University of Alberta

Determining the Critical Weight of the Rocky Mountain Wood Tick *Dermacentor andersoni* Stiles (Acari: Ixodidae)

By

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Abstract

The feeding cycle of female ixodid ticks is divided into preparatory, slow and rapid feeding phases. Female ticks, virgin or mated, reach a 'critical weight' (CW; 10-13X the unfed weight) at the transition between the slow and rapid feeding phases, which is characterized by several physiological and behavioural changes. To date, the CW has been determined for *Amblyomma hebraeum* only. This study established that, in both mated and virgin *Dermacentor Andersoni*, salivary gland degeneration and ovarian development are triggered when the tick exceeds 10-13X the unfed weight. However, mated ticks undergo these changes 4 days earlier than virgin ticks. Unlike *A. hebraeum*, virgin *D. andersoni* regularly fed well beyond their CW, and occasionally fed up to 100X their unfed weight.

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LIST OF ABBREVIATIONS

20-HE	20-hydroxyecdysone
AV	Autophagic vacuoles
Bw	Body weight
CW	Critical weight
DA	Dopamine
E	Ecdysone
ES	Ecdysteroid
MF	Male factor
MOPS	Morpholinopropanesulphonic Acid
OGP	Ovarian growth phase
OsO_4	Osmium Tetraoxide
PD	Post detachment
PE	Post engorgement
RH	Relative humidity
SEM	Standard error of the mean
SG	Salivary gland
TEM	Transmission Electron Microscopy
TSGDF	Tick salivary gland degenerating factor
Vt	Vitellin
X DPD-M	X days post detached mated (X= 0, 1, 2, 3, 4)
X DPD-V	X days post detached virgin (X= 0, 1, 2, 3, 4, 6, 8)
X DPE-M	X days post engorged mated (X=0, 1, 2, 3, 4)
X DPE-V	X days post engorged virgin (X= 0, 1, 2, 3, 4, 6, 8)

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CHAPTER ONE

Introduction

1.1 General background

Ticks are obligate haematophagous arthropods that can transmit numerous pathogens including fungi, bacteria, viruses and protozoa to animals and humans (de la Fuente *et al.,* 2005). The phylogeny of ticks is presented in Figure 1.1. The family Ixodidae has a tough sclerotized plate (a scutum) on their dorsal body surface and a non-sclerotized (though still somewhat 'hard') alloscutum. The family Argasidae lacks a scutum and possess a tough, highly folded leathery cuticle. The family Nuttalliellidae exhibits features from both the Ixodidae and the Argasidae, the females having a pseudoscutum, a plate-like structure that resembles the scutum, but is not clearly distinct from the alloscutum (Sonenshine, 1991).

Dermacentor andersoni Stiles (the Rocky Mountain wood tick) is widely distributed in western North America. These ticks are commonly found in mountainous areas and sagebrush country near streams. During the larval and nymphal stages they infest wild rodents and other small mammals (*e.g.* mice, ground squirrels, woodrats, jackrabbits and chipmunks etc.). During the adult stage they infest large mammals (*e.g.* horses, cattle, mule deer, mountain goat, elk and humans). This tick is a vector of the agents of Rocky Mountain spotted fever, tularaemia, Q-fever, anaplasmosis, and tick paralysis (James *et. al.,* 2006).

1.2 The life cycle of ixodid ticks

All ixodid ticks pass through four distinct stages: egg, larva, nymph and adult. Males and females are easily distinguishable in their adult stage

because of their marked sexual dimorphism (Figure 1.2). In most species, a tick feeds on a distinct host during each active stage, engorges and drops off (3-host life cycle; Sonenshine, 1991). Some tick species feed on one host during the larval and nymphal stages and on another host during the adult stage. These ticks are known as 2-host ticks (*e.g Hyalomma anatolicum excavatum*). Some ticks feed and develop the three stages of their life cycle on one host, and thus are known 1-host ticks (*e.g Boophilus microplus, Dermacentor albipictus*).

