3 Electron micrography of putative endosymbionts of A. hebraeum

Transmission electron micrography (TEM) of various tissues isolated from OC ticks revealed the presence of potential endosymbiotic bacteria in the salivary gland of an unfed male as well as in the Malpighian tubules of a partially fed virgin female (Fig. 5.4). The putative symbiont located in the salivary gland is approximately 2.3 μm in length, located intracellularly within a vesicle approximately 2.5 μm long. The potential endosymbiont found in the Malpighian tubule was smaller, approximately 1.1 μm long and located within a vesicle approximately 1.4 μm long. Despite strong PCR bands indicating the prevalence of bacteria in several tissues, including the Malpigian tubules, very few putative

bacteria were observed. No other potential endosymbiotic bacteria were observed in midgut, synganglion, trachea, accessory gland, testis or ovary.

5.4 Discussion

As well as serving as vectors for pathogenic microbes, ticks harbour a number of potentially endosymbiotic bacteria. *A. americanum* and *A. cajennense* harbour closely related symbiotic *Coxiella* spp. in all life stages (Jasinskas *et al.*, 2007; Klyachko *et al.*, 2007; Machado-Ferreira *et al.*, 2011). As in other *Amblyomma* spp., the *Coxiella* symbiont of *A. hebraeum* is present in eggs, unfed nymphs, and adults of both sexes at all feeding stages. However, in unfed ticks, the presence of *Coxiella* almost exclusively in the Malpighian tubules is different from what has been observed in either *A. americanum* or *A. cajennense*. Those *Coxiella* symbionts are prominent in the salivary glands and ovary, but are not present in the Malpighian tubules in large numbers (Zhong *et al.*, 2007; Machado-Ferreira *et al.*, 2011). *C. burnetii* is known to infect all of these tissues as well as the midgut and haemolymph (Noda *et al.*, 1997). The reason for the differences in which tissues are most heavily infected is unknown. However, small sample sizes, inter-tick and population-level variation may explain some of these differences.

Multiple pathogenic and potentially symbiotic microbes are vertically transmitted in ticks, including multiple species of *Rickettsia* (Burgdorfer & Brinton, 1975; Macaluso *et al.*, 2002; Stromdahl *et al.*, 2008), *Babesia* (Bock *et al.*, 2004; Bonnet *et al.*, 2007; Boldbaatar *et al.*, 2008), *Borrelia* (Rich *et al.*, 2001), *Coxiella* (Reeves *et al.*, 2006; Klyachko *et al.*, 2007; Machado-Ferreira *et al.*, 2011; Almeida *et al.*, 2012), *Anaplasma* (Baldridge *et al.*, 2009), and

Francisella (Niebylski *et al.*, 1997*a*; Goethert & Telford III, 2005). It should be noted, however, that the mere presence of endosymbionts in the ovary or eggs, is not sufficient to demonstrate vertical transmission. *Francisella tularensis*, subspecies *holarctica*, is found in the ovary and eggs, but was not detected in the larvae (Mani *et al.*, 2012). A similar situation applies to *I. ricinus* and its symbiont *Bartonella henselae* (Cotté *et al.*, 2008). The presence of the *A. hebraeum Coxiella* symbiont in unfed nymphs as well as in the ovaries and eggs indicates that it was transovarially transmitted, because both the larval and nymphal ticks were fed on tick-naïve rabbits and were not co-fed with any other tick stages. Additionally, both the unfed and fed immature ticks were housed in separate containers from the adults. All this indicates that horizontal transmission of the microbes is unlikely and matches well with what is seen in the other *Amblyomma* associated *Coxiella* spp. (Zhong *et al.*, 2007; Machado-Ferreira *et al.*, 2011).

The mechanisms by which transovarial transmission occurs in ticks remain poorly understood. At least two possibilities have been suggested:

(A) The primordial ovarian tissue is colonized at some point during development, and the population is able to expand with the female's blood meal and subsequent period of ovarian development.

(B) The microbe may colonize the oocytes only following the final blood meal, entering the oocytes along with Vg and other yolk proteins (Klyachko *et al.*, 2007).

These hypothesized mechanisms are not mutually exclusive. For example, when the vitellogenin receptor (VgR) was silenced by RNA interference, the

ability of the *Babesia gibsoni* parasite to invade the ovary of *H. longicornis* was severely impaired (Boldbaatar et al., 2008). On the other hand, Burgdorfer and Brinton (1975) showed high levels of *Rickettsia rickettsii* in the ovaries of even unfed adult *D. andersoni*. These *Rickettsia* appeared to be in a state of arrested growth, none of the cells undergoing division. Only during feeding did they enter a renewed period of growth, dividing cells being abundant, and the cellular cytoplasm being filled with various metabolic products (Burgdorfer & Brinton, 1975). Following feeding on an infected guinea pig, previous uninfected females developed a limited infection, with fewer tissues containing high levels of *Rickettsia*. The prevalence of the bacteria in the germinal cells in particular, not being as high as that of females infected transovarially (Burgdorfer & Brinton, 1975). Additionally, only some of the early eggs oviposited by these females were infected. The authors speculated that the formation of the vitelline membrane prevented the entry of the Rickettsiae into the oocytes (Burgdorfer & Brinton, 1975). Eggs produced shortly after engorgement would have developed too quickly to be infected, and only those eggs that matured later in the ovipositional period would have a chance to be infected, this made possible due to the asynchronous nature of egg development in ticks (Burgdorfer & Brinton, 1975).

Although very low levels of *Coxiella* were detected in the ovaries of unfed *A. hebraeum*, high levels were observed in the ovaries of both a partially fed (~10X the unfed weight) and an engorged female. At these times, the ovary is still relatively undeveloped; the oocytes generally do not begin to take up significant amounts of vitellogenin (Vg) and other yolk proteins until 3 – 4 days following

engorgement (Seixas *et al.*, 2008). Moreover, expression of the VgR in *A. hebraeum* begins only on the day of engorgement, with levels increasing subsequently (see Chapter 6). The presence of *Coxiella* in the ovary prior to the expression of the VgR, indicates that it is able to infiltrate the oocytes by a mechanism other than involving the VgR. It is possible that the *Coxiella* symbiont is present in the ovary of unfed adults or even nymphs at low enough levels that it was not detectable by my PCR amplifications. It is also possible that the *Coxiella* symbiont is able to also use the VgR or other receptors on the oocyte surface, to enter the developing egg during development.

The discovery of a *Rickettsia* sp. in the tissues of fed NC males, the salivary glands and fat body of fed NC females, as well as in the eggs, was unexpected, as this had not been previously observed in OC ticks. The *Rickettsia* sp. was present in much lower densities than the *Coxiella* sp., possibly explaining why it was never observed in the gDNA samples of whole ticks. The detection of this rickettsial symbiont in the eggs suggests that transovarial transmission may also occur in this species.

Phylogenetic analysis of partial 16S rDNA sequences indicated that the *A. hebraeum Rickettsia* symbiont is closely related to *Rickettsia conorii*, the causative agent of Mediterranean spotted fever (Yagupsky & Wolach, 1993; Raoult & Roux, 1997). This microbe is generally transmitted by *Rhipicephalus* spp., although *A. triste* has also been implicated as a possible vector in Uganda (Raoult & Roux, 1997; Conti Díaz, 2003). Like many *Rickettsia* spp., it is transovarially transmitted in ticks (Burgdorfer & Brinton, 1975; Rovery & Raoult,

2008). Although a 100% match to *R. conorii* was observed over the 1070 bp of 16S rDNA obtained, the 16S rDNA genes in various *Rickettsia* spp. are highly conserved, and the high degree of similarity seen with one gene does not necessarily mean that the microbe detected in *A. hebraeum* is *R. conorii*. We must obtain the complete 16S sequence as well as those of other genes commonly used for rickettsial phylogenetics, such as the membrane proteins *rOmpA* and *rOmpB*, and the citrate synthase gene *gltA* (Roux *et al.*, 1997; Fournier *et al.*, 1998*b*; Roux & Raoult, 2000), before a more accurate determination can be made. Interestingly, Zhong et al. (2007), working on the *A. americanum Coxiella* symbiont, also identified a *Rickettsia* sp., that they tentatively suggested is related to *R. amblyommii* on the basis of the partial sequence of *gltA*. This suggests that various *Amblyomma* spp. normally carry multiple symbionts. The failure to detect the *Rickettsia* symbiont in the OC *A. hebraeum* suggests that the association of the rickettsial symbiont and *A. hebraeum* may be relatively recent, or that various populations of *A. hebraeum* may possess distinct collections of symbionts. Despite the discovery of a *Coxiella* symbiont in both OC and NC ticks, the agent responsible for the deaths of three rabbits used to feed OC nymphs, whose deaths prompted this study, remains unknown.

The *A. hebraeum Coxiella* symbiont clusters with other *Coxiella* symbionts of *Amblyomma* spp., and is clearly separate from the *Coxiella* symbionts found in other hard ticks, or the *Coxiella burnetii*-like symbionts found in the soft ticks. As suggested by Machado-Ferreira et al. (2011), it is possible that the *Ambylomma Coxiella* symbionts may have arisen from an ancient infection in

the genus that has subsequently diversified along with its hosts into distinct populations. There is evidence in *A. americanum* that its *Coxiella* symbiont has undergone genome reduction, and that antibiotic treatments to reduce the population of *Coxiella* symbionts in the tick result in decreased reproductive fitness; this points to a long term association (Jasinskas *et al.*, 2007; Zhong *et al.*, 2007). It is currently unknown whether the *Coxiella* spp. found in *A. hebraeum* or *A. cajennense* also exhibits a reduced genome, or whether it plays a role in maintaining the fitness of its host.

Table 5.1 Taxa used for phylogenetic analysis of eubacterial 16S DNA sequences

Class	Species	Accession number
α-proteobacteria	*Amblyomma hebraeum* rickettsial symbiont	JX846591
	Dermacentor andersoni rickettsial symbiont	AY375424
	Dermacentor hunteri rickettsial symbiont	AY375426
	Dermacentor variabilis rickettsial symbiont	AY375427
	Ixodes scapularis rickettsial symbiont	D84558
	Rickettsia sp. (strain S)	U25042
	Rickettsia africae	L36098
	Rickettsia amblyommii	U11012
	Rickettsia canadensis	L36104
	Rickettsia conorii	L36107
	Rickettsia rhipicephali	L36216
	Rickettsia rickettsii (strain R)	L36217
	Rhodospirillum rubrum	D30778
	Agrobacterium tumefaciens	M11223
	Anaplasma marginale	M60313
	Brucella abortus	X13695
	Ehrlichia equi	M73223
	Ehrlichia ruminanteum	NR_044831
	Orientia tsutsugamushi	U17257
	Wolbachia pipientis	U23709
β-proteobacteria	*Burkholderia cepacia*	GQ359110
	Burkholderia pseudomallei	U91839
	Burkholderia mallei	AF110188
	Chromobacterium violaceum	M22510
	Comamonas testosteroni	M11224
	Neisseria polysaccharea	NR_041988
γ-proteobacteria	*Amblyomma americanum* symbiont	AY939824
	Amblyomma cajennense (Brazil) symbiont	HM133589
	Amblyomma cajennense (US) symbiont	HM133590
	Amblyomma hebraeum (NC) symbiont	JX846589
	Amblyomma hebraeum (OC) symbiont	JX846590
	Argas monolakensis symbiont	EF114360
	Carios capensis symbiont	DQ100452
	Cherax quadricarinatus symbiont	EF413063
	Dermacentor andersoni symbiont	AF001077
	Haemaphysalis concinnae symbiont	AF521888
	Haemaphysalis longicornis symbiont A	AB001519
	Haemaphysalis longicornis tick 47	AY342035
	Haemaphysalis longicornis tick 66	AY342036
	Haemaphysalis shimoga symbiont	HQ287535
	Rhipicephalus sanguineus symbiont	D84559
	Ornithodoros moubata symbiont A	AB001521
	Ornithodoros moubata symbiont B	AB001522
	Ornithodoros rostratus symbiont	JN887879
	Coxiella burnetii	D89800
	Francisella novicida	L26084
	Francisella philomiragia	L26085
	Francisella tularensis	L26086
	Legionella anisa	AY744776
	Legionella pneumophila	AF122885
	Wolbachia persica	M21292
Firmicutes	*Bacillus thuringienis*	D16281

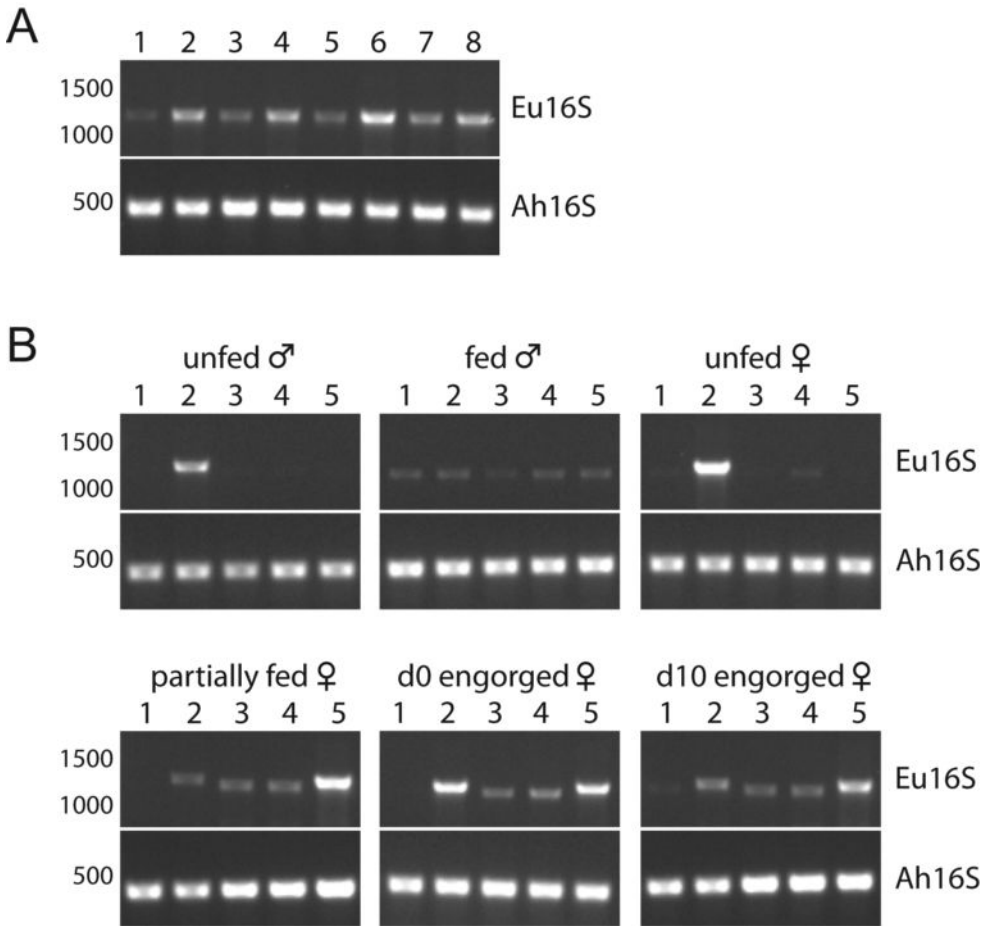

Figure 5.1. Eubacterial 16S PCR products amplified from *A. hebraeum* genomic DNA. (A) Eubacterial 16S (Eu16S) and *A. hebraeum* specific 16S (Ah16S) DNA was amplified from whole tick genomic DNA. Lane 1, OC eggs; lane 2, OC nymph; lane 3, OC unfed male; lane 4; OC unfed female; lane 5, NC eggs; lane 6, NC nymph; lane 7, NC unfed male; lane 8, NC unfed female. (B) Eu16S and Ah16S DNA was also amplified from specific tissues isolated from individual ticks at different stages of feeding. Lane 1, midgut; lane 2, Malpighian tubules; lane 3, salivary gland; lane 4, fat body; lane 5, testis/vas deferens or ovary in male and female ticks respectively. The partially fed female was ~15X the unfed weight (251 mg), whereas the engorged female was ~87X the unfed weight (2955 mg).

Figure 5.2. Alignment of the partial *Coxiella* 16S rDNA nucleotide consensus sequences isolated from OC and NC *A. hebraeum*. Identical nucleotides are unmarked and the two sites of difference between the sequences are indicated by black shaded frames.

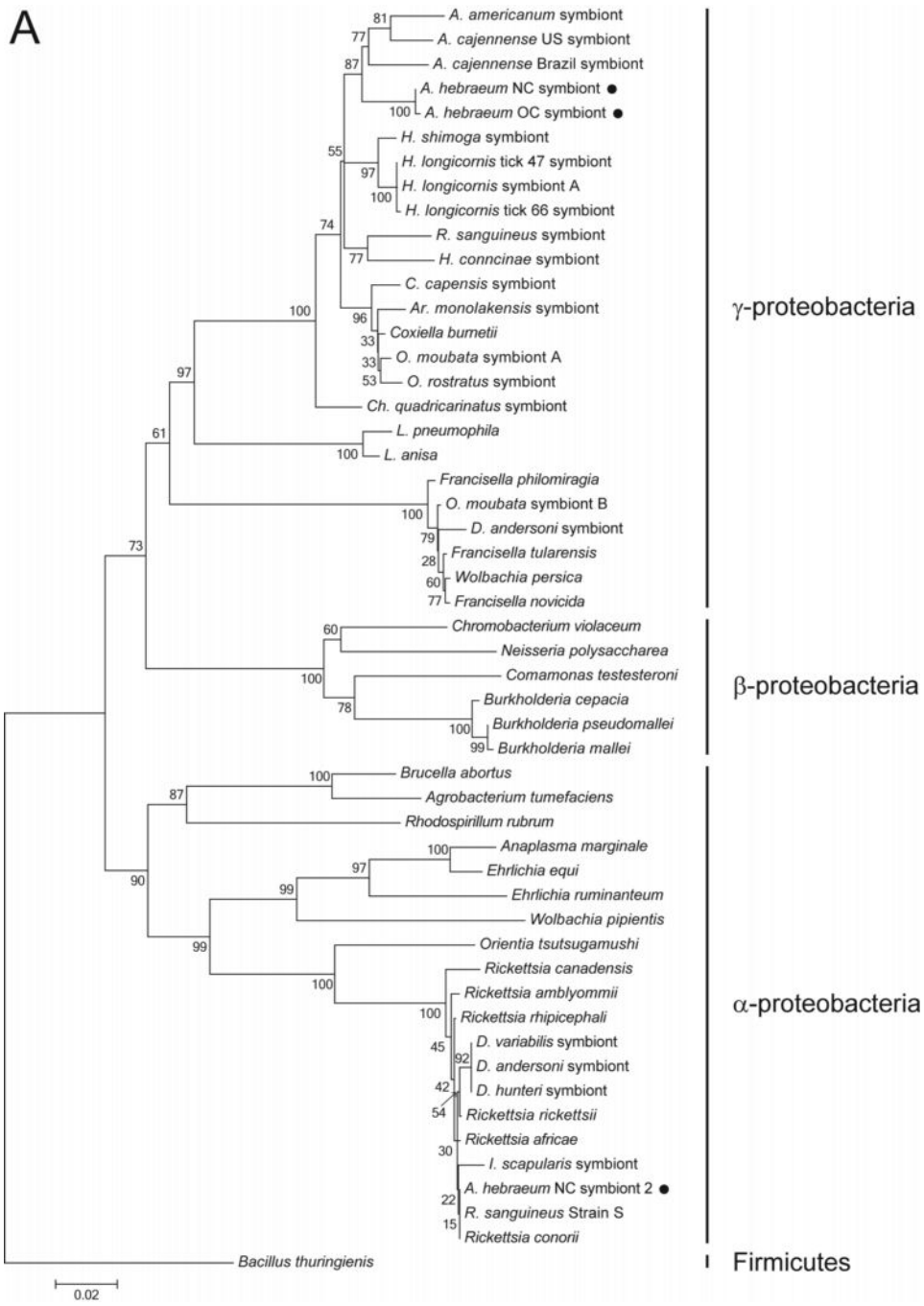
A
A. americanum symbiont
A. cajennense US symbiont
A. cajennense Brazil symbiont
A. hebraeum NC symbiont ●
A. hebraeum OC symbiont ●
H. shimoga symbiont
H. longicornis tick 47 symbiont
H. longicornis symbiont A
H. longicornis tick 66 symbiont
R. sanguineus symbiont
H. conncinae symbiont
C. capensis symbiont
Ar. monolakensis symbiont
Coxiella burnetii
O. moubata symbiont A
O. rostratus symbiont
Ch. quadricarinatus symbiont
L. pneumophila
L. anisa
Francisella philomiragia
O. moubata symbiont B
D. andersoni symbiont
Francisella tularensis
Wolbachia persica
Francisella novicida
Chromobacterium violaceum
Neisseria polysaccharea
Comamonas testesteroni
Burkholderia cepacia
Burkholderia pseudomallei
Burkholderia mallei
Brucella abortus
Agrobacterium tumefaciens
Rhodospirillum rubrum
Anaplasma marginale
Ehrlichia equi
Ehrlichia ruminanteum
Wolbachia pipientis
Orientia tsutsugamushi
Rickettsia canadensis
Rickettsia amblyommii
Rickettsia rhipicephali
D. variabilis symbiont
D. andersoni symbiont
D. hunteri symbiont
Rickettsia rickettsii
Rickettsia africae
I. scapularis symbiont
A. hebraeum NC symbiont 2 ●
R. sanguineus Strain S
Rickettsia conorii
Bacillus thuringienis
0.02
γ-proteobacteria
β-proteobacteria
α-proteobacteria
Firmicutes

Figure 5.3.