The duration of the life cycle of ixodid ticks depends on the prevailing environmental conditions, such as temperature, day length, and relative humidity (RH; Randolph, 2008). Under our laboratory conditions, adult *D. andersoni* engorge within ~6-8 days and then detach; oviposition begins 4-6 days later and continues for ~2 weeks. The eggs require a minimum of 3 weeks to hatch. The larvae require ~4 weeks to become active again and willing to feed. After feeding (~1-2 weeks) they engorge and detach from the host. The fed larvae require ~2-3 weeks to moult. The nymphs are ready to feed within ~3-4 weeks. They feed for ~1-2 weeks, engorge and drop off. The engorged nymphs moult to adults within ~2-3 weeks. If the adults are held at ~5-7°C and high RH, they can survive for up to a year. From the foregoing, the life cycle can be completed in as little as 70 days, although it tends to take about 1-2 years in the wild depending on the geographical location (Sonenshine, 1991; James *et al.,* 2006).

1.3 The feeding phases of ixodid ticks

The feeding period in mated ixodid ticks is divided into three distinct phases: in the preparatory phase (the first 24-36 hours), the female inserts its

mouthparts into the host skin, creates a feeding lesion and secretes a cement-like substance that secures it to the feeding site. During the slow feeding phase (7-10 days), the female attains a body weight of approximately 10 times its unfed weight. The female then gains up to a further 10 times in weight during the next 12-24 h (rapid feeding phase) and then detaches from the host.

In most ixodid species, mating is required for full engorgement. For example, virgin female *Amblyomma hebraeum* remain attached to the host indefinitely, most of them not exceeding ~10 times their unfed weight (Kaufman & Lomas, 1996; Weiss & Kaufman, 2001). If fed males are introduced and if copulation occurs, the females feed rapidly to engorgement (Snow, 1969; Weiss & Kaufman, 2001). However, ~15% of virgin *A. hebraeum* may feed up to about 20X the unfed weight, but do not proceed to full engorgement (Kaufman & Lomas, 1996).

1.4 The concept of 'critical weight' (CW)

In the wild, tick feeding can be interrupted because of host grooming. Also, if the host dies for any reason, ticks will detach spontaneously. Finally many hosts acquire immunity against tick feeding following repeated infestations. Ticks attaching to such hosts fail to engorge to repletion, and sometimes die *in situ* (Brossard & Wikel, 2008). The tick's subsequent behaviour depends on how much it has fed in the interim.

If feeding females are removed or detached from an experimental host prior to engorgement, their subsequent behaviour will depend on two factors: (1) the amount of blood they have ingested up to that point, and (2) whether or not they have mated (Lomas & Kaufman, 1992a; Weiss & Kaufman, 2001). If

a virgin or a mated female *A. hebraeum* is forcibly removed from the host before it achieves ~10 times its unfed weight, it will reattach and continue to feed if it is offered a suitable host. But if a female is removed from the host after exceeding approximately 10 times its unfed weight, it will not reattach (Lomas & Kaufman, 1999). Instead its salivary glands (SG) will degenerate within 4 days (mated) or 8 days (virgin) of detachment, followed by laying an egg mass, the size of which is approximately proportional to the amount of blood imbibed (Snow, 1969; Kaufman *et al.*, 1986).

The weight at which a partially fed *A. hebraeum* removed from the host switches from the so-called 'feeding strategy' (up to ~10X the unfed weight) to an 'egg development strategy' (beyond ~10X the unfed weight) was termed the 'critical weight' (CW) by Harris & Kaufman (1984). The following physiological and behavioural changes occur once a tick exceeds the CW, (1) the tick does not reattach if given the opportunity, (2) the SGs degenerate, (3) ovarian development accelerates and (4) haemolymph ecdysteroid (ES) titre increases dramatically. Although the CW can be determined from any of the latter parameters, the absolute value differs slightly depending on the parameter measured. The CW as measured by failure to reattach, by SG degeneration, by haemolymph ES titre and by ovary maturation was 9, 10, 10 and 12-13 times respectively (Weiss & Kaufman, 2001).

1.5 The tick salivary gland

1.5.1 Salivary gland structure

The SGs are paired organs that extend from the anterior of the body cavity to the single pair of spiracles, posterior to the fourth pair of walking legs (Figure 1.3). The SGs of adult ixodid ticks comprise three types of acini

(also known as alveoli) in females, and four types in males (Fawcett *et al.*, 1981a). The type I acini drain mostly into the anterior portion of the main salivary duct. This type of acinus is 'agranular' and does not change its histological appearance much during the feeding period (Sauer *et al.*, 1995). Types II and III acini consist of both 'granular' and 'agranular' cell types, and are situated throughout the posterior two-thirds of the glands along secondary and tertiary duct branches (Figure 1.4). Types II and III acini develop substantially in size during feeding (Fawcett *et al.*, 1981b), although apparently without an increase in cell number (Sauer *et al.*, 2000). Type IV acini, associated with the male only, are not apparent in the larva and nymph (Sauer *et al.*, 1995).

According to Fawcett *et al.*, (1981a and 1981b) the type III acinus comprises three types of secretory cells (d, e and f; Figure 1.5). Cell types d and e are large, located at the hilus (the upper region of the acinus, where it is connected to the salivary duct) of the acinus. At the unfed stage, both cell types contain large numbers of secretory granules. The f cells, located at the fundus (the lower/distal portion of the acinus), are relatively small. At the onset of feeding, these cells hypertrophy and accumulate secretory granules rapidly. There are also some slender interstitial cells containing pale cytoplasm in the clefts between the glandular cells. These adluminal and abluminal interstitial cells are central components of a "fluid secretory labyrinth" (Fawcett *et al.*, 1981a). The fluid secretory labyrinth plays an essential role in excreting excess water taken in during the blood meal. The SGs of larvae and nymphs have not been studied as much as those of the adults.

1.5.2 Physiological roles of salivary glands

Salivary glands are very much 'vital organs' in ticks. During the preparatory feeding phase they secrete the cytolysins (enzymes needed to form the feeding lesion) and the 'cement' used to attach the mouthparts securely to the skin. The SGs also play a major role in osmoregulation. Unfed ticks are able to survive for a long period of time because they can take up water vapour directly from the atmosphere. The mechanism involves a hygroscopic saliva that is secreted by the type I acinus. This hygroscopic saliva is deposited onto the mouthparts where it absorbs water vapour from the atmosphere; the tick then swallows it and thereby can re-hydrate (Knülle & Rudolph, 1982).

However, the salivary gland also functions as an excretory organ. Kaufman & Phillips (1973) showed that unfed female *D. andersoni*, weighing 7-10 mg, can imbibe approximately 4000 mg of host blood over the course of the meal. Throughout this time, excess fluid from the blood meal is secreted back into the host. This excretory mechanism enables the tick to concentrate the nutrient components of the blood and regulate haemolymph volume and ionic composition (reviewed by Kaufman, 1989).

1.5.3 Salivary gland development and degeneration

Salivary fluid secretory competence in adults varies according to the sex and stage of feeding. The adult male secretes very little fluid throughout the feeding cycle (Kaufman, 1990). *In vitro* salivary glands of unfed *D. andersoni* females secrete fluid at only a few nl/min. As feeding progresses, salivary fluid secretory competence increases, and it peaks at about 220 nl/min by the end of the slow feeding phase (Kaufman, 1976).

The SGs undergo extensive developmental changes during the slow phase of engorgement (Fawcett *et al.,* 1981b). After feeding for several days, the d and e cells lose most of their secretory granules. At this stage they are described as “vacuolar cells” or “depleted cells”. Between the cells, there are broad pale staining areas extended from the periphery of the acinus to the lumen and laterally between the bases of the glandular cells and the basal lamina. This pale area is regarded as a labyrinthine system which is marked by darker staining linear structures, interpreted as strands of cytoplasm among elongated vacuoles or intercellular canaliculi within greatly hypertrophied interstitial cells (Figure 1.6). At the onset of feeding the f-cells hypertrophy, an extensive endoplasmic reticulum is developed, large numbers of dense secretory granules are produced and the density of mitochondria increases. At the highest point of their secretory phase, autophagic vacuoles appear which contain concentric cisternae of endoplasmic reticulum in various stages of degradation by lysosomal enzymes. Most of the membranous elements of the reticulum are eliminated due to the intense autophagic activity (Fawcett *et al.*, 1981b).