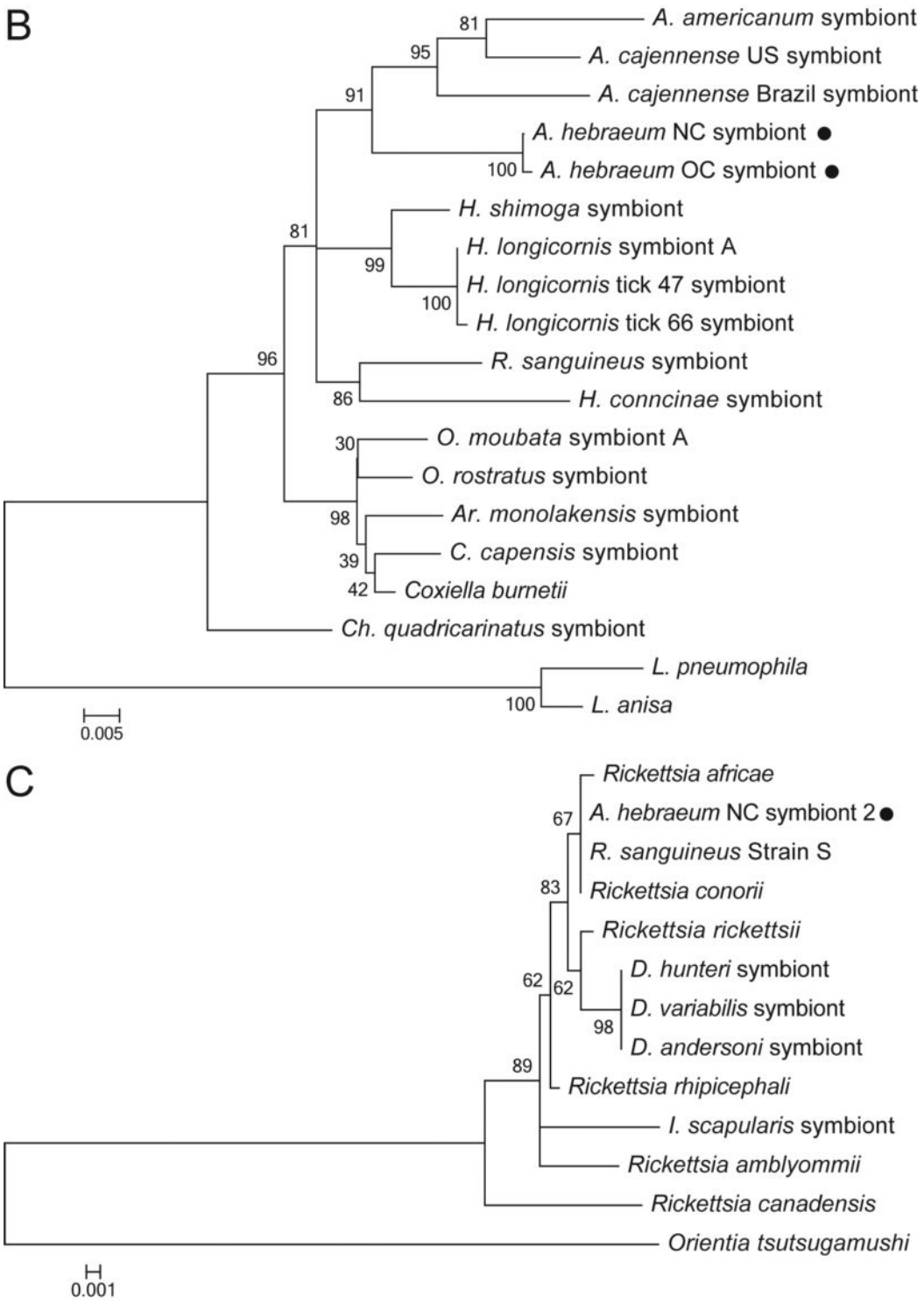

Figure 5.3. *(continued)*

Figure 5.3. *(continued)*

Phylogenetic trees of the 16S rDNA nucleotide sequences from a number of arthropod-associated bacterial species. The trees were constructed in MEGA5 using the neighbour-joining method, from distance matrices built from nucleotide sequences according to the Kimura 2-parameter method. Branches that were reproduced in less than 50% of the trees were collapsed, and bootstrap support, as a percentage of 1000 replicates, is indicated next to the branches. Sequences that are novel in this study are indicated by a ●. (A) Neighbour-joining tree of 52 putative endosymbionts, alpha-, beta- and gamma-proteobacteria with the gram-positive *Bacillus thuringienis,* from class Firmicutes, used as an outgroup. (B) Neighbour-joining tree of 16 putative *Coxiella* endosymbionts and *Coxiella burnetii* with the gamma-proteobacteria *Legionella pneumophila* and *L. anisa* used as an outgroup clade. (C) Neighbour-joining tree of six putative *Rickettsia* endosymbionts, and six *Rickettsia* sp. using the alpha-proteobacteria *Orientia tsutsugamushi* as an outgroup. Accession numbers can be found in Table 5.1.

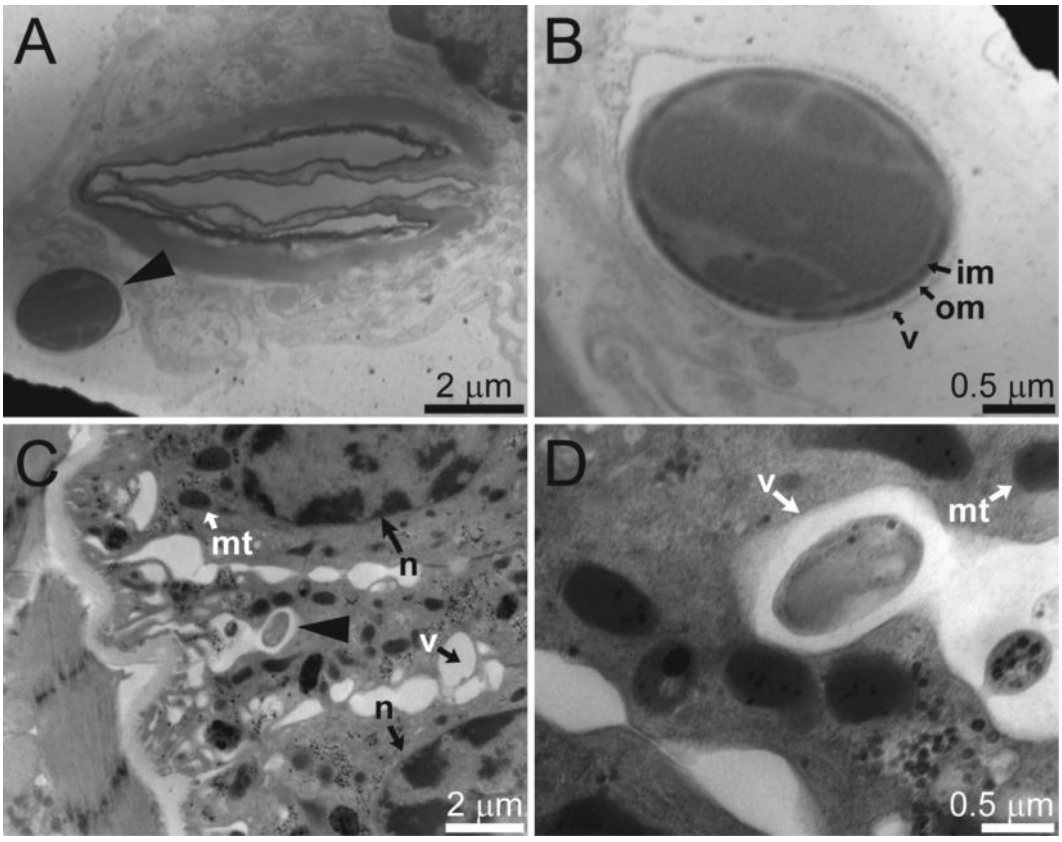

Figure 5.4. Transmission electron micrographs of the salivary glands and Malpighian tubules of OC *A. hebraeum*. (A) A putative endosymbiotic bacterium present in the salivary gland of an unfed male, indicated by an arrow head. (B) The putative endosymbiotic bacterium from the unfed male salivary gland at increased magnification. (C) A potential endosymbiotic bacterium (marked by an arrow head) found in the Malpighian tubule of a partially fed virgin female (~81 mg). (D) The putative endosymbiotic bacterium from the partially fed female Malpighian tubule at increased magnification. Abbreviations: mt, mitochondria; v, vacuole; om, outer cell membrane; im, inner cell membrane; n, nucleus.

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Chapter 6. The molecular characterization of the vitellogenin receptor and two vitellogenins from the southern African bont tick, *Amblyomma hebraeum*

6.1 Introduction

Because ticks are such important vectors of disease to humans and other animals, it is essential to develop ecologically sound methods to control them. The reproductive system is an obvious target for such research. In order to complete embryonic development, the eggs must be stocked with numerous nutrients, among them, yolk. In insects, the major yolk protein is vitellin (Vn), which is processed from vitellogenin (Vg). Vg is synthesized primarily in the fat body, post-translationally modified by glycosylation, phosphorylation, and sulphation into a number of glycolipoproteins, after which they may be further cleaved into large and small subunits. These proteins are then released into the haemolymph and taken up into the developing oocyte by receptor-mediated endocytosis, where the Vg may be further processed and stored (Raikhel & Dhadialla, 1992; Sappington & Raikhel, 1998; Tufail & Takeda, 2008).

In insects, an ecdysteroid (typically 20-hydroxyecdysone (20E)) or juvenile hormone (JH) is the vitellogenic hormone (Glinka & Wyatt, 1996; Engelmann, 2002; Engelmann & Mala, 2005). In at least some crustaceans, the JH precursors, methyl farnesoate and farnesolic acid, play the same role (Mak *et al.*, 2005; Chan *et al.*, 2005; Subramoniam, 2010). In ticks, Vg synthesis is commonly induced by a blood meal and regulated by ecdysteroids (Friesen & Kaufman, 2002, 2004; Thompson *et al.*, 2005; Seixas *et al.*, 2008). Vg is synthesized

primarily in the fat body in both insects (Melo *et al.*, 2000; Kokoza *et al.*, 2001; Raikhel *et al.*, 2002; Tufail & Takeda, 2002) and ticks (Diehl *et al.*, 1982; Chinzei & Yano, 1985; James *et al.*, 1999; Thompson *et al.*, 2007). However synthesis also appears to occur in the midgut, in at least some tick species (Coons *et al.*, 1989; Rosell & Coons, 1992; Thompson *et al.*, 2007; Boldbaatar *et al.*, 2010; Khalil *et al.*, 2011).

The vitellogenin receptors (VgRs) are large membrane bound proteins that make up a specialized subfamily of the low-density lipoproteins receptor (LDLR) superfamily (Schneider, 1996; Rodenburg *et al.*, 2006). They are situated within clathrin-coated pits on the external surfaces of developing oocytes, where they act to selectively transport macromolecules into the cell (Raikhel & Dhadialla, 1992; Sappington *et al.*, 1995; Grant & Hirsh, 1999). Vg taken up into the oocyte via the clathrin-coated pits accumulates in early endosomes, which fuse to form late endosomes, or transitional yolk bodies (Mukherjee *et al.*, 1997; Snigirevskaya *et al.*, 1997*a*, 1997*b*; Sappington & Raikhel, 1998). The Vg likely dissociates from the VgR during the formation of the transitional yolk bodies (Sappington & Raikhel, 1998). The VgR is then returned to the oocyte surface via tubular compartments and the Vg delivered to mature yolk bodies, where it is crystallized and stored until required for embryonic development (Mukherjee *et al.*, 1997; Snigirevskaya *et al.*, 1997*a*, 1997*b*; Sappington & Raikhel, 1998).

The molecular characteristics of VgRs have been described from multiple insects such as the fire ant *S. invicta* (Chen *et al.*, 2004) and the cockroaches *P. americana* and *L. maderae* (Tufail & Takeda, 2005, 2007), but have been best

characterized, both biochemically and molecularly, in the mosquito *A. aegypti* (Sappington *et al.*, 1995). VgRs have also been described from several crustaceans: the red mud crab *Scylla serrata* (Warrier & Subramoniam, 2002), the shrimp *Penaeus monodon* (Tiu *et al.*, 2008), the ticks *D. variabilis* (Mitchell *et al.*, 2007) and *H. longicornis* (Boldbaatar *et al.*, 2008), vertebrates such as chickens (Bujo *et al.*, 1994), *X. laevis* (Okabayashi *et al.*, 1996) and the rainbow trout, *Oncorhynchus mykiss* (Prat *et al.*, 1998) as well as the nematode *Caenorhabditis elegans* (Grant & Hirsh, 1999).

As members of the LDLR superfamily, both insect and tick VgRs are characterized by a highly conserved arrangement of various motifs. These modular elements consist of (i) extracellular, ligand-binding LDLR Class A cystein-rich repeats, (ii) the epidermal growth factor (EGF)-like LDLR Class B cysteine-rich repeats, (iii) repeats characterized by a YWXD motif that are proposed to form a β-propeller domain (Springer, 1998)), (iv) a serine/threonine-rich tract which may indicate an O-linked carbohydrate domain, which is present in some LDLR receptor types, (v) a transmembrane domain to anchor the receptor to the cell surface, and (vi) a cytoplasmic tail containing an internalization signal (Sappington & Raikhel, 1998; Tufail & Takeda, 2009).

Vgs, large (200-700 kDa) phosphoglycolipoproteins, occur in numerous arthropods, with those of most insects consisting of a large (>150 kDa) and a small (<65 kDa) subunit, produced from the cleavage of a single precursor in the fat body (Kunkel & Nordin, 1985; Raikhel & Dhadialla, 1992; Valle, 1993; Sappington & Raikhel, 1998). More recently, a number of complete Vg cDNAs

have been sequenced from various arthropod species. Insect Vg mRNAs are generally large: 5441 bp in the honey bee *Apis mellifera* (Piulachs *et al.*, 2003), 5722 bp in the cockroach *Periplaneta americana* (Tufail & Takeda, 2005), and 5764 bp in the fire ant *Solenopsis invicta* (Chen *et al.*, 2004). Vg mRNAs isolated from crustaceans tend to be even larger: 7970 bp in the Kuruma prawn *Penaeus japonicus* (Tsutsui *et al.*, 2000), and 8051 bp in the Red Crab *Charybdis feriatus* (Mak *et al.*, 2005).

Multiple Vg genes have been reported in vertebrates such as the chicken *Gallus gallus* (Wang *et al.*, 1983), and the African clawed frog *Xenopus laevis* (Wahli *et al.*, 1979), as well as several insects, such as the mosquito *Aedes aegypti* (Romans *et al.*, 1995), and the cockroaches *Leucophaea maderae* (Tufail *et al.*, 2007), and *Periplaneta americana* (Tufail *et al.*, 2001). Multiple Vgs have also been reported in several members of the Crustacea, such as the sea louse *Lepeophtheirus salmonis* (Dalvin *et al.*, 2011) and the shrimp *Metapenaeus ensis* (Tsang *et al.*, 2003).

Whereas there is a wealth of knowledge regarding vitellegenesis and its regulation in insects, our knowledge of this process is much more limited in ticks. The first complete Vg cDNA sequence from a tick was reported only in 2007 from the American dog tick *Dermacentor variabilis* (Thompson *et al.*, 2007). Vg sequences have now also been described for *Rhipicephalus microplus* (Granjeno-Colin *et al.*, 2008), *Ornithodoros moubata* (Horigane *et al.*, 2010) and *Haemaphysalis longicornis*, with multiple Vg genes described for *D. variabilis* (Khalil *et al.*, 2011) and *H. longicornis* (Boldbaatar *et al.*, 2010). Data from the

Ixodes scapularis genome project found in GenBank (Benson *et al.*, 2012) indicate the likelihood for at least two Vg genes present in that species. However, caution is required when identifying potential Vg genes due to the presence of various storage proteins in the Chelicerata, especially the common carrier protein (CP). CP is a haemelipoglyco-carrier protein expressed in both males and females throughout tick development (reviewed in Donohue *et al.*, 2009). In hard ticks, Vg is also a haem-binding protein, and as such shares many common features with CP, which can make differentiating between the two difficult.

To date, the majority of data on the process of vitellogenesis in arthropods comes from insects, with relatively little data from ticks. In this study, I report on the full-length cDNAs encoding two Vgs and the VgR from the ixodid tick *A. hebraeum*, and their molecular characteristics. I compare the structures of *A. hebraeum* Vg and VgR with those of other ticks and insects. The tissue and temporal specificity of expression of these genes is examined by RT-PCR, along with the effects of disruption of VgR expression using RNA interference.

6.2 Materials and methods

6.2.1 Tick rearing and feeding

As in previous chapters, all tick rearing and feeding was performed as described in Chapter 2.

6.2.2 Tick dissection and tissue collection for RNA extraction

Engorged female ticks were collected from the host, counting the day of drop-off as day 0 post-engorgement. Females were maintained in the colony

incubator until dissection at day 0, 4, 8 or 10 post-engorgement. After rinsing in sterile, RNase-free phosphate buffered saline (PBS, pH 7.4), engorged female ticks were immobilized on small sterile plastic petri dishes with a drop of cyanoacrylate glue (Loctite Corp., Rocky Hill, CT, USA), and chilled at 4°C for a minimum of 30 min to sedate the tick and to reduce the likelihood of puncturing the delicate gut while dissecting away the cuticle. The ticks were flooded with 1x PBS, the dorsal cuticle removed using a microscalpel, and various organs, including the ovary, midgut, fat body, salivary glands, Malpighian tubules and trachea harvested. Tissues were placed immediately into RNAlater (Ambion, Austin, TX, USA) and stored at -20°C until used for RNA extractions.

6.2.3 RNA isolation

The RNA used in the creation of the initial cDNA used to obtain the Vg and VgR sequences by degenerate PCR and perform 5'/3' RACE was isolated using the RNeasy Mini RNA extraction kit (Qiagen, Valencia, CA, USA). The ovaries and fat bodies of engorged females, 4 days post-engorgement, were each pooled to obtain ~30 mg of total tissue, and RNA extracted according to the manufacturer's directions. The total RNA was eluted in 100 µl of DNase and RNase-free water; then the ovary and fat body RNA samples were concentrated using 7.5 M lithium chloride (Ambion) according to the manufacturer's directions. RNA quantity and purity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

To examine the expression of Vg1, Vg2 and VgR, RNA was isolated from dissected tissues, and whole ticks, including unfed males and females, fed males,

partially fed females and engorged female ticks 0, 4 and 10 days post-engorgement. Whole ticks were washed in RNase-free PBS then ground in a sterile mortar and pestle under liquid nitrogen, with the resulting powders stored at -80°C, until RNA extraction. Both the dissected tissues and whole tick samples were placed in microcentrifuge tubes containing 1 ml of Trizol (Invitrogen, Grand Island, NY, USA), per 50 mg of tissue to be extracted. Tissue samples were homogenized using a hand-held plastic homogenizer in a microcentrifuge tube at room temperature, and RNA isolated according to the manufacturer's instructions. RNA samples were resuspended in 100 µl of sterile, DEPC-treated water, and then treated with the DNA-Free kit (Ambion), according to the manufacturer's specifications, to eliminate any DNA contamination. Following this, the nucleic acid concentration of each sample was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and then run out on a 0.8% agarose gel for visual confirmation of quality.

6.2.4 cDNA synthesis

One µg of total RNA was reverse-transcribed using either random hexamer primers (for degenerate PCR or gene expression analysis), or gene-specific primers (for 5'/3' RACE; see below) and SuperScript III reverse transcriptase (Invitrogen) to synthesize first-strand cDNA, according to the manufacturer's recommendations. All gene-specific primers were examined using OligoCalc (Kibbe, 2007) to check for possible self-complementarity, and to ensure that primer pairs would have similar melting temperatures.

6.2.5 Degenerate PCR

A consensus degenerate hybrid oligonucleotide primer (CODEHOP) strategy (Rose *et al.*, 1998) was used to design the primers for the initial amplification of the Vg1, Vg2 and VgR cDNA sequences from *A. hebraeum*. Protein sequences, from several tick species, for each gene of interest (Table 6.1) were downloaded from the National Centre for Biotechnology Information (NCBI) database and imported into BlockMaker (Henikoff *et al.*, 1995) to identify conserved regions of amino acids in the protein sequences of Vg1, Vg2 and VgR. These blocks were used as input to the CODEHOP program so that primers specific to each of the target genes could be produced, following reverse translation of the protein sequence, using the *R. microplus* codon usage table. Primers designed using the CODEHOP strategy contain a 5' non-degenerate consensus region that acts to stabilize a short 3' degenerate core (Rose *et al.*, 1998). The resulting primers were aligned with *D. variabilis* sequences to choose primer pairs that would yield amplicons of appropriate size for cloning, with only those primers that had less than 64-fold degeneracy being selected for use (Table 6.2).

Degenerate PCR reactions were performed using 0.5 µl of cDNA template, 1 unit of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), 2.5 mM $MgCl_2$, 12.5 pmol of each primer, 0.2 mM of each dNTP, and the buffer supplied with the enzyme (final concentration 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 50 mM $(NH_4)_2SO_4$). Amplification of VgR was carried out using touchdown PCR, consisting of an initial denaturation step at 94°C for 4 min, 10 cycles of

94°C for 30 s, 60°C for 30 s, and 72°C for 3.5 min; another 10 cycles with an annealing temperature of 59°C, followed by a further 15 cycles at 58°C, and ending with a final extension step at 72°C for 10 min. Amplification of Vg1 was performed similarly with the exception of the extension time being reduced to 2 min. Vg2 amplification was performed as for the VgR, but the denaturation and annealing times were reduced to 15 s each.

6.2.6 5′/3′ RACE

To obtain the 5'-regions of the genes of interest, 5' RACE was performed according to the protocols in Sambrook & Russell (2001). Briefly, following first-strand cDNA synthesis, residual RNA was digested using RNase H (Invitrogen), and then the cDNA was purified by two rounds of precipitation with ammonium acetate and ethanol. A 5' poly-A tail was added to the cDNAs using dATP (Roche) and terminal transferase (Invitrogen), according to the manufacturer's specifications.

Second strand cDNA synthesis and VgR amplification was performed in a single reaction using the 5' dA-tailed cDNA, 16 pmol of a $(dT)_{17}$-universal adaptor primer (UAP), 32 pmol of the abridged universal adaptor primer (AUAP) and 32 pmol of a gene-specific VgR primer (Table 6.3) in a reaction containing 1.5 units of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and the buffer supplied with the enzyme. Cycling conditions consisted of an initial denaturation at 94°C for 5 min followed by 1 cycle of denaturation at 94°C for 40 s, a 50°C annealing step for 5 min and extension at 72°C for 40 min to synthesize the second strand cDNA. This was

immediately followed by a touchdown PCR consisting of 94°C for 40 s, an annealing temperature decreasing from 58-54°C for 30 s, and 72°C for 4.5 min. Five cycles of amplification were performed at each annealing temperature, except for the 54°C annealing, in which 10 cycles were performed for a total of 30 cycles of amplification.

The 5' ends of Vg1 and Vg2 were obtained similarly: 5' dA-tailed cDNA was generated using a Vg1 or Vg2 specific primer, a UAP and an AUAP (Table 6.3) as described above. The second strand cDNA was immediately further amplified by a touchdown PCR consisting of 10 cycles of 94°C for 40 s, 58°C for 30 s, and 72°C for 2 min. Three additional sets of 10 cycles were performed using annealing temperatures of 57°C, 56°C and 55°C, then a final cycle of 94°C for 40 s, 58°C for 1 min and 72°C for 15 min was performed. Band-stab PCR (Bjourson & Cooper, 1992), was then used to specifically amplify a specific amplicon from each reaction, using cycling conditions identical to the initial touchdown reactions.

Amplification of the 3' cDNA ends was performed using a 3' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen) according to the manufacturer's directions. First strand cDNA synthesis was performed using UAP and RNA isolated from either the ovary or fat body of female *A. hebraeum* 4 days post-engorgement. VgR and Vg1 cDNAs were then amplified using 10 pmol each of AUAP and a gene-specific primer (Table 6.3) in a reaction containing 2.5 units of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), 1.5mM $MgCl_2$, 0.2 mM of each dNTP, and the buffer supplied with the enzyme. Cycling

conditions consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The 3' end of Vg2 was amplified similarly, but using 30 pmol each of AUAP and a gene-specific primer (Table 6.3), 1 unit of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), and 2.5 mM $MgCl_2$ in the reactions. Cycling conditions consisted of an initial denaturation at 94°C for 5 min followed by 10 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 2 min. This was followed by 10 cycles with a 59°C annealing temperature, then 15 more cycles with a 58°C annealing temperature and ended with a final 10 min extension at 72°C.

6.2.7 Cloning and sequencing of putative Vgs and VgR

The amplified cDNA fragments were purified either using a QIAquick PCR Purification Kit (Qiagen), gel purified using the QIAEX II, QIAquick Gel Extraction Kits (Qiagen), or by the 'freeze and squeeze' method which consists of freezing an excised gel slice, placing the slice in a piece of Parafilm™, then manually squeezing the DNA out of the frozen slice into a sterile microfuge tube. The sequences were then determined by either directly sequencing the purified PCR products, or after subcloning into either a pGEM-T (Promega, Madison, WI, USA), a pJET1.2 (Thermo Fisher Scientific) vector or a homemade TA cloning vector derived from pUC19 cut with SmaI restriction endonuclease with dT-overhangs added using dTTP and Taq polymerase. The resulting plasmids were transformed into *E. coli* OmniMAX 2 T1^R cells (Invitrogen) by electroporation and plasmids purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics

GmbH, Mannheim, Germany).

The plasmids and PCR product sequences were determined using BigDye® Terminator v3.1 Cycle Sequencing mix (Applied Biosystems, Foster City, CA, USA) and T7, SP6, pJET1.2 forward and reverse sequencing primers and/or gene-specific primers (Table 6.4). Reactions consisted of approximately 200 ng of plasmid DNA or 20 ng of PCR template, 1.6 pmol of primer, 0.5x BigDye dilution buffer and 2 μL BigDye Sequencing premix in a 10 μL reaction volume. Cycling conditions consisted of 25 cycles: 95°C for 20 s, 50°C for 15 s, and 60°C for 60 s.

6.2.8 RT-PCR for expression analysis

Levels of Vg1, Vg2 and VgR mRNA present at various times and tissues were evaluated by semi-quantitative RT-PCR. Following cDNA synthesis, each cDNA was diluted 1/10 with sterile, DEPC-treated water prior to use as template for PCR-based gene expression analysis. Vg1, Vg2 and VgR were amplified in reactions containing 0.5 units of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 15 pmol of each gene-specific primer (Table 6. 5) and the buffer supplied with the enzyme. Cycling conditions consisted of an initial denaturation at 94°C for 5 min followed by 28 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 1 min followed by a final 10 min 72°C extension.

6.2.9 Sequence and bioinformatic analysis

Following the removal of all primer and vector sequences from the raw sequence data, sequences were assembled using GeneTool (Biotools, Inc.

Edmonton, Canada), then compared against the non-redundant GenBank nucleotide database using BLASTN (www.ncbi.nlm.nih.gov) searches (Altschul *et al.*, 1997) to determine putative gene identity. Open reading frames (ORFs) were predicted using the NCBI ORF finder, and the putative protein sequences compared against the non-redundant GenBank protein database using BLASTP searches (Altschul *et al.*, 1997) to confirm putative protein identity, and the NCBI Conserved Domain Database (CDD; Marchler-Bauer *et al.*, 2011) to identify conserved domains. Tools available at the Center for Biological Sequence Analysis at the Technical University of Denmark (CBS) were used to identify signal peptide cleavage sites, identified using the SignalIP (Petersen *et al.*, 2011) prediction server (http://www.cbs.dtu.dk/services/SignalP/), potential transmembrane regions using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), as well as possible phosphorylation and N-linked glycosylation sites using the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) and the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) respectively. The GPP Prediction Server (http://comp.chem.nottingham.ac.uk/glyco/) was used to evaluate potential O-linked glycosylation sites (Hamby & Hirst, 2008). The tool PATTINPROT (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html) was used to identify specific conserved motifs in the protein sequences (Combet *et al.*, 2000). Various tools available at the ExPASy: SIB Bioinformatics Resource Portal were also used. Specifically, the predicted molecular weights and isoelectric points (pIs) were determined using the Compute pI/Mw tool

(http://web.expasy.org/compute_pi/), potential sulfation sites using Sulfinator (http://web.expasy.org/sulfinator/), and domain structure analyzed using the ScanProsite tool (http://prosite.expasy.org/scanprosite/).

6.2.10 Sequence alignments and phylogenetic analysis

Homology of the *A. hebraeum* VgR and Vgs were compared to the amino acid sequences of other arthropod VgRs, Vgs and CPs. Sequences were aligned using MUSCLE (Edgar, 2004), and phylogenetic relationships inferred using MEGA5 (Tamura *et al.*, 2011). Distance matrices based on the protein sequences were constructed according to the Jones-Taylor-Thornton model (Jones *et al.*, 1992), and trees constructed using the neighbour-joining method (Saitou & Nei, 1987), with bootstrap values assessed at 1000 replicates. Sequences were compared in a pairwise fashion, with all ambiguous positions removed for each sequence pair. All non *A. hebraeum* amino acid sequences were obtained from GenBank, accession numbers of the sequences used can be found in Table 6.6.

6.2.11 RNA interference (RNAi)

To produce regions of VgR, Vg1, Vg2 and β-lactamase (Bla) genes that include a T7 promoter region on both the sense and antisense strands, 2 µl of 1/10 diluted cDNA (from the ovary or fat body of females 4 days post-engorgement) or purified pBluescript II SK+ (Stratagene, Santa Clara, CA, USA) were amplified in a PCR containing 1 unit of Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 30 pmol of each gene-specific primer (Table 6.7) and the buffer supplied with the enzyme, using the

following cycling conditions: 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and a final extension of 72°C for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) then used as template to synthesize VgR, Vg1, Vg2 and Bla dsRNA *in vitro* using the MEGAscript RNAi kit (Ambion) according to the manufacturer's specifications. Quality of the dsRNA was evaluated by running 0.5 µl of each dsRNA on a 0.8% agarose gel. The targeted regions were specifically chosen because A) a BLAST search revealed no significant homology to other genes in GenBank, thus decreasing the likelihood of off target effects, and B) analysis of each gene using the IDT RNAi oligo design tool (http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx) indicated the presence of a potential dicer recognition site in the targeted regions.

Unfed adult female ticks were divided into weight-matched groups and injected with 1 µg of each of the *A. hebraeum* VgR, Vg1, Vg2, or Vg1+Vg2 dsRNAs, in a total volume of 2 µl. Control ticks were injected with 1 µg of Bla dsRNA in 2 µl, or the equivalent volume of the TE buffer used to suspend the dsRNA. All ticks were marked by tying coloured threads to one or more legs and anchoring it in place with a small drop of cyanoacrylate glue (Loctite Corp.). Injections were made through the fourth coxa into the haemocoel of the females, using 33 G, ½ inch hypodermic needles and 1.00 cc DB Yale Tuberculin syringes (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) placed in a microapplicator (Instrumentation Specialities Co., Lincoln, Neb.; model M). Following injection, ticks were stored overnight in the colony incubator at 26°C, to determine

mortality. Ticks from both the experimental and control groups, along with an equal number of males, were placed on rabbit hosts, with an equal number of each treatment group placed on each rabbit.

The ticks were monitored and removed and weighed upon engorgement. Feeding success in ticks that survived injection overnight was determined by measuring the number of engorged ticks, the engorged weight, the extent of oviposition and egg hatching success. At 8 days post-engorgement, females were dissected, and various tissues harvested. Each ovary was weighed, its developmental stage assessed (Seixas *et al.*, 2008), and the eight largest oocyte lengths recorded. Total RNA was then extracted from the ovary and fat body of each female and cDNA synthesized from those tissues to examine gene-specific dsRNA mediated knockdown.

6.2.12 Nucleotide sequence accession numbers

The nucleotide sequence data reported in this work will be deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers JX846592 (AhVgR), JX846593 (AhVg1), and JX846594 (AhVg2).

6.3 Results

6.3.1 Sequence and structural analysis of the AhVgR

The full-length putative AhVgR (*A. hebraeum* VgR) cDNA is 5703 bp, comprising a 99 bp 5' untranslated region (UTR), a 5406 bp ORF, encoding 1801 amino acid (aa) residues, and a 153 bp 3' UTR with a 45 bp poly-A tail occurring 20 bp downstream of the AATAAA polyadenylation signal. The full-length

nucleotide sequence has 75% and 73% identity to the *D. variabilis* and *H. longicornis* VgRs respectively, (accession numbers DQ103506 and AB299015 respectively; E-values: 0.0).

The predicted amino acid sequence of the translated AhVgR ORF is 1801 aa and is shown aligned with the *D. variabilis* and *H. longicornis* VgR aa sequences (Fig. 6.1). The AhVgR has a 22 aa predicted signal peptide, with the cleavage site located between residues 22 and 23, at the ASS-ECP aa junction (Fig. 6.1). Following cleavage of the signal peptide, the mature protein was predicted to have a molecular weight of 196.5 kDa, with a pI of 5.21. Analysis of conserved domains indicated that the AhVgR is a member of the LDLR superfamily, containing the conserved arrangement of five modular elements that characterize the superfamily (Fig. 6.2). As shown in Fig 6.1, AhVgR possesses twelve LDLR class A (LDLRA) repeats, arranged in two binding domains with four repeats in the first site and eight in the second. Each repeat contains six cysteine residues and a conserved SDE acidic residue region between the fifth and sixth cysteine residues. A total of eight epidermal growth factor (EGF)-like domains (class B repeats), each also containing six cysteine residues, are present, two of which are calcium-binding EGF-like domains. Each LDLRA domain was followed by one EGF-like domain and one calcium-binding EGF-like domain. A total of 18 repeats containing a YWXD motif were present in three groups of six. The first two groups were followed by a single EGF-like domain, with the last group followed by two EGF-like domains. A hydrophobic transmembrane region encompassing residues 1670 – 1692 is followed by a cytoplasmic domain

containing potential internalization signals at residues 1742 – 1747 (following the consensus sequence FXNPXF), and at 1719 – 1720 and 1754 – 1755 (dileucine (LL) motif). A putative *O*-linked sugar domain (OLSD) was predicted in a serine-rich stretch of 17 aa (residues 1653 – 1669, 29% Ser) between the final cysteine of the EGF-like domain and the beginning of the hydrophobic transmembrane domain. In addition to this potential post-translational modification, 11 putative N-linked glycosylation sites were recognized with the consensus sequence NX[S/T], and 99 potential phosphorylation sites (serine: 57, threonine: 18, and tyrosine: 24) were predicted in the AhVgR amino acid sequence.

Overall, the amino acid sequence of AhVgR had relatively high homology to other tick VgRs, the highest homology being to the VgR from *D. variabilis* with 78% identity and 88% similarity, but sequence homology was much lower when comparing AhVgR to those from insects, AhVgR having only 32% identify and 48% similarity to the VgR from the cockroach *Periplaneta americana*. Phylogenetic analysis was performed to compare AhVgR with the VgRs from hard ticks, insects and crustaceans (Fig. 6.3). AhVgR groups with the other tick VgRs, which themselves form a distinct clade, separate from the VgRs present in insects and crustaceans.

6.3.2 Sequence and structural analysis of AhVg1 and AhVg2

Two Vg cDNAs were obtained and based on sequence homology to the *D. variabilis* vitellogenins, designated AhVg1 (*A. hebreaum* Vg1) and AhVg2. The AhVg1 cDNA is 5866 bp in length and contains a 5715 bp ORF, as well as a 35 bp and 74 bp 5' and 3' UTRs. There is a putative polyadenylation signal located at

5800 – 5805 bp and is followed by a 42 bp poly-A tail 19 bp downstream of the signal. The AhVg1 nucleotide sequence has 79% identity to the *D. variabilis* Vg1 (DvVg1; AY885250: E-value: 0.0) and 77% to the *R. microplus* GP80 protein (BMU49934; E-value: 0.0).

The predicted amino acid sequence encoded by the ORF is 1904 residues long and contains a 21 aa N-terminal signal sequence, with the cleavage site located between residues 21 and 22 at the VLF-DPV junction (Fig. 6.4). The predicted molecular weight of the protein following removal of the signal peptide is 213.8 kDa, with a pI of 6.68. BLASTP homology searches indicated that the AhVg1 has 72% identity and 83% similarity with the DvVg1 but only 25% identity (42% similarity) with the *D. variabilis* Vg2 (DvVg2; EU204907; E-value: 0.0) and only 26% identify (42% similarity) with the *O. moubata* Vg (OmVg; BAH02666; E-value: 7e-77). Analysis of conserved domains indicated the presence of three domains typically found in insect Vgs, in AhVg1: an N-terminal lipoprotein domain (aa 33 – 719), a domain of unknown function DUF1943 (aa 751 – 962), and a von Willebrand factor type D domain (vWD) near the C-terminal end of the protein (aa 1479 – 1647). The AhVg1 sequence contains two putative RXXR cleavage sites: RLFR (aa 465 – 468) and RKIR (aa 1870 – 1873), and twelve KXXK sites, homologous to the KFKKAN site found in the Vg of the bean bug *Riptortus cavatus* (Hirai *et al.*, 1998). Similar to DvVg1, AhVg1 contains the G-[LI]-CG motif variant GLCS (aa 1631 – 1634, Fig. 6.5), but does not contain the conserved DGXR motif typically found ~20 aa upstream of the G-[LI]-CG site in most insect Vgs; rather, it contains a similar sequence, DGVN at

the same location. The DGVN site is also found at this location in DvVg1, OmVg, a putative *I. scapularis* Vg (XP_002415224), a putative *R. microplus* Vg (ABS88989) and the *R. microplus* GP80 protein (AAA92143), but not in any of the reported *H. longicornis* Vgs (Fig. 6.5). A total of 112 possible phosphorylation sites were detected (Ser: 49, Thr: 27, Tyr: 36), along with 20 possible N-glycosylation sites and 13 tyrosines that may undergo sulfation. Although serines made up 6.1% of all AhVg1 amino acids, no large polyserine tracts are present. Unlike what is seen in many insect Vgs, but similar to what is seen in other tick Vgs.

The full-length AhVg2 is 5963 bp in length and encodes a 1926 aa protein in its 5781 bp ORF. There is an 84 bp 5' UTR and a 76 bp 3' UTR that contains a 22 bp poly-A tail 24 bp downstream of the putative polyadenylation signal (bp 5912 – 5917). The AhVg2 has 78% identity with DvVg2 (E-value: 0.0), and 70% identity to a putative *I. scapularis* Vg (XM_002403922, E-value: 8e-178).

The 1926 aa protein contains a 16 aa signal peptide at the N-terminal end, with the cleavage site between potions 16 and 17 at the VVA-HDL junction (Fig. 6.6). Following removal of the signal sequence, the protein is predicted to have a molecular weight of 215.9 kDa and a pI of 6.67. AhVg1 and AhVg2 shared only 26% identity (42% similarity), but AhVg2 has 73% identity and 83% similarity to the DvVg2 and 39% identity (55% similarity) to OmVg, with E-values of 0.0 for both. Similarly to AhVg1, AhVg2 also contains the three domains typically found in Vgs: an N-terminal lipoprotein domain (aa 28 – 734), a DUF1943 domain (aa 768 – 983), and a C-terminal von Willebrand factor type D domain (aa 1590 –

1758). AhVg2 possesses one [RK]-X-[RK]-R cleavage motif at amino acids 852 – 855 (RVRR) as well as two RXXR cleavage motifs, RSLR (aa 855 – 858), and RVVR (1153 – 1156), in addition to 13 KXXK sites. Unlike AhVg1, AhVg2 does not have the variant GLCS motif, but like DvVg2, has a GLCG motif near the C-terminal end of the protein, (aa 1739 – 1742, Fig 6.5). Both DvVg2 and AhVg2 lack the either of the DGXR and DGVN sequences and instead have NGSS at this position, a sequence not shared by other arthropod Vgs (Fig. 6.5). AhVg2 contains 15 putative N-glycosylation sites, four possible sulfation sites, as well as 138 potential phosphorylation sites (Ser: 46, Thr: 46, Tyr: 46), and like other tick Vgs, contains no long polyserine tracts in its protein sequence.

In general, the *A. hebraeum* Vg amino acid sequences have relatively high homology to the corresponding *D. variabilis* Vgs, but low levels of homology to other tick and insect Vgs. Phylogenetic analysis comparing AhVg1 and AhVg2 with the Vgs from other arthropods, indicates that the tick Vgs form a separate clade from both the insects and the crustaceans, with Vg1 sequences from *D. variabilis, A. hebraeum* and *I. scapularis* clustering together, the Vg2 sequences from *D. variabilis, A. hebraeum, I. scapularis*, and *O. moubata* clustering together, and the three HlVgs forming their own cluster at the base of the tick Vg clade (Fig. 6.7). Both AhVg1 and AhVg2 lack the conserved FEVGKEYVY and DASAKERKEIED sequences found directly following the signal peptide and the RXXR cleavage site respectively, in haemoglycolipo-carrier proteins (CP; Fig 6.8). These sequences, or closely related sequences are found in tick CPs, but not generally in tick Vgs. These sequences are present in HlVg2, with similar

sequences present in the reported HlVg1 and HlVg3 (Fig 6.8). Phylogenetic analysis of tick Vgs and CPs, shows that both AhVg1 and AhVg2 cluster with other tick Vgs, specifically associated with other Vg1 or Vg2-like sequences respectively. The HlVgs do not fall into distinct groups as neatly, as HlVg2 forms a distinct clade along with other tick CPs, and HlVg1 and HlVg3 occupy intermediate positions between the two groups (Fig 6.9).

6.3.3 Developmental regulation and tissue specificity

AhVgR, AhVg1 and AhVg2 were all expressed in adult females only after they fed to repletion (Fig. 6.10). Levels of each mRNA appear low upon the day of engorgement, but visibly increase over time, with the highest levels observed in females ten days post-engorgement (d10pE). The positive control, 16S rRNA was detected in all samples. Tissue-specific expression of each mRNA was also examined by RT-PCR of total RNA collected from a number of tissues of engorged ticks zero (d0pE), four (d4pE) or 10 days post-engorgement (Fig. 6.10). AhVgR was only expressed in the ovary at all three stages, whereas AhVg1 and AhVg2 were not expressed in the d0pE or d4pE ovaries. AhVg1 and AhVg2 were not expressed in high abundance in any tissue from the d0pE females, though a very low level of expression of AhVg1 in the midgut was observed. In both the d4pE and d10pE tissue samples, AhVg1 and AhVg2 were prevalent in the miscellaneous tissues, fat body and midgut, as well as in the ovary in the d10pE sample. Miscellaneous tissues comprised all other tissues of the tick that were not tested separately. Specifically this included the Malpighian tubules, tracheae, salivary glands, Géné's organ, the synganglion, muscles and cuticular epithelia of

each tick tested. It is likely that both the miscellaneous tissues and the ovary samples were contaminated with fat body tissue due to the amorphous nature of this tissue and its tendency to grow around other tissues.

6.3.4 Gene silencing of AhVgR, AhVg1 and AhVg2 by RNA interference

Ovaries harvested from females injected with VgR-dsRNA eight days after engorgement showed decreased yolk uptake (Fig. 6.11) and had significantly smaller oocytes compared to the ovaries of the Bla-dsRNA or TE-injected control females ($p<0.000$, Table 6.8). The ovaries of the VgR-dsRNA injected females were also significantly developmentally delayed ($p<0.000$): these females were generally between OGP stages 2 and 3, whereas the controls were generally at approximately OGP 4, with some individuals at OGP 5. The ovaries and eggs masses produced by the VgR-dsRNA injected females were not significantly different from those of the controls, although they did take significantly more time to begin oviposition ($p=0.010$, Table 6.8). There were no significant differences between either the engorged weights or mortality rates of all treatment groups (Table 6.8). One of the rabbits used to feed the VgR-dsRNA, Bla-dsRNA and TE-injected ticks developed a strong immune response during tick feeding, leading to the deaths of 30% each of the Bla-dsRNA and TE-injected controls, and 40% of the VgR-dsRNA injected females, and 23% of the uninjected males. RT-PCR analysis of total RNA extracted from the ovaries of the VgR-dsRNA showed a decreased abundance of of VgR mRNA compared to the Bla-dsRNA and TE-injected controls (Fig. 6.12). Approximately similar amounts of the 16S rRNA positive control was detected in all samples.

The weights of ovaries of females injected with the various AhVg-dsRNAs were not significantly less than those of the controls, although the average oocyte length of the AhVg1 and AhVg1+2-dsRNA injected females was significantly smaller than that of the combined controls ($p=0.015$ and $p<0.000$ respectively; Table 6.8). The ovaries of the AhVg-dsRNA injected females did not vary significantly in weight compared to those of the controls, nor were these ovaries significantly less developed than those of the controls: the AhVg1 and AhVg2-dsRNA injected groups generally being at approximately OGP4 and the AhVg1+2-dsRNA injected ticks being at approximately OGP3 (Fig. 6.11). However, the single ovary harvested from the Vg2-dsRNA injected group had an abnormal appearance: although the oocytes had visible yolk uptake, unlike the control oocytes, the yolk did not appear densely packed, but rather appeared as though only a thin shell of yolk proteins were present around a mostly hollow core (Fig. 6.11). The AhVg-dsRNA injected ticks were not significantly different from the controls in terms of engorged weight, days to onset of oviposition, the mass of eggs produced, or mortality rate (Table 6.8). Compared to the controls, the level of AhVg1 present in total RNA isolated from the fat body of AhVg1-dsRNA injected females appeared slightly lower, as the levels of AhVg2 in samples from AhVg2-dsRNA injected females (Fig. 6.12). Levels of AhVg1 and AhVg2 did not appear to be substantially lower in the doubly injected ticks when compared to the controls. The 16S rRNA control was again detected at approximately similar levels in all samples.

6.4 Discussion

6.4.1 VgR structure

Like both the DvVgR and the HlVgR, the AhVgR has four cysteine-rich LDLRA repeats in the first ligand-binding site, and eight in the second, an arrangement that is quite different from those found in most insect VgRs, which generally have a total of 13 LDLRA repeats in a five- and eight-repeat arrangement (Tufail & Takeda, 2009), with the exception of the *S. invicta* VgR, which also has a four- and eight-repeat arrangement of the LDLRA domains (Chen *et al.*, 2004). The arrangement found in ticks is also different from that found in classical LDLRs, which have a single, seven-repeat domain (Yamamoto *et al.*, 1986) and in vertebrate VgRs (Bujo *et al.*, 1994; Okabayashi *et al.*, 1996) and VLDLRs (Takahashi *et al.*, 1992; Sakai *et al.*, 1994), both of which have a single eight-repeat domain. Each LDLRA repeat is approximately 40 aa long and contains six conserved cysteine residues that form a specific disulfide bond pattern ($C_I - C_{III}$, $C_{II} - C_V$, and $C_{IV} - C_{VI}$) that are required for proper folding and binding of the receptor to its ligand (Goldstein & Brown, 1974). The correct folding and disulfide bond formation of the ligand-binding region is dependant on proper coordination of a Ca2+ ion (Blacklow & Kim, 1996; Atkins *et al.*, 1998) chelated by a cluster of conserved acidic residues (in bold, C**D**XXX**D**CX**D**GS**DE**) between the fourth and sixth cysteines of each repeat (Fass *et al.*, 1997).

The distribution of the EGF-like and YWXD repeats between the LBDs in the AhVgR is identical to that seen in the *D. variabilis* VgR (Fig. 6.2). Interestingly, in the *H. longicornis* VgR, only seven EGF-like repeats are present,

there being only three in the first EGF-precursor domain (Fig. 6.1 and 6.2). This also differs from what is seen in insect VgRs. Although insect VgRs also have only seven EGF-like repeats, they generally have four in the first domain and three in the second (Tufail & Takeda, 2009). Each EGF-like repeat contains three internal disulphide bonds, though in a different bond pattern from that seen in the LDLRA repeats ($C_I - C_{III}$, $C_{II} - C_{IV}$, and $C_V - C_{VI}$; Tufail & Takeda, 2009). Similarly to what is seen in other VgR and LDLRs, the EGF-like repeats bind calcium with high affinity to the receptor (Selander-Sunnerhagen *et al.*, 1992; Rao *et al.*, 1995), which acts to induce a rigid conformation of the EGF-like domain, making it resistant to proteolytic degradation (Periz *et al.*, 2005). In the AhVgR, EGF-like repeats flank the β-propeller domains, regions comprising six YWXD motifs, that play a vital role in in ligand release and receptor recycling (Springer, 1998; Jeon *et al.*, 2001). The AhVgR, like both the DvVgR and HlVgR as well as various insect VgR sequences all possess three β-propeller domains, unlike vertebrate VgRs and LDLRs, which contain a single β-propeller domain (Tufail & Takeda, 2009).

Although an OLSD is present in many insect VgRs, such as those from *A. aegypti* (Sappington *et al.*, 1996), and the cockroaches *P. americana* (Tufail & Takeda, 2005), *B. germanica* (Ciudad *et al.*, 2006), and *L. maderae* (Tufail & Takeda, 2007); it is not present in the VgRs reported from *S. invicta* (Chen *et al.*, 2004), and *D. melanogaster* (Schonbaum *et al.*, 1995). Both *A. hebraeum* and *D. variabilis* possess a 17 aa stretch that is rich in serine and threonine residues directly N-terminal to the transmembrane domain, which may form an OLSD in

these species. This region is much shorter than those found in insects, which are generally ~30 residues long, but the proportion of S/T residues is similar despite the size difference: *A. hebraeum* and *D. variabilis* each possess a total of five S/T residues in this region (29%), compared to the six residues found in *P. americana* and *L. maderae* (19%), the eight in *B. germanica* (29%) and the nine in *A. aegypti* (32%). The only other fully sequenced tick VgR, from *H. longicornis*, also has a 17 residue stretch directly N-terminal to the transmembrane domain that is enriched in S/T residues, possessing a total of four S/T residues (24%), but does not possess an OLSD (Boldbaatar *et al.*, 2008). Experimental verification of protein glycosylation sites can be time and laboratory-intensive processes. Although there are multiple computational approaches to identifying *O*-linked glycosylation sites, unlike N-linked glycosylation sites, *O*-linked glycosylation sites lack a consensus sequence indicating a potential glycosylation site (Chen *et al.*, 2010). The exact role of the OLSD is still uncertain, when this region of a LDLR is deleted experimentally, there appears to be no effect on ligand-biding, endocytosis, recycling, or degradation (Davis *et al.*, 1986). However, it has been suggested that the OLSD may be involved in promoting receptor stability on the cell surface or in the regulation of the cytoplasmic domain (Rodenburg *et al.*, 2006). Similarly to the DvVgR, the AhVgR has a 22 amino acid transmembrane domain as well as a C-terminal cytoplasmic domain that contains two LL internalization signals as well as the conserved FXNPXF internalization signal.

In terms of both sequence similarity and conserved features that have been described, the AhVgR is similar to other tick and insect VgRs and LDLRs. The

biological significance of the structural variation observed in various arthropods is still unknown.

6.4.2 Vg structures

As described above, both AhVgs possess a number of features common to other tick and insect Vgs. The AhVgs, along with all other tick Vgs sequenced to date, lack the long polyserine domains common to insect Vgs. However, they do contain a large number of possible phosphorylation sites. The role of the polyserine domains in insects is not clear, however it has been suggested that they may be highly phosphorylated (Tufail *et al.*, 2001; Tufail & Takeda, 2002). Evidence showing the removal of phosphate reduces the affinity of Vg for its receptor (Dhadialla *et al.*, 1992) and that dephosphorylation of the vertebrate Vg component phosvitin decreases uptake into oocytes (Miller *et al.*, 1982). This suggests that the polyserine tracts may play a role in receptor-ligand interactions on the oocyte surface (Hirai *et al.*, 1998).

Insect Vgs contain a conserved G[L/I]CG motif present near the C-terminus (Tufail & Takeda, 2008), a shorted version of the TCG[L/I]CG motif found in vertebrates (Mouchel *et al.*, 1996). Although crustacean Vgs contain a similar GLLG motif, it is located further towards the C-terminal end of the protein. Tick Vgs contain the conserved G[L/I]CG motif found in insect Vgs and at a similar location. AhVg2, DvVg2, both putative *I. scapularis* Vgs, and the OmVg all containing the typical GLCG domain, but AhVg1, like DvVg1, the *R. microplus* GP80 protein and the partial *R. microplus* Vg (ABS88989) all possess a modified GLCS domain at this site (Fig. 6. 5). In insect Vgs, the conserved

G[L/I]CG motif is followed by a number of cysteine residues, which, in conjunction with the G[L/I]CG motif are thought to be necessary for the oligomerization of Vns in vertebrates (Mayadas & Wagner, 1992; Mouchel *et al.*, 1996). Many of the tick Vgs also have a number of cysteine residues following the conserved GLC[G/S] site, but their positions and number appear to be somewhat variable between species. DvVg1, RmGP80, and RmVg have a single cysteine residue following their GLCS sites, DvVg1 instead having a series of ~20 P[S/T]H[E/H/P/S/K][E/K]Y repeats and RmGP80 and RmVg having ~13 and ~12 PT[H/R][H/R][E/K]Y sites respectively. AhVg1 has a similar series of 13 P[T/S/E]HH[K/Q]Y repeats, but also has seven cysteine residues downstream of the GLCS site. These repeats do not appear to be present in other Vgs, though a number of the proline, histidine and tyrosine sites appear to be conserved in one of the putative IsVgs (XP_002415224). As noted by Thompson *et al.* (2007), although these repeats are found only in haem-binding Vgs, they are not present in all haem-binding Vgs, nor are they associated with the closely related haem-binding CPs. The mechanism by which haem binds to Vg in ticks is unknown, as is the function of the above repeats. Nearly all insect Vgs, also contain a DGXR motif approximately 18 amino acids upstream of the G[L/I]CG site. The DG residues of this motif work together with the G[L/I]CG motif and cysteine residues to produce the secondary structure required for the proper functioning of Vns during embryogenesis (Tufail *et al.*, 2001). A similar motif, DGVN, was found in AhVg1, and it, or a similar DGXX motif, is present in most tick Vgs. Notably, however, both AhVg2 and DvVg2 lack this sequence, and instead

possess a NGSS sequence at this location. The significance of this alteration and its potential effect on the protein's structure is unknown.

The vast majority of insect Vgs are cleaved into two subunits, in the fat body (Chen & Raikhel, 1996), by pro-protein convertases (PCs), a type of subtilisin-like endoproteases (Barr, 1991; Rouillé *et al.*, 1995). These endoproteases usually cleave the precursor proteins at the C-terminal side of a conserved [R/K]–$[X]_n$–[R/K] motif, where X_n can be any 0, 2, 4, or 6 amino acids, following a single, paired or quartet of basic amino acids in the recognition motif (Matthews *et al.*, 1994; Rouillé *et al.*, 1995; Rholam & Fahy, 2009). In insects, the recognition site is generally [R/K]-X-[R/K]-R, although RXXR is also used (Sappington & Raikhel, 1998). This site has also been found in tick Vg sequences, but there is currently no conclusive evidence confirming that the tick Vgs described are actually cleaved at these sites (Thompson *et al.*, 2007; Horigane *et al.*, 2010; Boldbaatar *et al.*, 2010; Khalil *et al.*, 2011). Generally, in order for cleavage to occur, the recognition site alone is not sufficient; rather, PC mediated processing occurs only if the recognition site is directly adjacent to a predicted β-turn, or is part of an Ω-loop and, as such, is more readily accessible to the PC (Brakch *et al.*, 2000; Rholam & Fahy, 2009).

Previous studies have suggested that there are approximately seven Vn subunits in *A. hebraeum* (Friesen & Kaufman, 2002, 2004). The authors described 211, 171, 157, 148, 98, 66 and 62 kDa proteins present in the ovaries of females d10pE and isolated 211 kDa and 148 kDa Vg proteins from the haemolymph of females d10pE, which where then used to produce an anti-Vg antibodies that

recognized 219, 211, 169 and 78 kDa proteins in d10pE female fat body (Friesen & Kaufman, 2002, 2004). The large 219 kDa protein present in the fat body may correspond to the unprocessed form of AhVg2, which is estimated to be ~218 kDa prior to removal of the signal peptide and ~216 kDa afterwards. AhVg1 has two RXXR sites, the first, RLFR (aa 465 – 468), is homologous to the single cleavage motif present in the DvVg1 sequence and, if cleaved, would produce ~50 and 164 kDa subunits, neither of which match the previously described Vn fragments. In neither species is this site directly adjacent to a predicted β-turn, though it does appear to be located in an ~14 residue region between two type VIII predicted β-turns. In *D. variabilis*, the size of the predicted cleavage products matche the size of two experimentally obtained Vn fragments (Thompson *et al.*, 2007), suggesting that this site is cleaved in *D. variabilis*. The second cleavage motif in AhVg1, RKIR (aa 1870 – 1873), is directly followed by a β-turn, and if cleaved would produce ~210 and 3 kDa subunits, the larger of which is similar in size to the 211 kDa Vg protein present in the haemolymph of females 10 days post-engorgement, but not in non-engorged female haemolymph (Friesen & Kaufman, 2002). If both sites present in *A. hebraeum* were to be cut, three subunits ~3, 50 and 161 kDa would be produced, none of which match the previously described *A. hebraeum* Vn band sizes.

AhVg2 has three RXXR sites, the first, RVRR (aa 851 – 854), is homologous to the RVRR and RIRR sequences in DvVg2 and OmVg respectively, and is adjacent to the second RXXR site, RSLR (aa 854 – 857), which aligns with the RGVR site in DvVg2. In all three species these sites are not

adjacent to a predicted β-turn; however, they are within an ~15 residue region between two potential type VIII β-turns, possibly indicative of an Ω-loop region. If either site of the AhVg2 were cleaved, ~94 and 122 kDa subunits would be produced, sizes that do not match well with the Vn bands previously described. The third RXXR site in AhVg2 is RVVR (aa 1153 – 1156), similar to the DvVg2 RLVR site. In both species this site is adjacent to a predicted β-turn and upon cleavage at this position, ~128 and 88 kDa subunits would be produced. In the event all three sites were to undergo cleavage, four subunits ~0.4, 34, 88 and 94 kDa would be produced. In general, the predicted cleavage products from the AhVgs do not match the Vn band sizes observed in either the ovary or the fat body of replete female ticks, nor do any of the predicted products match the 148 kDa Vg band seen in the haemolymph. It is possible that one or more Vgs are present in *A. hebraeum* that have yet to be identified. Both AhVgs identified in this study were isolated from the fat body. It is possible that additional Vgs expressed only in the midgut could account for some of the missing bands. Of the three Vgs isolated from *H. longicornis*, HlVg1 is expressed only in the midgut (Boldbaatar *et al.*, 2010). Both AhVgs also possess many other potential dibasic cleavage sites in addition to numerous KXXK sites that could be potentially cleaved by other dibasic endoproteases, to produce a large array of possible Vn subunits. Determination of Vg and Vn subunits via SDS-PAGE is difficult due to the possibility of dimers (Friesen & Kaufman, 2002), the potential for the presence of artifacts (Sappington & Raikhel, 1998), and the difficulty in conclusively determining the identity of a given band.

Vitellogenin is only one of the two major haem-binding storage proteins present in ticks, the second being a haemoglycolipo-carrier protein (CP). Both CP and Vg are high molecular weight (~200 kDa) members of the lipid transfer protein superfamily, able to bind lipids, carbohydrates and haem. Additionally, based on sequence homology, these two genes are likely derived from a common ancestral gene (Donohue *et al.*, 2009). CPs have been characterized in *A. americanum* (Madden *et al.*, 2004; Cordill, 2005), *D. variabilis* and *O. parkeri* (Gudderra *et al.*, 2001, 2002), with similar HeLP and HLGP proteins identified in *R. microplus* (Maya-Monteiro *et al.*, 2000) and *D. marginatus* (Dupejova *et al.*, 2011), respectively. Consensus sequences homologous to the *D. variabilis* CP have been identified in *R. appendiculatus* (Nene *et al.*, 2004), *A. variegatum* (Nene *et al.*, 2002) and *A. cajennense* (Batista *et al.*, 2008). EST databases and sequence data from the *I. scapularis* genome project also show two putative CPs present in that species. Compared to Vgs, such as those in *D. variabilis* (Thompson *et al.*, 2007; Khalil *et al.*, 2011) and *O. moubata* (Horigane *et al.*, 2010), which are expressed exclusively in females after they have mated and fed to repletion, CPs are generally expressed in both sexes following feeding (Donohue *et al.*, 2008). Comparison of the AhVg sequences identified in this work with those of other tick Vgs and CPs, suggests that both sequences are most likely Vgs and not CPs; both are expressed only in females following mating and engorgement, with the main tissue sources being the fat body and midgut. As well, both BLAST searches and phylogenetic analysis of the protein sequences, group the AhVgs with other tick Vgs and not with CPs (Fig. 6.9). Interestingly, the

HlVgs do not group cleanly with the other tick Vgs. HlVg2, as has been previously noted by Donohue *et al.* (2009), groups closely together with the tick CPs and contains the conserved sequences present in other CPs (Fig 6.8, 6.9). The HlVg2 mRNA, along with that of HlVg3 appears to be weakly expressed in fed males (Boldbaatar *et al.*, 2010), which although common for CPs, is not usual for tick Vgs. Due to the similarities between CPs and Vgs in ticks, it is difficult to determine identity based solely upon homology searches. As a result, Khalil *et al.* (2011) proposed that CPs and Vg could be distinguished by (1) examining expression patterns, because Vgs are usually only present in engorged females whereas CP is more ubiquitously expressed throughout feeding and (2) by careful examination of the protein sequence for the highly conserved N-terminal amino acids, which typically follow the signal peptide cleavage site and the single RXXR cleavage site. Although HlVg2 appears to be a CP, and HlVg3 has elements of both Vgs and CPs, by these criteria, both AhVgs appear to be Vgs.

6.4.3 Localization and developmental regulation of the AhVgR, AhVg1 and AhVg2 messages

As previously mentioned, the ecdysteroid 20-hydroxyecdysone (20E), rather than JH, appears to be the main regulator of vitellogenesis in ticks. Injection of 20E into *D. variabilis* (Thompson *et al.*, 2005; Khalil *et al.*, 2011) and *A. hebraeum* (Friesen & Kaufman, 2002; Seixas *et al.*, 2008) stimulates the production of Vg, whereas injection or topical applications of JH had no effect on vitellogenesis (Friesen & Kaufman, 2004; Thompson *et al.*, 2005). Moreover, Neese *et al.* (2000), in a comprehensive study, failed to show the presence of JH

or its biosynthesis in *D. variabilis* and *O. parkeri*.

Treatment of *D. variabilis* with 20E is sufficient to stimulate uptake of the Vg into the oocytes, implying that the VgR is also expressed at this point and is to some degree synchronized with Vg expression (Thompson *et al.*, 2005; Mitchell *et al.*, 2007). This is not the case in *A. hebraeum*. Injection of 20E is able to stimulate the production of Vg, but is not sufficient to stimulate Vg uptake into the oocytes (Friesen & Kaufman, 2004; Seixas *et al.*, 2008). Only when haemolymph collected from engorged females was co-injected with 20E was a significant increase in ovary weight, oocyte size and ovary Vn content observed, suggesting that there is some sort of 'Vg uptake factor' (VUF) that is required to promote the uptake of Vg into the oocyte (Seixas *et al.*, 2008). Like in *D. variabilis* (Mitchell *et al.*, 2007), the AhVgR is expressed only in the ovaries of females following engorgement (Fig. 6.10). In contrast, in parthenogenic *H. longicornis*, the VgR is expressed at low levels in the larvae, nymphs, unfed and partially fed adult females in addition to the ovaries of engorged females (Boldbaatar *et al.*, 2008). Currently, it is not clear how expression of the VgR is regulated in ticks. In the mosquito *Aedes aegypti*, translation of the VgR begins during previtellogenic development, coinciding with elevated levels of juvenile hormone (JH) and is strongly correlated with ecdysteroid titre (Sappington *et al.*, 1996; Cho & Raikhel, 2001; Cho *et al.*, 2006). The role of ecdysteroids in the regulation of the mosquito VgR is supported by the presence of two binding sites for the ecdysone-responsive early gene proteins, E74 and BR, in the AaVgR gene regulatory region 1.5 kb upstream of the VgR gene (Cho *et al.*, 2006).

Additionally, the level of their expression is are correlated with the ecdysteroid titre in the female following her blood meal. The ability of partially fed virgin *D. variabilis* females to transport Vg into their oocytes following injection with 20E implies that the DvVgR must also be upregulated, possibly by 20E, despite the fact that the DvVgR is not normally expressed in partially fed females, but only in females following engorgement. However, regulation of the AhVgR may be different because despite an increase in haemolymph Vg content in females following injection of 20E, no Vg was taken up into the oocytes. This suggests that either the receptor is not expressed at this time, or that some other factor is necessary to allow the AhVgs to bind and be taken into the cell.

Both of AhVg mRNAs were also detected only in mated females that had fed to repletion, with levels of expression increasing as time to oviposition decreased. As seen in other tick species, the AhVgs were expressed predominantly in both the fat body and midgut. Unexpectedly, expression of both AhVg1 and AhVg2 was seen in a mixture of pooled tissues including the synganglion, muscle, Géné's organ, Malpighian tubules, salivary glands, and tracheae four days following engorgement (Fig. 6. 10). By day 10 post-engorgement, there was also a low-level of expression of these genes in the ovary.

Although most insects synthesize Vg in the fat body, some also exhibit Vg synthesis in the follicle cells of the ovary (Brennan *et al.*, 1982; Isaac & Bownes, 1982; Melo *et al.*, 2000). The crustacean *Penaeus japonicus* expresses Vg in the ovary follicle cells in addition to the parenchymal cells of the hepatopancreas (Tsutsui *et al.*, 2000). Whereas low levels of AhVg1 and AhVg2 expression were

observed in the ovaries of females 10 days post engorgement, and very low levels of DvVg1 expression have been observed in the ovary of *D. variabilis* (Thompson *et al.*, 2007; Khalil *et al.*, 2011), whole mount *in situ* hybridization done in *O. moubata* indicated that the OmVg was expressed in the fat body cells associated with the ovary, rather than in the ovary itself (Horigane *et al.*, 2010). Tick ovaries are panoistic, lacking nurse or follicle cells, the developing oocytes sheathed only by the thin, acellular tunica propria, which would seem to make synthesis of Vg in the ovary unlikely. One of the major difficulties in determining the source of Vg synthesis is harvesting most tissues without including fat body and tracheae. In ticks, the fat body is an amorphous tissue, consisting of highly dispersed cells that often cling to the tracheae. It is particularly abundant near the basal tracheal trunks in close proximity to the spiracular plate, but it is often also found growing around all the major organs, and in the case of the ovary and salivary gland, infiltrates between the acini and oocytes respectively, making it exceedingly difficult to see and essentially impossible to remove. Likewise, tracheae are associated with all organs, and it is impossible to completely separate them from the dissected tissues. Although the overall amount of fat body contamination for a given piece of tissue is generally quite small compared to the size of the organ being harvested, Vg is very highly expressed at certain times of development, and thus a misleading picture of Vg expression may be generated due to this contamination.

6.4.4 The effects of RNA interference on vitellogenesis

RNA interference is a powerful tool for studying gene function and

disrupting gene expression in ticks (de la Fuente *et al.*, 2007; Smith *et al.*, 2009). Whereas complete silencing of the AhVgR was not achieved, a fair degree of gene knock-down was, resulting in significantly decreased oocyte length, delayed ovarian development, and a longer latency to oviposition, demonstrating the importance of the AhVgR in the transport of Vg into the developing oocytes. Unlike in *D. variabilis* or *H. longicornis*, complete abolition of yolk-uptake was not observed in the VgR-dsRNA injected females, despite using four times the amount of dsRNA construct than was used in the *D. variabilis* experiments. Following engorgement, *A. hebraeum* requires about 6 – 7 days to fully develop its ovary (OGP 4), and 10 – 11 days to begin oviposition under our colony conditions. It is possible that during this developmental period, the tick produces sufficient AhVgR transcript to overcome the effects of silencing, resulting in a developmental delay as opposed to complete abolition of expression. Although it is possible that there could be a second VgR, or another lipoprotein receptor able to transport Vg into the oocyte, this seems unlikely for two reasons. (1) Only one VgR has been found in both *D. variabilis* (Mitchell *et al.*, 2007) and *H. longicornis* (Boldbaatar *et al.*, 2008). (2) In a small number of the AhVgR-dsRNA injected ticks, a high degree of knock-down of the AhVgR was observed, resulting in no yolk uptake into the oocyte.

The results of the AhVg1 and AhVg2 knock-down were less impressive; only a slight decrease in oocyte length was observed in the AhVg1 and AhVg1+2-dsRNA-injected ticks. It may be that the ticks were able to overcome silencing because of the abundant Vg transcript, resulting in only a very slight

developmental delay that was quickly made up. It is also possible that, as previously mentioned, additional, unidentified Vg-genes are present in this species, which could have compensated for the silencing of AhVg1 and AhVg2.

6.4.5 Conclusions

The full-length sequences of the vitellogenin receptor and two vitellogen mRNAs are presented in this study. As discussed above, the AhVgR sequence displays the characteristic features of a vitellogenin receptor and both AhVg sequences possess the various features generally found in other Vgs, but not those specific to the closely related CPs. All three mRNAs were shown to be female specific, and expressed only after mating and engorgement. The AhVgR was expressed only in the ovary, whereas both AhVgs were expressed primarily in the fat body and midgut, but with unexpected levels of expression in other tissues as well. Much work remains to be done to understand the process of vitellogenesis in *A. hebraeum*, particularly with regard to the regulation of the VgR and the uptake of Vg into the oocytes.

Table 6.1 Sequences used in degenerate primer design

Target gene	Species	NCBI Accession number	
		GenBank	GenPept
VgR	*D. variabilis*	DQ103506	AAZ31260
	H. longicornis	AB299015	BAG14342
	I. scapularis	XM_002400052	XP_002400096
Vg1	*D. variabilis*	AY885250	AAW78557
	I. scapularis	XM_002415179	XP_002415224
	R. microplus	EU086096	ABS88989
Vg2	*D. variabilis*	EU204907	ABW82681
	I. scapularis	XM_002403922	XP_002403966
	O. moubata	AB440159	BAH02666

Table 6.2 Primers used for initial gene amplification by degenerate PCR

Target	Primer ID	Direction	Sequence (5'-3')*	Degeneracy	AA consensus***	Primer combinations	Amplicon (bp)
AhVgR	ADS125	Forward	GCACCAGAACCTGTAC**TGGGTNGAYGC**	8	HQNLYWVDA	ADS125 + ADS130	2037
	ADS130	Reverse	CGACGTGGTAGTCCATG**CCRTRCATRTC**	8	DMHGMDYHVA	ADS125 + ADS131	2379
	ADS131	Reverse	CCGGGCGGTG**CCRTCCATRTT**	4	NMDGTTR	ADS125 + ADS132	2628
	ADS132	Reverse	CGGCCGGTGTGCTTG**TTRCANGCCAT**	8	MACNKHTGR		
AhVg1	ADS134	Forward	GCACGTGCTGAACGTG**ACNYTNGARGG**	64	HVLNVTLEG	ADS134 + ADS139	1269
	ADS136	Forward	CGAGCCATTCGTGGTG**ACNTAYAAYGC**	16	EPFVVTYNA	ADS136 + ADS139	1008
	ADS139	Reverse	ACGAACTCGTGGTATGGC**TCRTTRTCRTA**	8	YDNEPYHEFV	ADS200 + ADS203	816
	ADS189**	Reverse	CGAAAGTGGGCTTCACGAGC	N/A	N/A	ADS136 + ADS139	1008
	ADS200	Forward	TTCAAGGCCTACCCG**GANAARGARAC**	16	FKAYPDKET	ADS201 + ADS189	1100
	ADS201	Forward	GAAGACCGTGCCAACC**AARTGGGAYYT**	8	KTVPTKWDL		
	ADS203	Reverse	CAGGACCACCTGCTGC**ARRTCCCAYTT**	8	KWDLQQVVL		
AhVg2	ADS194	Forward	CCGAGCACCCAGTG**GAYGARTAYAA**	8	QHPVDEYK	ADS194 + ADS198	1308
	ADS195	Forward	CACGACCTGCAGACC**CCNRTNTAYGA**	64	HDLQTPIYE	ADS195 + ADS198	1170
	ADS198	Reverse	CCAGGGGGTAGGTCACG**TTRTCRAANGT**	16	TFDNVTYPLE		

*Boldfaced sequence indicates the primers' 11 nucleotide degenerate core.

**ADS189 was designed from AhVg1 sequence following the initial round of degenerate PCR and is not degenerate itself.

Table 6.3 Primers used for cloning and 5'/3' RACE

Target	Primer ID	Direction	Sequence (5'-3')	Purpose	Location on gene
UAP	ADS146	Reverse	GGCCACGCGTCGACTAGTAC$(T)_{17}$	5'/3'RACE	NA
AUAP	ADS147	Reverse	GGCCACGCGTCGACTAGTAC		NA
VgR	ADS150	Forward	GTTTCTCGATGCACGGCCACG	3'RACE	4043 – 4063
	ADS151	Forward	GCTGGCTGAAGACACTCTTGCG		4074 – 4095
	ADS166	Forward	CTGCGTAGAATCCGATGACCG		4902 – 4922
	ADS167	Forward	GTAGCCTTGCTTGTCCTCGG		5143 – 5162
	ADS168	Reverse	CCACCATTGAGGCAAACCGG		4988 – 4969
	ADS152*	Reverse	TAAGGCGAGTCCAACCGGG**T**G	5'RACE	2211 – 2191
	ADS153	Reverse	CCATTGGCTCGGCACACCTC		2156 – 2137
	ADS148	Forward	GGATCGCGCCTGCTTCTGC	Cloning	2703 – 2721
	ADS149	Reverse	CAGGCGATGCACTCGTGAGATC		3689 – 3668
	ADS185	Forward	GACTTGGCCGCCGGACTT		6 – 23
	ADS186**	Reverse	TTTCAGTGTAAAAAGTAT**R**CGACTTT		5661 – 5636
	ADS191	Forward	CATCGGTTCCGTCTTCGGCATG		2058 – 2079
Vg1	ADS158	Forward	ACCACCCAGGACGTCGTGC	3'RACE	4740 – 4758
	ADS159	Forward	GACCTTCAAGCTGCTGTTCGACG		4853 – 4875
	ADS177	Forward	CTAGCCACCACCAGTACCCTG		5365 – 5385
	ADS160	Reverse	CACGAACCAGTGCAGCAGCG	5'RACE	4058 – 4039
	ADS161	Reverse	ACGTACCAGAAGGGGCTCGG		3898 – 3879
	ADS188	Reverse	GACGGCGCTAAGCAGGTAAC		3164 – 3145
	ADS189	Reverse	CGAAAGTGGGCTTCACGAGC		3141 – 3122
	ADS207	Reverse	CACGGGCACGGAGTAGTAG		1616 – 1598
	ADS208	Reverse	CGACCTTGGAGGGGCAGGC		1596 – 1578
	ADS225	Reverse	GCACACGCCGTAGTAGCCGG		728 – 709
	ADS226	Reverse	CGGGTGACCTTGCAGTCGTCC		676 – 656
	ADS187	Forward	CTACGACACCACCGTCGAG	Cloning	3089 – 3106
	ADS204	Forward	GAGGGTCGCTGTCTGCGAC		1535 – 1553
	ADS206	Reverse	CAGAAGGCCTCCCTTGGAG		2516 – 2498
Vg2	ADS221	Forward	GGACCTCTACACCGGATATAC	3'RACE	4683 – 4703
	ADS222	Forward	GCCAACCTTACCGTGTACAC		4708 – 4727
	ADS227	Forward	GGTCATGCGTCCTAAGTACACGG		5265 – 5287
	ADS209	Reverse	CACGTTGTACCTCACGGTGAG	5'RACE	945 – 925
	ADS210	Reverse	GTTTCCTTCGGGCATCCGG		893 – 875
	ADS219	Forward	TCTGCTCTGGGTCCCGTGG	Cloning	3901 – 3919
	ADS220	Reverse	GGCTGACAAGGCAGGTCGG		4864 – 4846
	ADS211	Forward	CTTGTCGAGCCCGAGTACTC		832 – 851
	ADS212	Reverse	CCTTCTTCTTGAATTCAGTGCTG		1732 – 1710

*This primer was generated with an error in the second to last base, the T should have been a C.

**The 19th base is degenerate due to its design prior to all sequence ambiguities being resolved.

Table 6.4 Sequencing primers

Target	Primer ID	Direction	Sequence (5'-3')	Location on gene
T7 promoter	ADS15	Forward	TAATACGACTCACTATAGGG	N/A
SP6 promoter	ADS16	Reverse	TATTTAGGTGACACTATAG	N/A
M13 (-20)	ADS73	Forward	GTAAAACGACGGCCAG	N/A
M13	ADS74	Reverse	CAGGAAACAGCTATGAC	N/A
pJET1.2 F seq	ADS238	Forward	CGACTCACTATAGGGAGAGCGGC	N/A
pJET1.2 R seq	ADS239	Reverse	AAGAACATCGATTTTCCATGGCAG	N/A
VgR	ADS151	Forward	GCTGGCTGAAGACACTCTTGCG	4074 – 4095
	ADS153	Reverse	CCATTGGCTCGGCACACCTC	2156 – 2137
	ADS166	Forward	CTGCGTAGAATCCGATGACCG	4902 – 4922
	ADS167	Forward	GTAGCCTTGCTTGTCCTCGG	5143 – 5162
	ADS168	Reverse	CCACCATTGAGGCAAACCGG	4988 – 4969
	ADS169	Forward	GTTCCTGCTCTACATGCTTCCG	4008 – 4029
	ADS170	Reverse	GCCGTGCATATCCGCAAGAG	4107 – 4088
	ADS171	Forward	GCTGCGGCGATGGTCAGTG	3395 – 3413
	ADS172	Reverse	CCAGTAGAGGGGAAGGCAC	3060 – 3042
	ADS183	Forward	GTCACAACAATGAGTGCATCCC	428 – 449
	ADS184	Reverse	GGTGGCAAGGTCCAAGTACTC	1497 – 1477
Vg1	ADS158	Forward	ACCACCCAGGACGTCGTGC	4740 – 4758
	ADS159	Forward	GACCTTCAAGCTGCTGTTCGACG	4853 – 4875
	ADS161	Reverse	ACGTACCAGAAGGGGCTCGG	3898 – 3879
	ADS177	Forward	CTAGCCACCACCAGTACCCTG	5365 – 5385
	ADS178	Reverse	GAGGGTACTGGTGGTGCTC	5424 – 5406
	ADS179	Forward	GAACCCTTACGTGACCGTGG	4319 – 4338
	ADS180	Reverse	GAGCTTCTTGGTTCCGATGGTG	4640 – 4619
	ADS188	Reverse	GACGGCGCTAAGCAGGTAAC	3164 – 3145
	ADS205	Forward	CCTGGTCGCCTACCTGAAC	1814 – 1832
	ADS226	Reverse	CGGGTGACCTTGCAGTCGTCC	676 – 656
Vg2	ADS213	Forward	GTCTCGTCTTCTCGTCATCC	1631 – 1650
	ADS214	Reverse	CCGCGGACACATGAAGGAG	2557 – 2539
	ADS215	Forward	GCAACACCTCCCTCGTGCC	2468 – 2486
	ADS216	Reverse	GCCAGGTAAAGAGGGTTCAC	3460 – 3442
	ADS217	Forward	CGTTCGAGTACAAGAAGCTCC	3362 – 3382
	ADS218	Reverse	CAGGGGTCCGTACTTCGGG	4152 – 4134
	ADS221	Forward	GGACCTCTACACCGGATATAC	4683 – 4703
	ADS227	Forward	GGTCATGCGTCCTAAGTACACGG	5265 – 5287

Table 6.5 Primers for gene expression analysis

Target	Primer ID	Direction	Sequence (5'-3')	Amplicon (bp)
Ah16S	ADS89	Forward	CTGCTCAATGATTTTTTAAATTGCTGTGG	456
	ADS90	Reverse	CCGGTCTGAACTCAGATCAAGT	
AhVgR	ADS151	Forward	GCTGGCTGAAGACACTCTTGCG	915
	ADS168	Reverse	CCACCATTGAGGCAAACCGG	
AhVg1	ADS205	Forward	CCTGGTCGCCTACCTGAAC	703
	ADS206	Reverse	CAGAAGGCCTCCCTTGGAG	
AhVg2	ADS217	Forward	CGTTCGAGTACAAGAAGCTCC	791
	ADS218	Reverse	CAGGGGTCCGTACTTCGGG	

Table 6.6 Sequences and accession numbers used for phylogenetic analyses

Subphylum	Species	Gene					
		VgR	Vg1	Vg2	Vg3	CP1	CP2
Chelicerata	*Dermacentor variabilis*	AAZ31260	AAW78557	ABW82681		ABD83654	CF35055
	Haemaphysalis longicornis	BAG14342	BAJ21514	BAG12081	BAJ21515		
	Amblyomma hebraeum	JX846592	JX846593	JX846594			
	Amblyomma americanum					ABK40086	
	Ixodes scapularis	XP_002400096	XP_002415224	XP_002403966		XP_002415017	XP_002411435
	Ornithodoros moubata		BAH02666				
	Rhipicephalus microplus		ABS88989	AAA92143*			
Hexapoda	*Bombyx mori*	ADK94452	BAA06397				
	Periplaneta americana	BAC02725	BAA86656	BAB32673			
	Solenopsis invicta	AAP92450	AAP47155	AAY22960	AAY22961		
	*Aedes aegypti***	AAC28497	AAA18221	AAQ92367	AAQ92366		
	Leucophaea maderae	BAE93218	BAB19327	BAD72597			
	Blattella germanica	CAJ19121	CAA06379				
	Antheraea pernyi	AEJ88360	BAB16412				
	Spodoptera litura	ADK94033	ABU68426				
	Nilaparvata lugens	ADE34166	BAF75351				
	Plautia stali		BAA88075	BAA88076	BAA88077		
	Drosophila melanogaster	AAB60217					
	Apis mellifera	XP_001121707	CAD56944				
Crustacea	*Penaeus monodon*	ABW79798	ABB89953				
	Macrobrachium rosenbergii	ADK55596	BAB69831				
	Pandalus hypsinotus		BAD11098				
	Litopenaeus vannamei		AAP76571				
	Cherax quadricarinatus		AAG17936				
	Fenneropenaeus merguiensis		AAR88442				
	Marsupenaeus japonicus		BAB01568				
	Metapenaeus ensis		AAT01139	AAM48287	AAN40701		

*This sequence is actually that of the *R. microplus* GP80 protein

**Vg1, Vg2 and Vg3 are actually VgA, VgB and VgC in *Aedes aegypti*.

Table 6.7 Primers used for the generation of dsRNA template DNA

Target	Primer ID	Direction	Sequence (5'-3')	Amplicon (bp)
VgR	ADS228	Forward	TAATACGACTCACTATAGGCGGGAGTTCTTTTCGTTCTGAG	839
	ADS229	Reverse	TAATACGACTCACTATAGGCCAGTAGAGGGGAAGGCAC	
Vg1	ADS240	Forward	TAATACGACTCACTATAGGCAAGGGCTACCCGCACCAGCC	528
	ADS241	Reverse	TAATACGACTCACTATAGGTCCAAAGTCGGCGTCACGAATTC	
Vg2	ADS242	Forward	TAATACGACTCACTATAGGCCACTCAGCTTGTTGCTGTTGC	576
	ADS243	Reverse	TAATACGACTCACTATAGGCGACTTCGGTAACCACGCACTTCTC	
Bla	ADS234	Forward	TAATACGACTCACTATAGGGCTTAATCAGTGAGGCACCTATC	841
	ADS235	Reverse	TAATACGACTCACTATAGGCATTTCCGTGTCGCCCTTATTC	

Table 6.8 Effects of RNAi treatment on female *A. hebraeum*

Treatment group	Engorged weight (mg)	Ovipositional delay (days)	Ovary weight (% bw)	Ovarian growth phase	Oocyte length (µm)	Egg mass (% bw)	Hatching success (%)	Mortality (%)
Bla	1482 ± 130	10 ± 0.6	4.2 ± 0.7	3.9 ± 0.2	545 ± 7.4	51.6 ± 2.34	75.8 ± 9.2	40.9
TE	1569 ± 199	10.5 ± 0.8	4.7 ± 0.6	3.9 ± 0.2	578 ± 6.7	46.2 ± 7.02	60 ± 14.3	27.8
AhVg1	1363 ± 106	11 ± 0.3	4.3 ± 1.0	4.0 ± 0.4	518 ± 15.4*	49.5 ± 2.65	47.8 ± 19.2	11.1
AhVg2	1487 ± 229	13.5 ± 0.5	8.4 ± 0	4.0 ± 0.0	598 ± 16.2	49.6 ± 0	50 ± 35.0	66.7
AhVg1+2	1691 ± 142	13 ± 1.2	3.5 ± 1.5	3.1 ± 0.4	487 ± 21.9**	52.4 ± 0	40 ± 21.8	28.6
AhVgR	1473 ± 153	15 ± 1.9*	2.8 ± 0.8	2.5 ± 0.2**	359 ± 10.1**	40.9 ± 3.52	40.8 ± 16.2	42.1

* and **: Level of significance with respect to the combined controls is indicated: *$0.01 < p < 0.05$, **$p \leq 0.01$

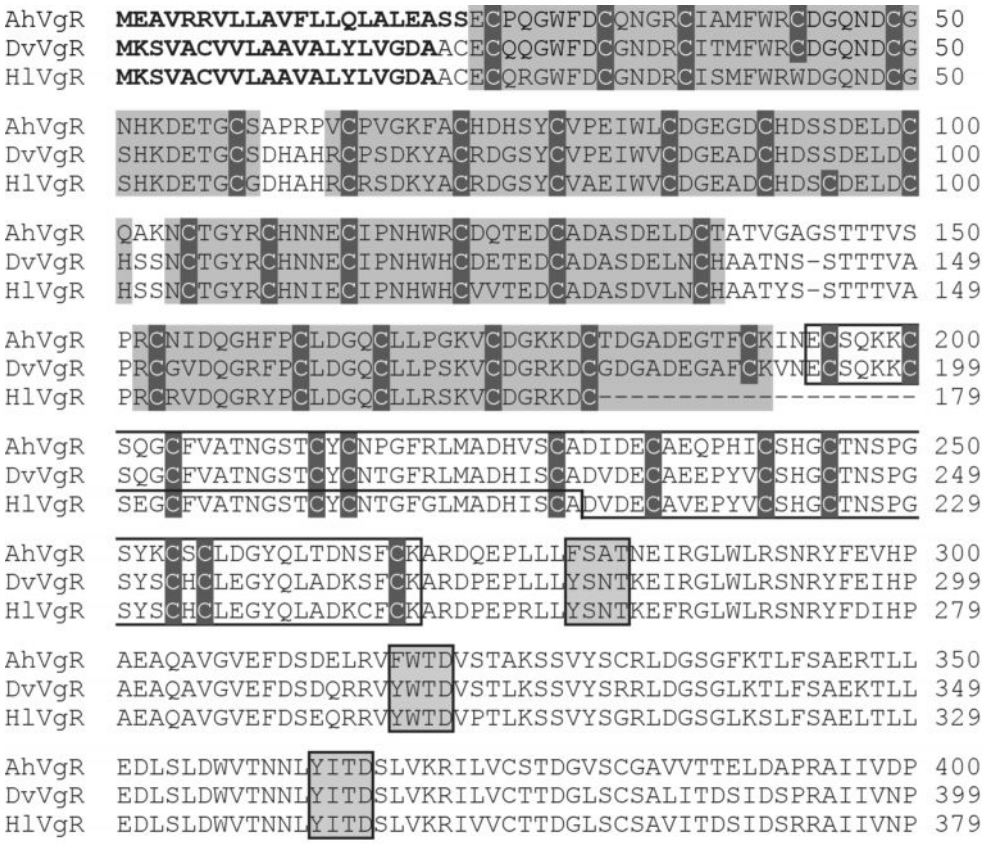

Figure 6.1. The deduced amino acid sequence of the *Amblyomma hebraeum* vitellogenin receptor (JX846592) aligned with those from *Dermacentor variabilis* (DQ103506) and *Haemaphysalis longicornis* (AB299015). The signal peptide sequence is indicated by bold letters, the $LDLR_A$ repeats shown in grey shading and the EGF-like domains enclosed in unshaded boxes. The conserved cysteine residues of these domains are indicated by dark grey shading. The YWXD-like repeats that make up the β-propeller domains are enclosed in grey shaded boxes. The predicted transmembrane domain is marked by a light grey box with grey border and the location of the putative O-linked sugar domain is underlined. The potential internalization signals homologous to the consensus sequence FXNPXF and the di-leucine motif are shown in black shaded boxes.

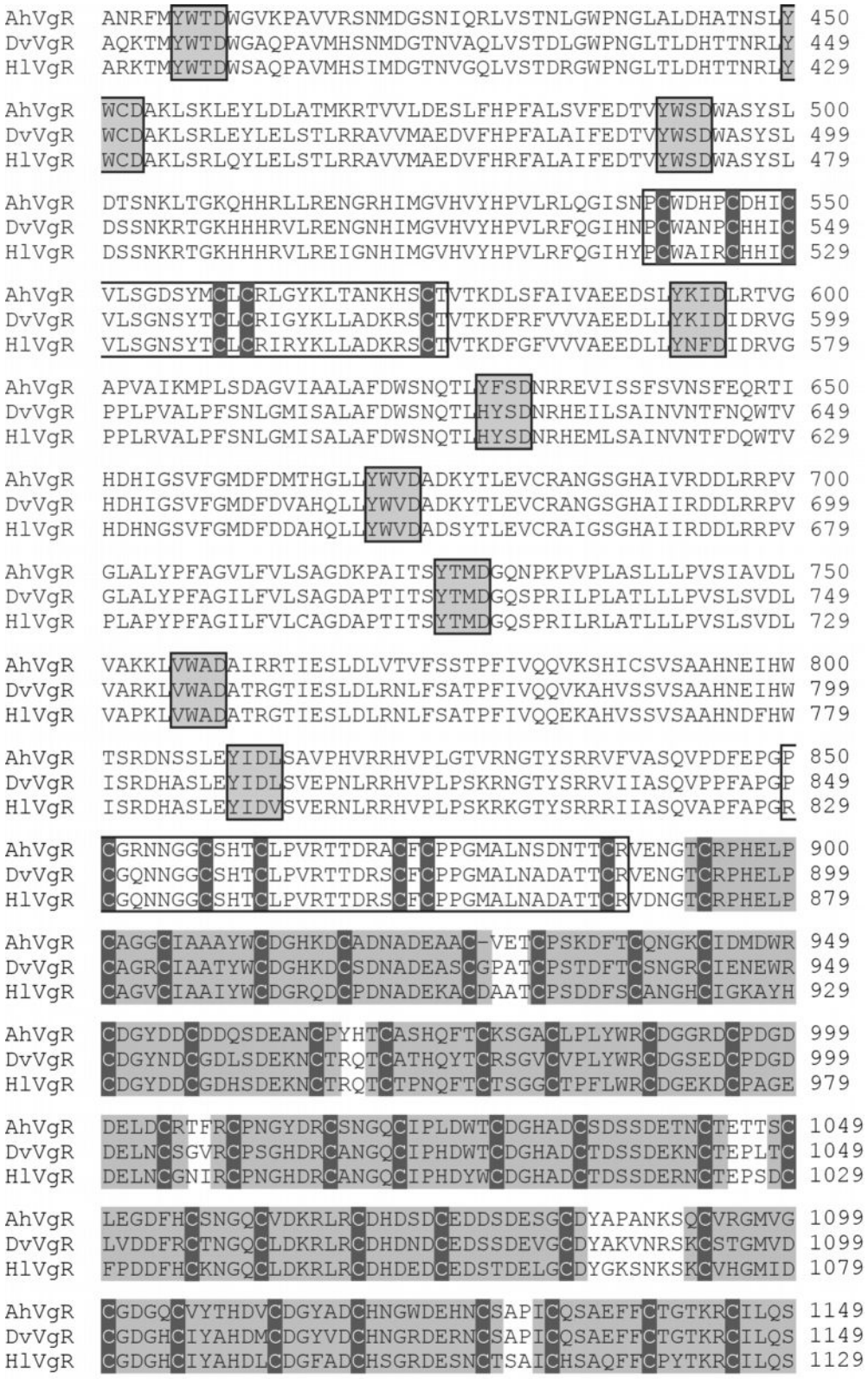

Figure 6.1. (*Continued*)

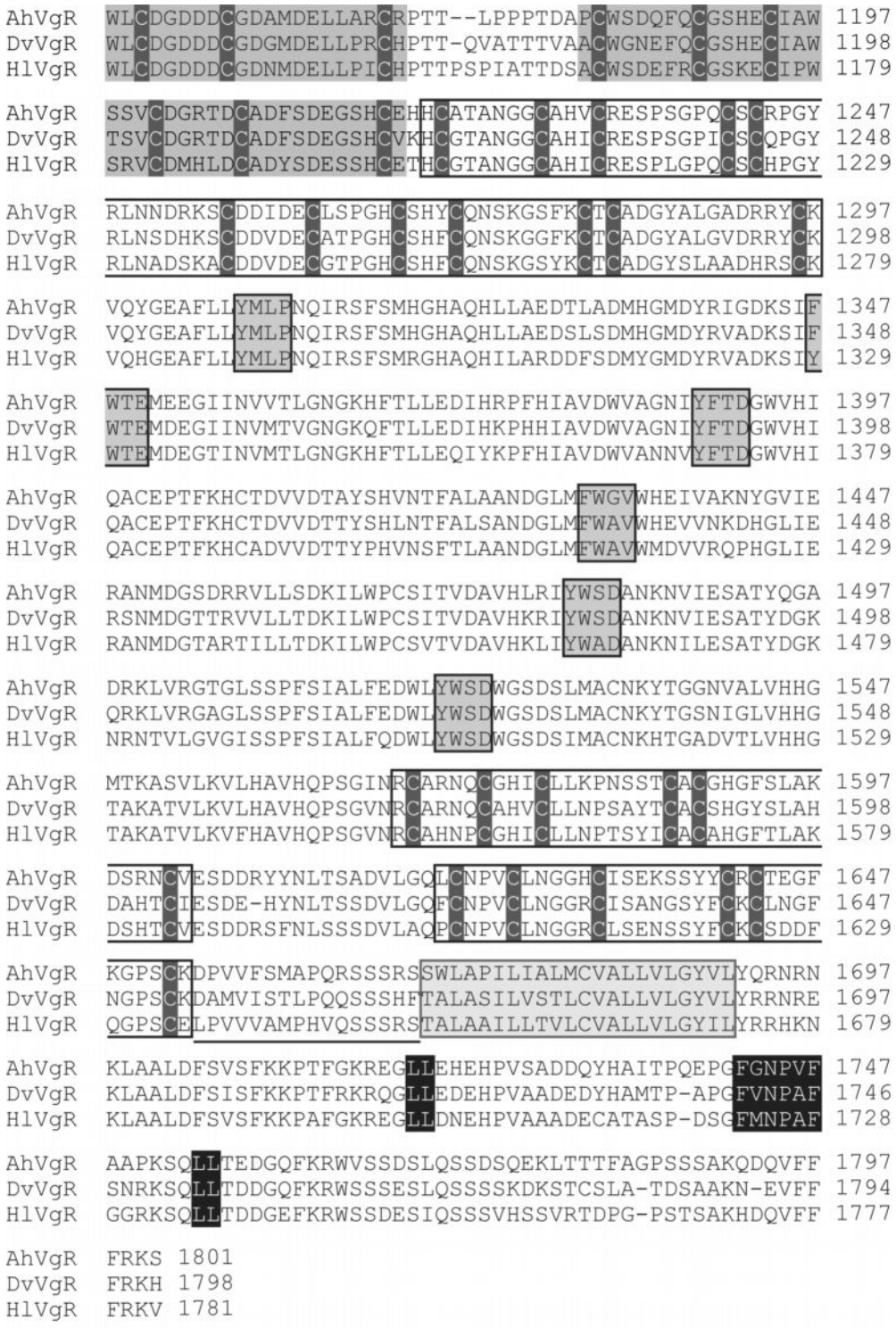

Figure 6.1. (*Continued*)

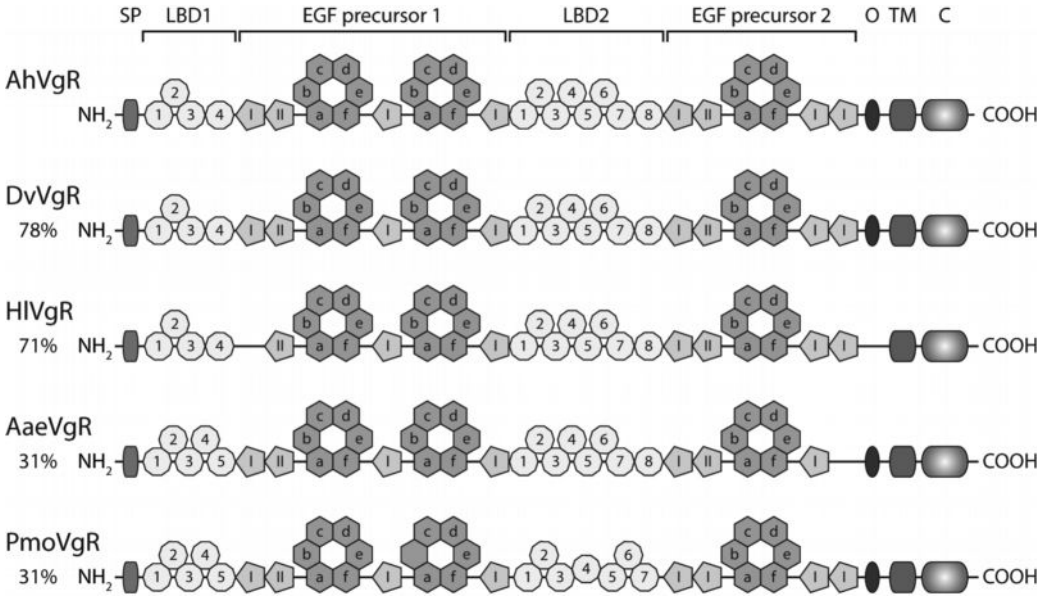

Figure 6.2. Schematic comparison of the *Amblyomma hebraeum* VgR (AhVgR) with those of *Dermacentor variabilis* (DvVgR), *Haemaphysalis longicornis* (HlVgR), *Aedes aegypti* (AaeVgR), and *Penaeus monodon* (PmoVgR). The ligand-binding LDLRA repeats of the lipid binding domains are indicated with the numbers 1-8, while the EGF-like repeats are marked with roman numerals I and II indicating non-calcium binding and calcium-binding repeats respectively. The YWXD containing repeats that form the β-propeller domains are labelled with the letters a-f. The percentages on the left indicate the overall identity of each protein compared to AhVgR. SP, signal peptide; LBD, lipid binding domain; O, potential *O*-linked sugar domain; TM, transmembrane domain; C, cytoplasmic domain. Sequences were obtained from GenBank: DvVgR(AAZ31260), HlVgR (BAG14342), AaeVgR (AAC28497), and PmoVgR (ABW79798).

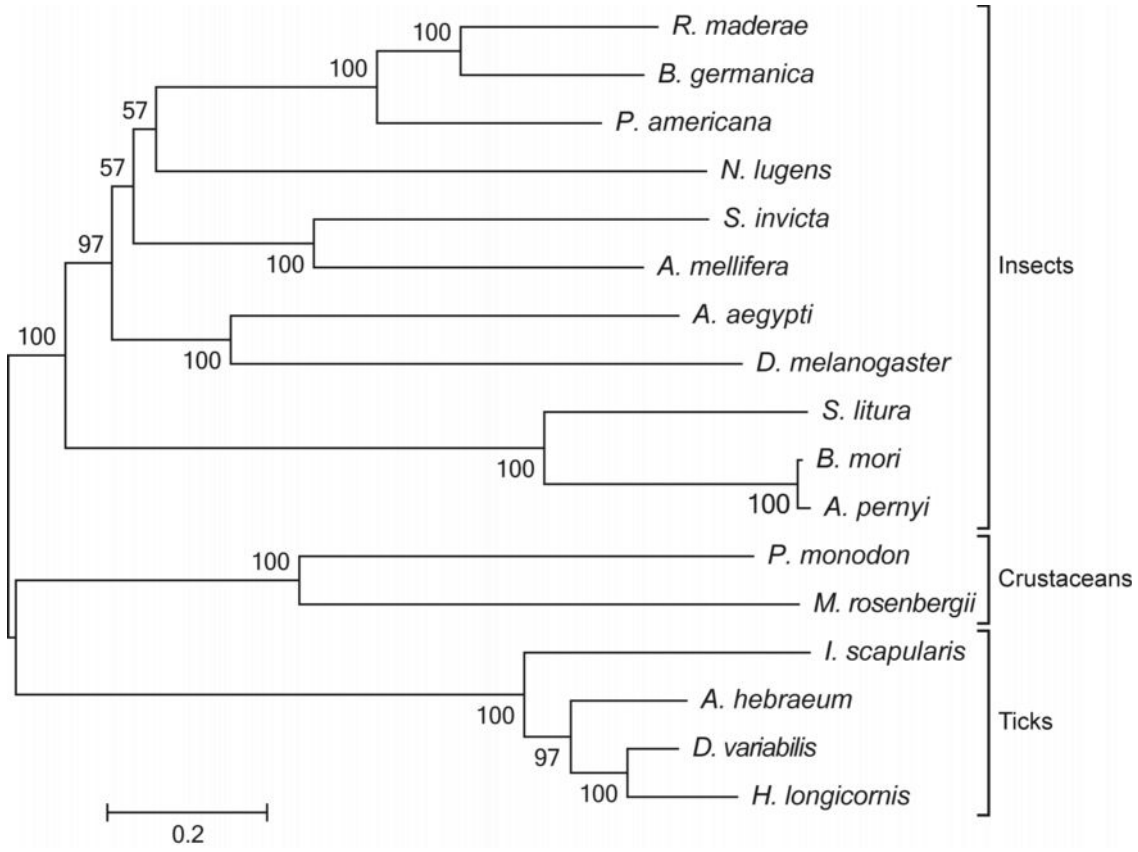

Figure 6.3. Phylogenetic tree of the vitellogenin receptors (VgRs) from various arthropod species. The tree was constructed using the neighbour-joining method, from distance matrices built from amino acid sequences according to the Jones-Taylor-Thornton matrix-based model, using MEGA5. Branches corresponding to partitions reproduced in fewer than 50% of the bootstrap replicates are collapsed. The units of distance are the number of amino acid substitutions per site. Bootstrap support values from 1000 replicates are indicated at the nodes. Sequences were compared pairwise with all ambiguous positions removed for each pair, resulting in a total of 2369 positions in the final dataset. All non *A. hebraeum* amino acid sequences were obtained from GenBank. Tick amino acid sequences were obtained from *Ambylomma hebraeum* (JX846592), *Dermacentor variabilis* (AAZ31260), *Haemaphysalis longicornis* (BAG14342), and *Ixodes*

scapularis (XP_002400096). Insect amino acid sequences were from *Aedes aegypti* (AAC28497), *Antheraea pernyi* (AEJ88360), *Apis mellifera* (XP_001121707), *Blattella germanica* (CAJ19121), *Bombyx mori* (ADK94452), *Drosophila melanogaster* (AAB60217), *Leucophaea maderae* (BAE93218), *Nilaparvata lugens* (ADE34166), *Periplaneta americana* (BAC02725), *Solenopsis invicta* (AAP92450), and *Spodoptera litura* (ADK94033). Crustacean amino acid sequences were from *Macrobrachium rosenbergii* (ADK55596), and *Penaeus monodon* (ABW79798).

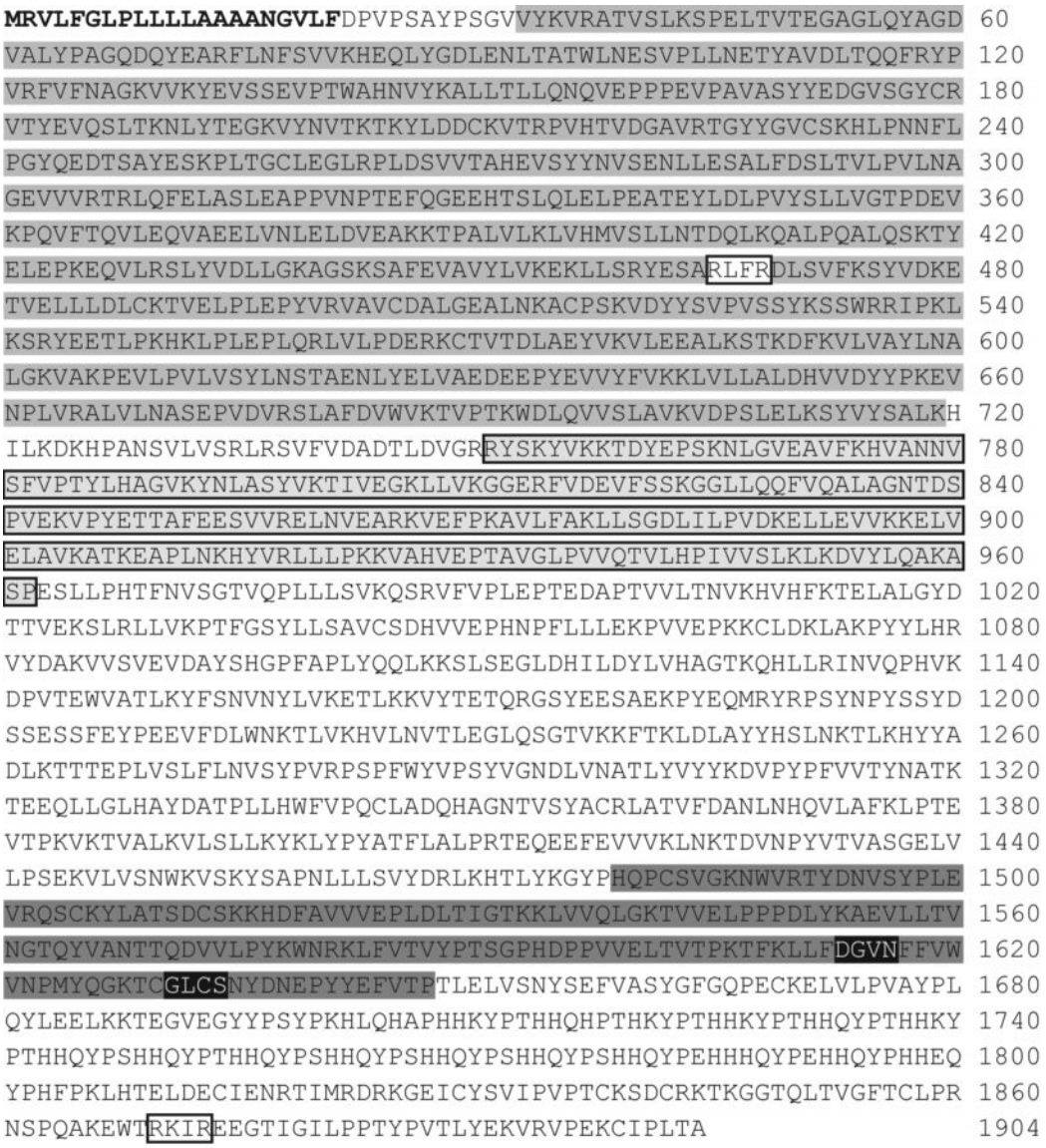

Figure 6.4. Complete deduced amino acid sequence of AhVg1. The predicted signal peptide sequence is in bold text and the putative RXXR cleavage sites are indicated by open boxes. The conserved GLCS and DGVN sites are shown with black backgrounds. The N-terminal lipoprotein domain is shown in grey shading, the DUF1943 domain is boxed in light grey with black borders, and the von Willebrand factor type D domain marked by dark grey shading.

Figure 6.5. Alignment of the partial amino acid sequences of AhVg1 and AhVg2 with other arthropod Vg sequences available in the GenBank database. The alignment shows the conserved DGXR and GLCG regions present in tick and insect Vgs, but not in crustacean Vgs. Identical residues are shown with black backgrounds while similar residues are shown with grey backgrounds. Tick amino acid sequences: AhVg1: *Amblyomma hebraeum* Vg1 (JX846593), AhVg2: *A. hebraeum* Vg2 (JX846594), DvVg1: *Dermacentor variabilis* Vg1 (AAW78557), DvVg2: *D. variabilis* Vg2 (ABW82681), HlVg1: *Haemaphysalis longicornis* Vg1 (BAJ21514), HlVg2: *H. longicornis* Vg2 (BAG12081), HlVg3: *H. longicornis* Vg3 (BAJ21515), IsVg1: *Ixodes scapularis* putative Vg (XP_002415224), IsVg2: *I. scapularis* putative Vg (XP_002403966), OmVg: *Ornithodoros moubata* Vg (BAH02666), RmVg: *Rhipicephalus microplus* partial Vg (ABS88989), RmGP80: *R. microplus* GP80 partial precursor (AAA92143). Insect amino acid sequences:

AaeVgA: *Aedes aegypti* VgA (AAA18221), AaeVgB: *A. aegypti* VgB (AAQ92367), AaeVgC: *A. aegypti* VgC (AAQ92366), ApeVg: *Antheraea pernyi* Vg (BAB16412), BgeVg1: *Blattella germanica* Vg (CAA06379), BmoVg: *Bombyx mori* Vg (BAA06397), LmaVg1: *Leucophaea maderae* Vg1 (BAB19327) LmaVg2: *L. maderae* (BAD72597), NluVg: *Nilaparvata lugens* Vg (BAF75351), SliVg: *Spodoptera litura* Vg (ABU68426). Crustacean amino acid sequences: CquVg: *Cherax quadricarinatus* Vg (AAG17936), FmeVg: *Fenneropenaeus merguiensis* Vg (AAR88442), LvaVg: *Litopenaeus vannamei* Vg (AAP76571), PmoVg: *Penaeus monodon* Vg (ABB89953).

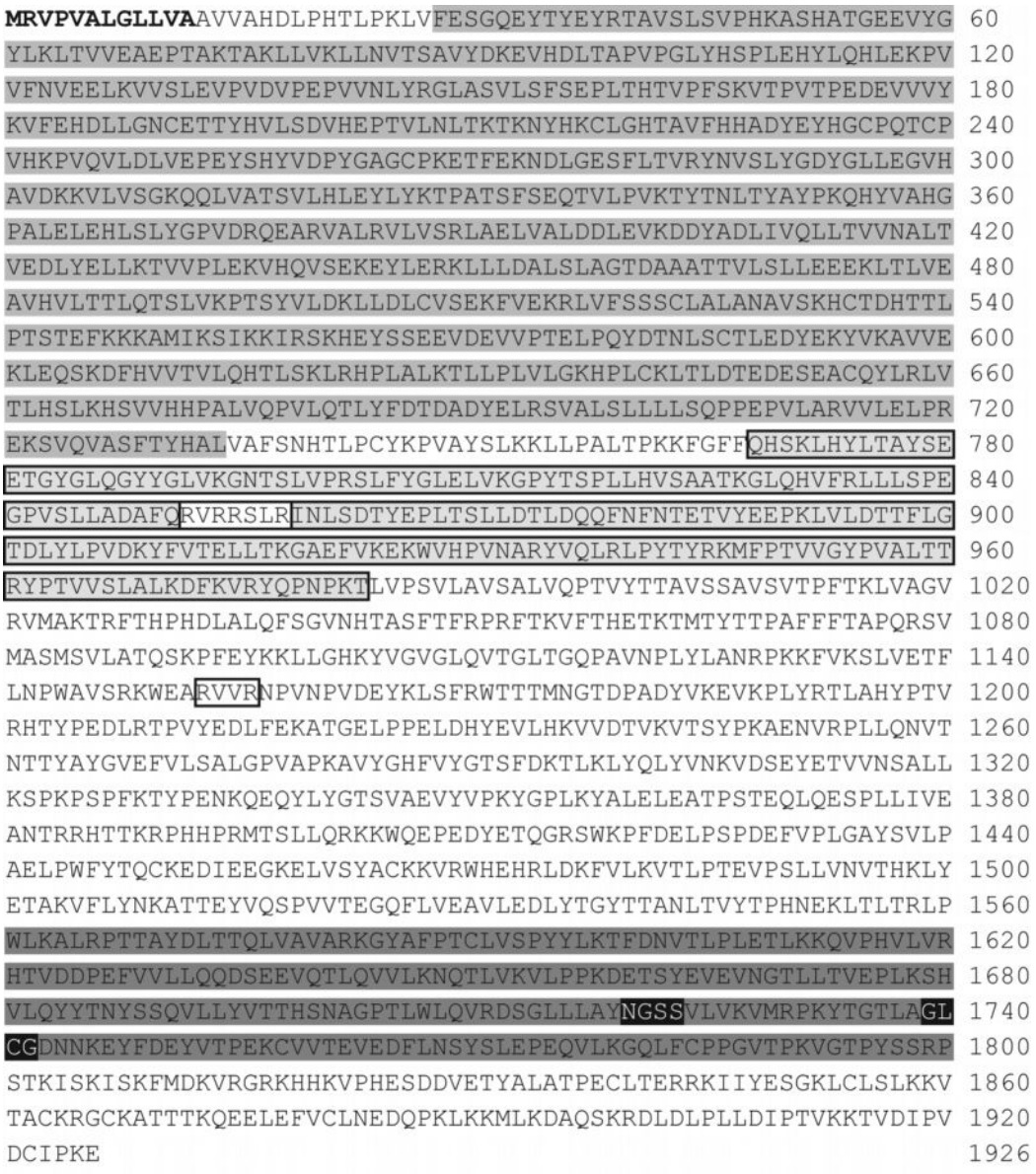

Figure 6.6. Complete deduced amino acid sequence of AhVg2. The consensus RXXR sites of potential cleavage are indicated by open boxes and the conserved GLCG site as well as the NGSS variant of the DGXR site shown with black backgrounds. The predicted signal peptide sequence is marked by boldface type and the N-terminal lipoprotein domain is shaded in grey. The DUF1943 domain is boxed in light grey with black borders, and the von Willebrand factor type D domain marked by dark grey shading.

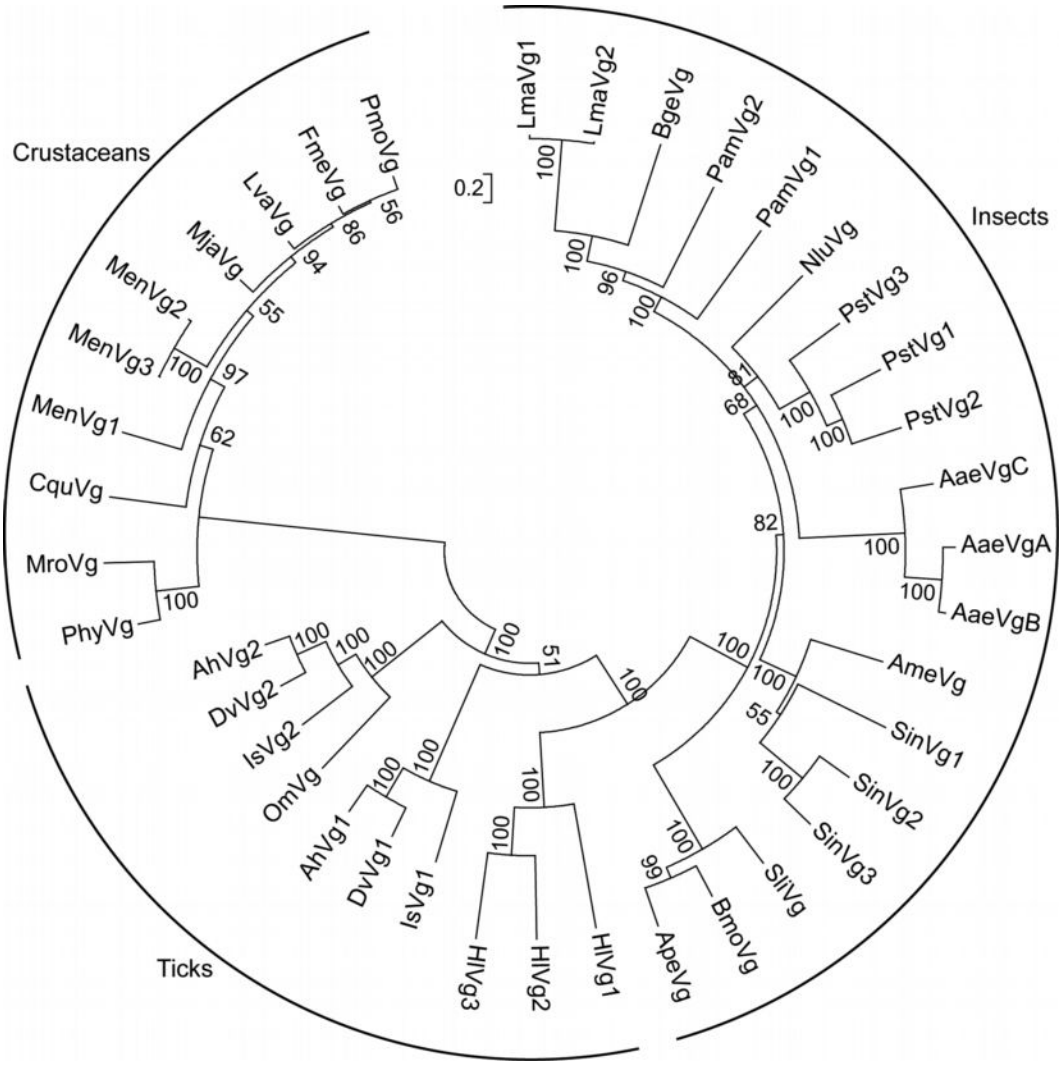

Figure 6.7. Phylogenetic tree of the vitellogenins (Vg) from various arthropod species. Distance matrices built from amino acid sequences, according to the Jones-Taylor-Thornton model, were used to construct a tree using the neighbour-joining method in MEGA5. Numbers at the nodes indicate bootstrap support from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths distances measured in the number of amino acid substitutions per site. All 39 sequences were compared pairwise with all ambiguous positions removed for each sequence pair, resulting in a total of 3070 positions in the final dataset. Tick

amino acid sequences are from Ah, *Amblyomma hebraeum*; Dv, *Dermacentor variabilis*; Hl, *Haemaphysalis longicornis*; Is, *Ixodes scapularis*; Om, *Ornithodoros moubata*; and Rm, *Rhipicephalus microplus*. Insect amino acid sequences are from Aae, *Aedes aegypti*; Ape, *Antheraea pernyi*; Bge, *Blattella germanica*; Bmo, *Bombyx mori*; Lma, *Leucophaea maderae*; Nlu, *Nilaparvata lugens*; Pam, *Periplaneta americana*; Pst, *Plautia stali*; Sli, *Spodoptera litura*; and Sin, *Solenopsis invicta*. Crustacean amino acid sequences are from Cqu, *Cherax quadricarinatus*; Fme, *Fenneropenaeus merguiensis*; Lva, *Litopenaeus vannamei*; Men, *Metapenaeus ensi*; Mja, *Marsupenaeus japonicus*; Mro, *Macrobrachium rosenbergii*; Phy, *Pandalus hypsinotus*; and Pmo, *Penaeus monodon*. Accession numbers for all sequences are located in Table 6.6.

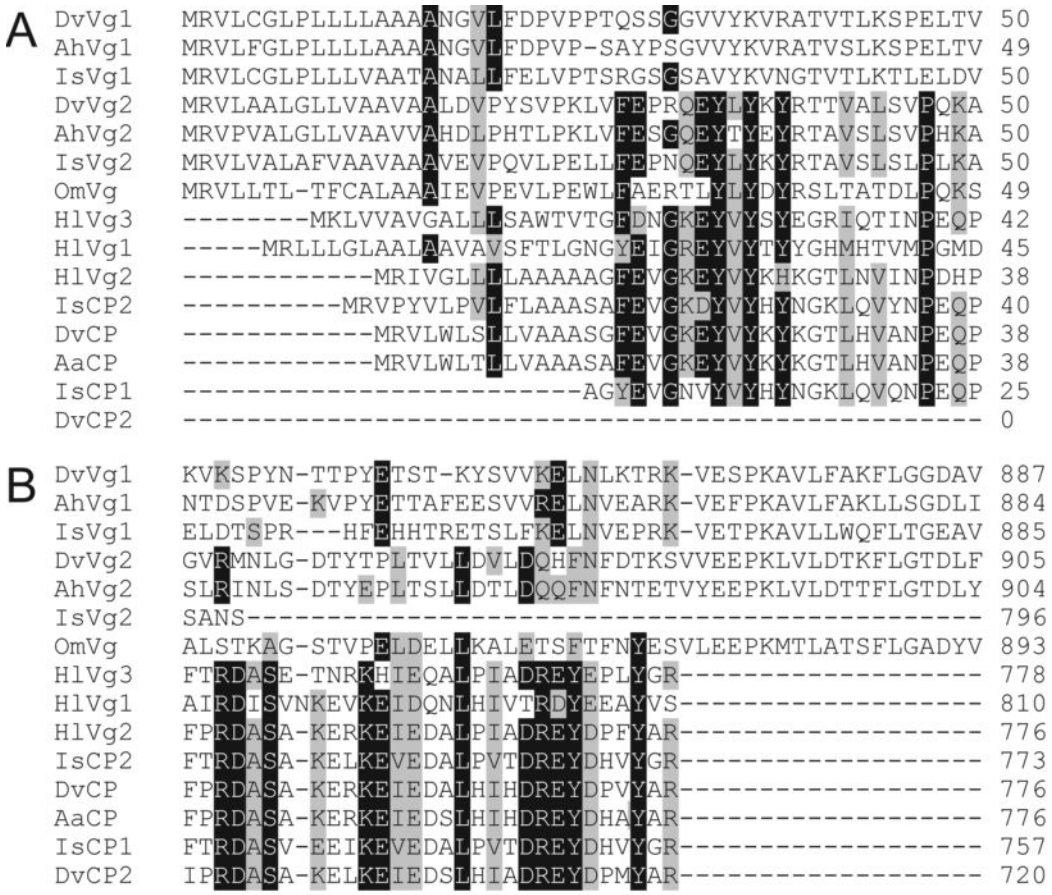

Figure 6.8. Alignment of tick vitellogenin (Vg) and carrier protein (CP) amino acid sequences. (A) Alignment showing the conserved FEVGKEYVY sequence present in CP immediately following the signal peptide cleavage site. (B) Alignment showing the conserved DASAKELKEIED sequence present in Cps following the conserved RXXR cleavage site. Identical residues are shown with black backgrounds while conserved residues are shown with grey backgrounds. Aa, *Amblyomma americanum*; Ah, *A. hebraeum*; Dv, *Dermacentor variabilis*; Hl, *Haemaphysalis longicornis*; Is, *Ixodes scapularis*; Om, *Ornithodoros moubata*; Rm, *Rhipicephalus microplus*. Accession numbers for each gene are provided in Table 6.6.

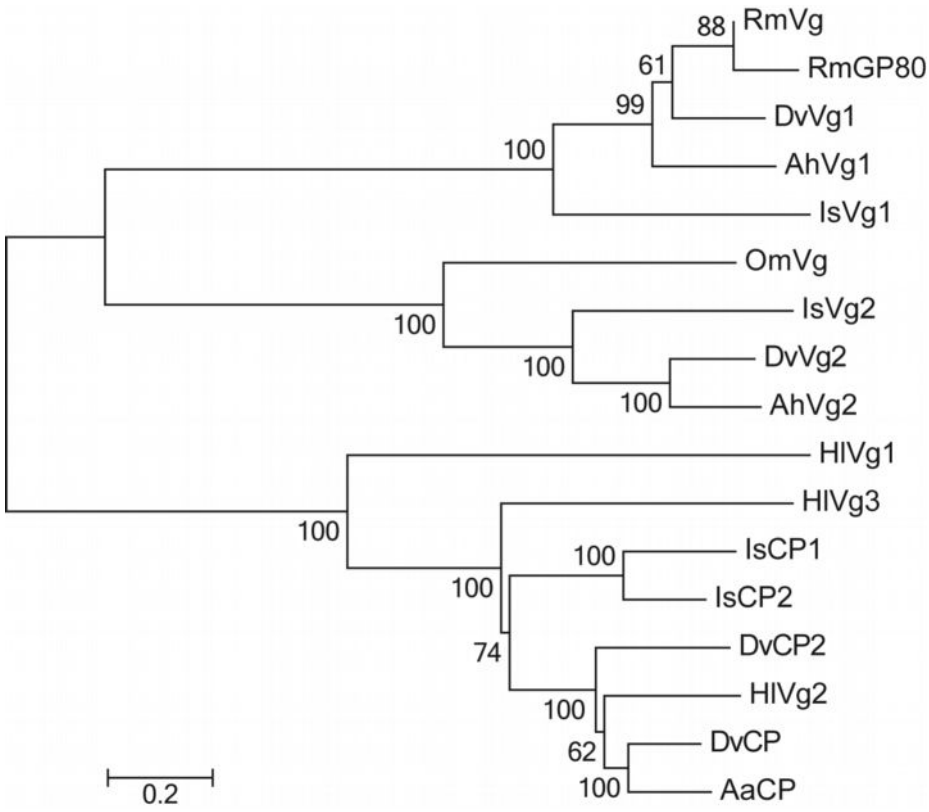

Figure 6.9. Phylogenetic tree of the vitellogenins (Vg) and carrier proteins (CPs) from various tick species. Neighbour-joining trees were constructed from distance matrices built from amino acid sequences according to the Jones-Taylor-Thornton model using MEGA5. Bootstrap support (1000 replicates) is indicated at the nodes and branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The tree is drawn to scale, with the distances reported as the number of amino acid substitutions per site. Sequences were compared in a pairwise fashion with all ambiguous positions removed for each pair. A total of 2182 positions remained in the final dataset. Aa, *Amblyomma americanum*; Ah, *A. hebraeum*; Dv, *Dermacentor variabilis*; Hl, *Haemaphysalis longicornis*; Is, *Ixodes scapularis*; Om, *Ornithodoros moubata*; Rm, *Rhipicephalus microplus*. Accession numbers for all genes are in Table 6.6.

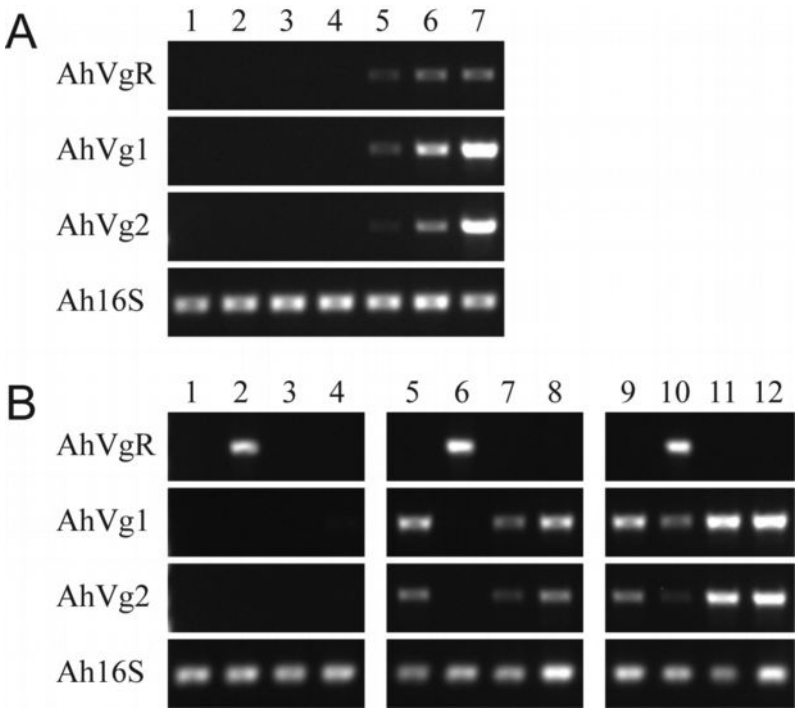

Figure 6.10. Spacial and temporal expression of the *Amblyomma hebraeum* vitellogenin receptor (VgR), vitellogenin 1 (AhVg1) and vitellogenin 2 (AhVg2) mRNAs. (A) Levels of AhVgR, AhVg1, AhVg2 and Ah16S rRNA (Ah16S) expression were analyzed by RT-PCR on total RNA isolated from whole ticks. Lane 1, unfed male; lane 2, fed male; lane 3, unfed female; lane 4, partially fed female; lanes 5 – 7, females 0, 4, and 10 days following engorgement respectively. (B) Levels of expression were also analyzed by RT-PCR on total RNA isolated from specific tissues taken from females 0 days (lanes 1 – 4), 4 days (lanes 5 – 8), and 10 days (lanes 9 – 12) post-engorgement. Lanes 1, 5 and 9: pooled synganglion, muscle, Géné's organ, Malpighian tubule, trachea, and salivary gland tissues; lanes 2, 6, and 10: ovary; lanes 3, 7 and 11: fat body; and lanes 4, 8 and 12: midgut. Ah16S was used as an internal control.

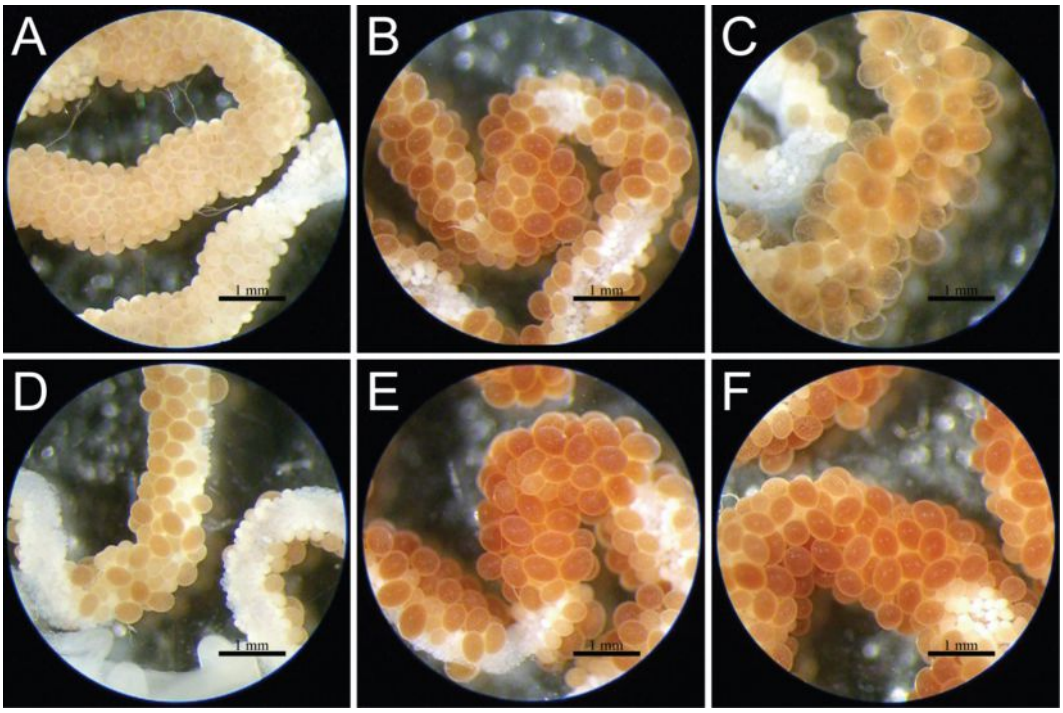

Figure 6.11. The effect of RNAi on the ovaries of *A. hebraeum* females injected with AhVgR-dsRNA, AhVg1-dsRNA, AhVg2-dsRNA, Bla-dsRNA, or TE-buffer. (A) An ovary between OGP 2 and 3, from a female treated with AhVgR-dsRNA shows some yolk uptake, but all oocytes are still very small and poorly developed; (B) AhVg1-dsRNA treated ovary, in OGP 4 with well developed oocytes full of large yolk granules; (C) AhVg2-dsRNA treated ovary, in OGP 4, but with abnormal oocyte morphology, the oocytes appearing almost hollow containing only a thin lining of large yolk granules; (D) AhVg1+2-dsRNA treated ovary, in OGP 3, some oocytes are large, well developed and full of yolk, but many are still underdeveloped and lacking much yolk uptake; (E) Bla-dsRNA treated ovary, in OGP 4, with the vast majority of oocytes being very well developed, filled with large yolk granules; (F) TE-buffer treated ovary, also in OGP 4, with the majority of oocytes highly developed and filled with large yolk granules.

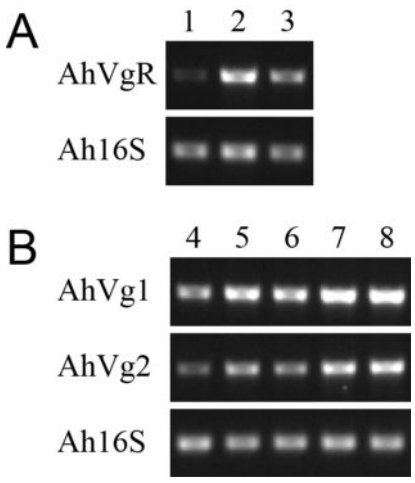

Figure 6.12. RT-PCR analysis of the AhVgR, AhVg1 and AhVg2 mRNA expression levels following RNA interference. The ovary and fat body of each tick was dissected out eight days following engorgement, and the total RNA extracted and subjected to RT-PCR. (A) AhVgR and Ah16S expression levels in the ovaries of dsRNA-injected ticks. Lane 1, AhVgR-dsRNA; lane 2, Bla-dsRNA; lane 3, TE-buffer injected control. (B) AhVg1, AhVg2 and Ah16S expression levels in the fat body of ticks injected with dsRNA. Lane 4, AhVg1-dsRNA; lane 5, AhVg2-dsRNA; lane 6, AhVg1+2-dsRNA; lane 7, Bla-dsRNA; lane 8, TE-buffer injected control. The levels of Ah16S were examined as an internal control.

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Chapter 7. General Discussion

7.1 General conclusions

An increased understanding of reproduction and feeding is necessary for developing biological control methods for ticks. The original aims of my thesis were to have been directly related to this topic. Initial results indicated that the *A. hebraeum* engorgement factor (EF), voraxin, was a promising target as a component of a future anti-tick vaccine (Weiss & Kaufman, 2004). As recorded in Chapters 2 and 3, a great deal of effort was expended attempting to replicate the results of Weiss and Kaufman, using a bacterial expression system to generate the recombinant proteins, but to no avail.

My inability to confirm Weiss & Kaufman's findings that the injection of fed male testis/vas deferens (TVD) into partially fed virgin females stimulates engorgement was particularly disconcerting, as this was to have been the positive control for the voraxin bioassay (see Chapter 2). After 4 rather frustrating years, it became necessary to abandon this project and turn my attention elsewhere. Chapters 4-6 record the results of these other endeavours. In this concluding chapter, I shall outline several areas that I believe worthy of further investigation.

7.1.1 The tick engorgement factor

Several factors could have accounted for my inability to stimulate an engorgement response in injected females: (1) I generally injected the ticks through the posterior body wall, whereas Weiss and Kaufman injected through the camerostomal fold; and (2) there were substantial colony-level differences between the ticks used in the initial experiments by Weiss and Kaufman and the

ticks used in my experiments. Weiss and Kaufman's original success in stimulating partially-fed virgins to engorge following injection of fed male TVD into the haemocoel implies that in normally mated ticks voraxin is conveyed via the haemolymph from the seminal receptacle to its target, likely the synganglion. This should occur regardless of the original site of injection; however, it should be noted that injection through the camerostomal fold rarely breaks the gut wall, whereas injection through the posterior body wall often does. This has a number of implications: (1) First, the majority of voraxin would be injected into the gut from where a lesser amount than intended would make its way into the haemolymph. Although voraxin might eventually become evenly distributed throughout the tick, this would include the gut volume, resulting in a greater dilution than occurs when injecting into the haemolymph. (2) Gut proteins may also bind voraxin to an unknown extent, thus hindering its ability to reach its target tissue. (3) Regarding catabolism in the gut: in ticks, digestion of the blood meal occurs mostly intracellularly, the midgut lumen acting as a storage location for the undigested blood meal (Coons *et al.*, 1986). Nevertheless, it is certainly conceivable that the midgut contains an enzyme that inactivates voraxin. (4) The leakage of gut contents into the haemocoel could also interfere with a number of physiological processes, or synganglion signalling, that could mask or nullify the voraxin effect. Notwithstanding the foregoing, it should be noted that several undergraduate student researchers working in the lab since 2004 attempted injecting the fed male gonad homogenates through the camerostomal fold, but also failed to elicit an engorgement response.

The lab's tick colony is known to "crash" every five-to-ten years, exhibiting much lowered levels of engorgement and decreased reproductive success, necessitating the acquisition of a new colony on a regular basis. Such a crash happened in 2003, 2 years prior to my joining the laboratory, and a new colony of ticks was imported from a much larger colony that had been maintained on cattle in Switzerland for many years. As detailed in Chapter 4, this colony eventually exhibited numerous morphological defects. It also contained females that were able to engorge without mating, giving rise to a short-lived parthenogenic line, something never before reported for this species. Because of the difficulties encountered in using this colony, plus the unexplained deaths of several rabbits while feeding the nymphs, we decided to import a "new" colony in 2007. This time the founder ticks came directly from South Africa, and were only one generation removed from the wild. Surprisingly, this colony also exhibited a high degree of morphological abnormalities, as well as a greatly reduced life-span compared to the previous colony. To make matters worse, I was unable to stimulate an engorgement response in partially fed virgins by injecting male gonad homogenates (Chapter 2). As these injections were also performed through the posterior body wall, I cannot rule out the possibility that the problems in replicating Weiss and Kaufman's work may be multi-factorial in nature. Interestingly, although Donohue *et al.* (2009) were able to stimulate feeding virgin *D. variabilis* to engorge following injection of male gonad homogenates, they injected the ticks either into the gonopore, or like Weiss and Kaufman, through the capitular formamen. Matsuo *et al.* (2002) also demonstrated that the injection of

MAG homogenates or spermatophores into the haemocoel of virgin *H. longicornis*, stimulated a significant increase in body volume, but did not indicate the site of injection.

Although I attempted to silence voraxin in *A. hebraeum*, it was ultimately unsuccessful, with little apparent knock-down of the mRNA transcript, and no apparent inhibition of female engorgement (Smith *et al.*, 2009; Chapter 3). On the other hand, although silencing of voraxinα in *D. variabilis* resulted in a 96% knockdown of the gene, it did not significantly affect the ability of females to feed to repletion or lay viable eggs (Donohue *et al.*, 2009). It should be noted that virgin *D. variabilis* females will often feed to the same extent as small, replete, mated females (~300 mg; Donohue *et al.*, 2009), and that the mechanisms controlling feeding and engorgement may be different between these species. I was unable to silence the *A. hebraeum* voraxin gene, even though I chose a similar region of the gene for knock-down as Donohue *et al.* (2009) used for *D. variabilis*. These authors also injected approximately twice the amount of dsRNA than I did, which might contribute to the difference observed.

7.1.2 Morphological abnormalities and parthenogenesis

As discussed in Chapter 4, I observed a high incidence of morphological abnormalities in our lab colony of *A. hebraeum*, as well as a number of virgin females that engorged and laid viable eggs. The cause(s) of these abnormalities are unknown, but one can speculate that the high degree of inbreeding may have played a role, considering that ticks collected from the field rarely exhibit major morphological defects (Guglielmone *et al.*, 1999; Dergousoff & Chilton, 2007).

Because the natural distribution of *A. hebraeum* is confined to the southern regions of Africa, and that very few other labs outside of South Africa work on this species, it was impractical to arrange for regular infusions of new ticks of varying genotypes into the colony. On the other hand, *A. hebraeum* colonies have been maintained, on rabbits, in our lab since the late 1970s. With the exception of a single incidence of bilateral gynandromorphy, and an engorged female showing a duplication of the posterior region, including what appear to be two additional spiracular plates[11], the morphological abnormalities I encountered have not been noticed here previously. However, it is possible that this is because they constituted such a small proportion of the population that they were not noticed previously.

Low levels of thelytoky have been reported in many normally bisexual ixodid ticks, with its prevalence varying dramatically among distinct populations of the same species. For example, in *D. variabilis* there is anywhere from 1.5-50% larval hatch from virgin eggs, but only very few of these survive to adulthood (Gladney & Dawkins, 1971; Oliver Jr., 1971; Homsher *et al.*, 1984). Similarly, Gunn & Hilburn (1991) found a low level of parthenogenesis in a Texan population of *A. cajennense*, but de Freitas *et al.* (2002) did not observe any instances of parthenogenesis in a Brazilian population of the same species. My observations of parthenogenesis in *A. hebraeum* were unusual in that the initial virgin females engorged to a large size and laid normal sized egg masses (Chapter 4). Only 125 female adults were ultimately obtained from a large number of larvae, because many of those larvae died before being offered a chance to feed.

11 see: http://www.biology.ualberta.ca/faculty/reuben_kaufman/index.php?Page=5638

The die-off of those larvae occurred much sooner than has been our normal experience. On the other hand, unlike what was reported in *D. variabilis* and *A. cajennense*, the surviving larvae appeared generally healthy and active.

It is possible that thelytoky is an adaptation to allow for reproduction in the event that con-specific males are unavailable. The distribution of *A. hebraeum* and *A. variegatum* partially overlap in sub-Saharan Africa (Peter *et al.*, 1998), and *A. hebraeum* females respond to male *A. variegatum* pheromones (Yunker *et al.*, 1990). Although early attempts by Rechav *et al.* (1982) to hybridize *A. hebraeum* and *A. variegatum* were unsuccessful, more recent attempts by Clarke & Pretorius (2005) resulted in viable offspring. Moreover, whereas the cross between male *A. hebraeum* and female *A. variegatum* failed to produce any viable eggs, the alternative cross resulted in exclusively female progeny, all of which resembled *A. hebraeum* in those morphological characters examined (Clarke & Pretorius, 2005). Regrettably, the authors did not report any further characterization of these hybrids using either classical or molecular techniques. However, the similarity to *A. hebraeum* may have been due to parthenogenesis.

7.1.3 Symbionts of A. hebraeum

As mentioned in Chapter 5, some studies have shown that treatment of *A. americanum* with antibiotics to reduce the numbers of its *Coxiella* symbionts resulted in decreased reproductive fitness – the ticks exhibiting a prolonged time to oviposition, decreased hatching success, and fewer viable larvae produced compared to PBS- and DMSO-injected controls (Zhong *et al.*, 2007). Although it is possible that the antibiotics used in this experiment had some sort of toxic effect

on the tick, leading to the observed decrease in fitness, two antibiotics with distinct mechanisms of action, tetracycline and rifampin, had similar effects, making this interpretation less likely (Zhong *et al.*, 2007). Moreover, these antibiotics have been used successfully in other arthropods to reduce or remove bacterial symbionts (Bressac & Rousset, 1993; Zchori-Fein *et al.*, 1995), and the observed decrease in tick fitness was correlated with a decrease in bacterial load in the ticks (Zhong *et al.*, 2007). Other endosymbiotic bacteria can affect the tick's ability to serve as a reservoir for other, more pathogenic bacteria. The presence of the non-pathogenic *Rickettsia peacockii* is able to prevent the transovarial transmission of the pathogenic *R. rickettsia* (Macaluso *et al.*, 2002).

In many insect species, bacterial endosymbionts affect the reproductive biology of the host, drastically altering sex ratios, and in some cases resulting in all-female parthenogenic populations (Werren *et al.*, 2008). Although *Wolbachia* spp. have long been known to induce parthenogenesis in various insects (reviewed by Werren *et al.*, 2008), more recent reports indicate that other bacteria can do the same. The leafminer parasitoid *Neochrysocharis formosa* (Hymenoptera: Eulophidae) harbours a *Rickettsia* sp. that induces parthenogenesis (Hagimori *et al.*, 2006), and a member of the Cytophaga-Flavobacterium-Bacteroides lineage induces parthenogenesis in both the parasitoid wasp *Encarsia pergandiella* (Zchori-Fein *et al.*, 2001), and the phytophagous mite *Brevipalpus phoenicis* (Weeks *et al.*, 2001). Interestingly, a similar *Cytophaga-Flavobacterium-Bacteroides* lineage bacterium has been found in *I. scapularis* (Benson *et al.*, 2004), although its effect on the tick has not yet been characterized. It is unknown

if either the *Coxiella* or *Rickettsia* symbionts present in *A. hebraeum* have any effect on the reproductive biology of the tick.

Although *A. hebraeum* is a known vector of various *Rickettsia* spp., the presence of *Coxiella*-like symbionts has not been previously reported in this species (Chapter 4). Its presence in *A. hebraeum* is particularly interesting due to its similarity to the *A. americanum Coxiella* symbiont that plays a role in tick fitness (Zhong *et al.*, 2007).

7.1.4 Vitellogenesis

In Chapter 6, I described the identification of the vitellogenin receptor and two of its ligands, AhVg1 and AhVg2 from *A. hebraeum*. Although efforts were made to determine the sites and timing of Vg expression, the locations of Vg synthesis are still uncertain. Contamination of most tissues with some fat body, due to the way it grows around and into other organs in the tick, makes it difficult to determine what other tissues may also play a role in Vg synthesis due to it being one of the major sources of Vg in the tick. Friesen & Kaufman (2004) identified an 86 kDa Vn-binding protein in the ovary, which they postulated may be the VgR. My results, as well as the protein's small size compared to all other known VgRs, and the fact that it did not lose its capacity to bind Vn following treatment with reducing agents, a common trait of members of the LDLR family (Sappington & Raikhel, 1998), do not support the view that this protein is a VgR. It is possible that it assists in folding of Vn, the assembly of the Vn macromolecule, or the further processing of Vn in the developing oocyte.

The currently accepted pathway by which vitellogenesis is regulated in

ixodid ticks begins with the transfer of a number of factors from the male to the female during copulation. Among them is voraxin, which stimulates the female to fully engorge (Weiss & Kaufman, 2004). Voraxin either stimulates the seminal receptacle to produce a factor that stimulates the synganglion, or acts directly on the synganglion to stimulate the release of an ecdysteroidogenic neuropeptide, the 'vitellogenin inducing factor' (VIF; Chinzei & Taylor, 1990; Chinzei *et al.*, 1992; Lomas *et al.*, 1997; Friesen & Kaufman, 2009). The VIF then acts on the epidermal cells to initiate ecdysteroid synthesis (Zhu *et al.*, 1991; Lomas *et al.*, 1997; Roe *et al.*, 2008), and these ecdysteroids then act on the fat body and midgut to stimulate Vg synthesis (Friesen & Kaufman, 2002, 2004; Thompson *et al.*, 2005). In *D. variabilis*, the uptake of Vg into the developing oocytes follows directly from Vg-synthesis (Thompson *et al.*, 2005). In contrast, in *A. hebraeum* an additional factor, the 'vitellogen uptake factor' (VUF), constitutes the signal for Vg uptake into the oocytes (Friesen & Kaufman, 2004; Seixas *et al.*, 2008). Very little is known about the VUF other than that it is a haemolymph-borne endocrine factor and is present in engorged ticks (Lunke & Kaufman, 1992; Friesen & Kaufman, 2004; Seixas *et al.*, 2008).

7.2 Future directions

7.2.1 The identity and role of the tick engorgement factor in A. hebraeum

(1) Before one can confirm the results of Weiss & Kaufman (2004), it is essential to re-demonstrate, in a fresh population of *A. hebraeum*, that the TVD of fed males, when injected into the haemocoel, can induce engorgement in virgin females. This would then serve as the positive control for the assessment of the

biological activity of any recombinant voraxin produced in the future. Alternatively, this work, as well as the experiments described below, could be done in another tick species, preferably using wild-caught ticks. Working on a genetically diverse wild population would likely alleviate some of the problems I encountered using an inbred lab colony.

(2) Determination of the full-length sequence of the *A. hebraeum* voraxinα and voraxinβ. As mentioned above and discussed previously in Chapter 2, the published sequence of voraxinα is much smaller in *A. hebraeum* than that of *D. variabilis* and alignments indicate that it is likely only the C-terminal region of the gene. Voraxinβ, has not yet been observed in any other tick-species, but like voraxinα, it was not subjected to 5'/3' RACE, and so is likely an incomplete sequence. Determining the full-length sequences is necessary to ultimately determine gene function, and could also offer more potential target sites for gene silencing.

(3) Production of new recombinant voraxin proteins. Although the published *A. hebraeum* voraxin sequence is not full length, it is likely to be in the correct reading frame (Donohue *et al.*, 2009). With new, full-length recombinant voraxin proteins, after assessment of their biological activity, additional work using various truncated forms could help to determine the active site(s) of the molecules.

(4) Production of a new recombinant antibody. The antibodies produced in this study were not able to confer any level of protection against tick feeding, possibly due to the incomplete purification of the initial antigen. A recombinant

antibody would allow the protective ability of immunizations against voraxin to be more fully assessed, compared to the single rabbit study performed by Weiss & Kaufman (2004). It would also enhance the ability of researchers to further characterize the expression and site(s) of action of voraxin in ticks.

(5) Characterization of voraxin expression in male ticks. Previous work has shown that mating and transfer of the spermatophore to the female is necessary for females to feed to repletion (Pappas & Oliver Jr., 1971, 1972). Additionally, voraxin is upregulated in the testis by feeding (Weiss & Kaufman, 2004). However, the exact timing of voraxin expression is still unknown, as are the cell types responsible for its production. The use of RT-PCR, *in situ* hybridization, along with other immunocytochemical techniques would allow one to identify cell-types and determine the onset of expression. A recombinant voraxin antibody would also be a necessary component of an enzyme-linked immunosorbent assay (ELISA), which would allow for the levels of protein production to be quantified at various stages of development, as well as in the spermatophore.

(6) Characterization of voraxin activity in the female. The role voraxin plays, as well as its site of action in the female, remain completely unknown. The first question is whether voraxin somehow passes through the wall of the seminal receptacle (SR) into the haemocoel and acts directly on its target, or whether it stimulates receptors in the SR to secrete the active material into the haemocoel. The ability of Weiss & Kaufman (2004) to obtain an engorgement response by the injection of voraxin directly into the haemocoel suggests that voraxin is normally transported from the SR to the haemocoel.

The second major question relates to the target of voraxin (or the SR secreted mediator). Voraxin stimulates feeding, a behavioural response that suggests the direct involvement of the synganglion. Additionally, the synganglion appears to be the source of various neuropeptides responsible for the stimulation of ecdysteroid production and regulation of salivary gland degeneration and vitellogenesis (Harris & Kaufman, 1984; Lomas *et al.*, 1997; Šimo *et al.*, 2009). It would be possible to monitor the progress of voraxin following its entry into the female reproductive tract using an ELISA or immunohistochemistry.

Tissues containing voraxin-binding proteins could be evaluated by means of an antigen-binding assay using recombinant voraxin and the anti-voraxin antibody. Any voraxin-binding proteins discovered could then be purified using affinity-based methods, allowing identification and further characterization of the role they play in the engorgement pathway.

7.2.2 Morphological defects and parthenogenesis in A. hebraeum

The appearance of a short-lived line of parthenogenic *A. hebraeum,* as well as the high prevalence of morphological abnormalities in our tick colony, raise a number of interesting avenues for future research.

(1) Determine the prevalence of parthenogenesis in the new colony (NC) of *A. hebraeum*, and if possible establish a parthenogenic line. As previous discussed (Chapter 4), a short-lived parthenogenic line of *A. hebraeum* arose from the ticks of our old colony (OC). However, we have not yet observed parthenogenesis among the ticks from our NC. To gain an understanding of the prevalence of parthenogenesis in this species, NC virgin females could be fed, any eggs

produced monitored for viability and, if possible, any resulting larvae fed to adulthood. Similar to what has been done with the OC parthenogenic ticks and in other species, the parthenogenically-produced ticks could then be compared in various ways to ticks from the bisexual population.

(2) Describe the mechanisms of parthenogenesis. As previously discussed in Chapter 4, it is uncertain whether thelytoky in ticks is due to apomictic or automictic parthenogenesis. Both types have been observed in various tick colonies (Oliver Jr. *et al.*, 1973; Homsher *et al.*, 1984). The mechanism of parthenogenesis used to produce the observed thelyokous *A. hebraeum* is unknown. Unlike what was observed in the thelytokous *D. variabilis*, the parthenogenetically produced female *A. hebraeum* exhibited no morphological abnormalities. This may indicate that they were produced through apomixis, as this would essentially make them clones of their mother. Oliver Jr. (1989) stated that *A. hebraeum* is one of only five metastriate tick species to possess an XY-XX sex determination system, the remainder possessing the XO-XX system, and has a diploid number of 20 chromosomes. Cytological studies to confirm the normal diploid number in our population of bisexual *A. hebraeum* as well as the number in the parthenogenic line may help clarify the mechanisms of parthenogenesis in this system.

Hybridization experiments between the parthenogenic females and bisexual males could provide further information on the mechanisms of parthenogenesis in this species. Examination of the offspring sex ratios, their ability to reproduce parthenogenetically, and their karyotypes to determine

cytological differences from the parents, would provide additional information on the mechanisms of parthenogenesis. In particular, examination of cytological differences of the offspring in conjunction with the ability to reproduce parthenogenetically may allow for the creation of a linkage map to determine which chromosome(s) carry the genes responsible for parthenogenesis.

(3) Comparison of the parthenogenic and bisexual races of *A. hebraeum*. As discussed in Chapter 2, the transfer of voraxin from the male to the female is normally required for engorgement to occur. However, the parthenogenic females obviously did not require voraxin. Although neither I nor anyone else in the lab was able to confirm that the fed male testis contains an EF (see Chapter 2), following future confirmation, it would be interesting to examine the parthenogenic females to determine how they have managed to bypass this requirement. The ability of these females to produce voraxin could be examined, as well as investigating the activity of the downstream regulators of engorgement, once they have been identified. It is possible that in the parthenogenic females, these downstream regulators are constitutively active allowing engorgement to occur in the absence of a mating stimulus. Additionally, the salivary glands of engorged mated females degenerate within 4 days of detachment from the host, but those of large partially fed virgin females, require 8 days to degenerate. Because the parthenogenic females engorge and detach from the host without mating, it would be interesting to see what impact this has on the salivary glands. Salivary gland degeneration is triggered by an ecdysteroid (Lomas *et al.*, 1998), which is also responsible for stimulating vitellogenesis (Friesen & Kaufman,

2002, 2004). It would thus also be interesting to compare ovarian development and the regulation of vitellogenesis in parthenogenic and bisexual strains of *A. hebraeum*. The parthenogenic race of *H. longicornis* expresses its VgR in unfed ticks of all life stages (Boldbaatar *et al.*, 2008), in contrast to the bisexually reproducing *D. variabilis* (Mitchell *et al.*, 2007) and *A. hebraeum* (see Chapter 6), where it is expressed only in the feeding female. Comparative studies examining the expression and regulation of the genes involved in ovarian development and egg maturation may further increase our understanding of how these processes work in the tick.

7.2.3 Characterization of the putative bacterial symbionts of A. hebraeum

(1) To date, the potentially symbiotic bacteria found in *A. hebraeum* have been identified using only the partial sequence of the 16S rDNA. The cloning and sequencing of *Coxiella* specific genes, commonly used in phylogenies of this genus, such as the *Coxiella* outer membrane protein com1, and the DnaJ-like protein djlA (Sekeyová *et al.*, 1999), would more accurately determine the symbiont's phylogeny. Similarly, ticks could be screened for genes commonly used in rickettsial phylogenetics, such as the membrane proteins *rOmpA* and *rOmpB*, and the citrate synthase gene *gltA* (Roux *et al.*, 1997; Fournier *et al.*, 1998; Roux & Raoult, 2000), so that the identity of the *Rickettsia* sp. could be more accurately determined. In a similar vein, it would also be enlightening to determine whether *Coxiella* symbionts are present in other members of the Amblyomminae, to help determine whether the *Coxiella-Amblyomma* association is ancestral to the genus. Intriguingly, a *Coxiella* sp. has also been identified from

Bothriocroton auruginans (Vilcins *et al.*, 2009), a member of a genus closely related to *Amblyomma*. Although it appears to bear more similarity to the *C. burnetii*-like symbionts found in several species of soft ticks (Vilcins *et al.*, 2009), analysis of additional members of this genus for *Coxiella* symbionts may also shed light on the history of its association with various hard ticks.

(2) It is currently unknown whether *A. hebraeum* is competent to transmit either the *Coxiella* or *Rickettsia* species to its host. To evaluate the transmission potential of the bacterium, the ticks could be fed on susceptible hosts such as guinea pigs, a common rodent model for human Q fever infection (Voth & Heinzen, 2007), and the animals then screened for signs of infection. The *Francisella*-like symbiont of *D. andersoni*, despite not being transmissible by the tick to its hosts, was able to cause a mild infection when cultured bacteria were injected into uninfected guinea pigs (Niebylski *et al.*, 1997). To determine whether this is also true of the *A. hebreaum* symbiont, both the *Coxiella* and *Rickettsia* symbionts could be cultured and injected directly into a host. Some pathogens, such as *Babesia*, require a period of development within the tick vector to produce infectious sporozoites that are then transmitted to a vertebrate host during tick feeding (Homer *et al.*, 2000). Other pathogens exhibit enhanced infectivity when in the presence of various factors present in tick saliva (Nuttall & Labuda, 2004). Injection of infected tick tissue homogenates in the presence of salivary gland extracts could be done to test if this would enhance any potential infectivity. Pure cultures of the *A. hebraeum Coxiella* and *Rickettsia* symbionts would also allow for the development of anti-*Coxiella* and anti-*Rickettsia*

antibodies that could be used in further studies of the bacterium's distribution within the tick.

(3) Even though both a *Rickettsia* and *Coxiella* sp. are present in *A. hebraeum*, we don't yet know the distribution of these symbionts within various tissues, and at what point they colonize specific tissues, the ovary in particular. Understanding the mechanisms of transovarial transmission also has implications for understanding pathogen transmission in the tick. *A. hebraeum* is the primary vector of *Rickettsia africae*, a pathogen known to be vertically transmitted in this tick (Kelly *et al.*, 1996). However, in *D. variabilis*, infection with one *Rickettsia* sp. can inhibit the transovarial transmission of a second *Rickettsia* sp. (Macaluso *et al.*, 2002). By better understanding the bacteria present endemically in the host tick, we can gain a better understanding of the transmission dynamics of various pathogens, which in turn has implications for human and animal health.

(4) Interestingly, as discussed in Chapter 5, a related *Coxiella* symbiont found in *A. americanum* possesses a reduced genome (Jasinskas *et al.*, 2007). Moreover, when the ticks are treated with antibiotics to reduce the symbiont load, there is a measurable decease in tick reproductive fitness (Zhong *et al.*, 2007). These suggest that the *A. americanum Coxiella* symbiont may be a primary endosymbiont, perhaps supplying the tick with nutrients it may not otherwise be able to synthesize, or by affecting reproduction in some other way. Investigating these areas in *A. hebraeum* would be of great interest, especially as preliminary results suggest that the *Coxiella* sp. present in *A. americanum* and *A. hebraeum* are related (Chapter 5). The development of an axenic line of *A. hebraeum* by

repeated treatments with antibiotics known to be effective against *Coxiella* and *Rickettsia*, such as rifampin, doxycycline, tetracycline and telithromycin (Raoult, 1989; Brennan & Samuel, 2003; Fenollar *et al.*, 2003; Boulos *et al.*, 2004; Zhong *et al.*, 2007) would allow for a number of additional questions to be addressed. In addition to determining the effect that these bacterial symbionts have on tick fitness, it would be possible to test whether they can be horizontally transmitted from infected to uninfected ticks while feeding. Examining the effect of re-infection of the axenic line with either of the bacterial species could also be done to determine whether (A) the ticks remain competent to acquire and pass on endosymbionts and (B) whether there is a restoration of tick fitness upon reintroduction of one or both bacteria.

7.2.4 The mechanisms and regulation of vitellogenesis

(1) Sequence analysis of the identified AhVgs indicate the presence of multiple potential cleavage sites, as well as many locations where the translated proteins could be potentially modified through glycosylation and phosphorylation. However, it is not currently known which, if any, of the sites are cleaved, and which post-translational modifications are done to Vg. As these modifications may impact the proteins' uptake and further processing in the oocyte, it would be of interest to determine exactly how they are modified. Various methods of protein analysis including native-, SDS- and 2D-PAGE, as well as the use of western blotting, stains to detect specific modifications and mass spectrometry could be used as part of the initial attempts to determine the cleavage sites used and any modifications that may occur.

(2) RT-PCR indicated that the two identified AhVgs are strongly expressed in the fat body and midgut, but it also indicated the unexpected expression of AhVg in a number of other tissues, including the ovary, the salivary glands, Malpighian tubules, trachea, synganglion, Gene's organ, and muscle. Due to the way that the fat body grows in ticks, it is not possible to rule out that the source of the Vg expression in all these other tissues was due to contamination by the fat body. Further testing to determine the actual source of the Vg message needs to be done: an *in situ* hybridization experiment, as was done in *O. moubata* (Horigane *et al.*, 2010), using probes specific to the two identified AhVgs could easily clarify this issue. As previously stated, it is also unclear whether all the AhVgs have been identified, because the midgut was not used as a template in the original degenerate PCR experiments; thus an AhVg expressed only in that tissue would not have been identified in this study. Examination of the proteins that make up the native Vg may reveal protein sequences that do not match the currently described Vgs, and could then be investigated. Additionally, new degenerate PCR reactions could be performed using midgut derived mRNA as the template to isolate Vgs specifically from the midgut.

(3) Although the VIF is an ecdysteroidogenic neuropeptide (Chinzei & Taylor, 1990; Chinzei *et al.*, 1992; Lomas *et al.*, 1997), its identity remains unknown. As this factor appears to be produced only in the synganglia of mated females following engorgement and detachment from the host, comparing the transcriptome and/or proteomes of synganglia from partially fed virgins and engorged, mated females may provide a clue as to the identity of this factor.

Additional comparisons of the obtained sequences to other known ecdysteroidogenic neuropeptides could also assist in the identification of the VIF in *A. hebraeum*.

(4) To date almost nothing is known about the VUF, tentative evidence pointing to its existence in *A. hebraeum*, but not in *D. variabilis*. If large amounts of engorged female haemolymph could be obtained, one could subject it to size fractionation and protein degradation and/or inactivation, and then bioassay the resulting samples for their ability to stimulate yolk uptake in the oocytes of partially fed virgin females. Once it is determined whether the factor is a protein or some other material, various techniques could be applied for further characterization, such as 2D-PAGE, mass spectroscopy and protein sequencing or NMR. Identification of the VUF would allow production of a recombinant or synthetic version that could then be used to further elucidate its role in the tick, the site of production and whether a similar factor is present in other tick species.

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