Coons & Kaufman (1988) demonstrated that SG development during feeding is triggered by a haemolymph-borne factor in *A. hebraeum.* They transplanted SGs of unfed females to the haemocoels of partially fed females and allowed the ticks to reattach to a host after implantation. Enlarged abluminal interstitial cells and formation of a fluid secretory labyrinth were apparent in the implanted glands. Autophagic vacuoles were also found in some of the cells of the implanted glands. The SGs of males do not undergo developmental changes during feeding nearly as elaborate as those of females.

Nevertheless, male SGs transplanted to the haemocoels of feeding females showed ultrastructural changes not much less than those occurring in transplanted female SGs. It is intriguing that the SGs of males can respond to a humoral signal that they are normally never exposed to. All this strongly suggests that SG development during feeding is triggered by a haemolymph-borne factor.

After the female feeds to repletion, the secretory tissues of the SGs are resorbed within 3-4 days (Kaufman, 1990). This process is characterized principally by the appearance of autophagic vacuoles in the degenerating tissue (Harris & Kaufman, 1981). Harris & Kaufman (1981) designed the following experiment in *A. hebraeum* to see whether SG resorption is triggered hormonally. They implanted SGs of small partially fed ticks into the haemocoels of ticks that had engorged 12-24 hours earlier. After 2 days, the implanted glands were examined by transmission electron microscopy (TEM) for ultrastructural signs of degeneration. Controls consisted of SGs from small partially fed ticks transplanted into the haemocoels of other small partially fed ticks. Numerous autophagic vacuoles were found in the fluid secretory tissue of the type III acini of the glands transplanted into the engorged ticks, but none were apparent in the glands transplanted into the partially fed ticks. These experiments indicated that there is a hormone secreted into the haemocoel of the engorged tick that triggers SG degeneration. Harris & Kaufman (1981) named this hormone "tick salivary gland degeneration factor" (TSGDF). This hormone is released once the tick has fed above the CW and removed from the host (Harris & Kaufman, 1984). The CW is around 10X the unfed weight in *A.*

hebraeum and *A. americanum* (Harris & Kaufman, 1984; Lindsay & Kaufman, 1988; Weiss & Kaufman, 2001).

So what is TSGDF? Diehl *et al.*, (1982a) were the first to identify the ecdysteroids, ecdysone (E) and 20-hydroxyecdysone (20-HE), in the haemolymph of *A. hebraeum* following engorgement. Harris & Kaufman (1985) tested whether E and 20-HE might trigger SG degeneration *in vivo* and *in vitro*. If an ES is injected into a tick below CW, the SGs are induced to degenerate. Furthermore, the concentration of ES needed to induce degeneration *in vitro* (organ culture assays) is within the same range that the tick produces normally within the first few days of engorgement (Harris & Kaufman, 1985, Kaufman, 1991). These studies indicate that TSGDF is an ES.

Although SG degeneration has been well known from histological studies for a long time (*e.g.* Till, 1961), the first physiological assays were developed by Harris & Kaufman (1981 & 1984). In 1981 they considered two biochemical assays; acid phosphatase (an autophagic vacuole marker enzyme) and succinate dehydrogenase (a mitochondrial marker enzyme). There was an increase in the acid phosphatase activity and decrease in the succinate dehydrogenase activity within 4 days post engorgement in *A. hebraeum*. The implication is that the number of autophagic vacuoles increases while the number of mitochondria decreases and these enzyme activities were taken as a quantitative index of SG degeneration.

Harris & Kaufman (1984) developed a third quantitative assay for SG degeneration: salivary fluid secretory competence, an assay which was much more convenient for extensive data collection. They measured the rate of

salivary fluid secretory competence as a function of days post removal for mated female *A. hebraeum* both above and below the CW. By 4 days post removal, females above the CW lost 95% of the fluid secretory competence prevailing on day 0 post removal (Harris & Kaufman, 1984), and autophagic vacuoles were in abundance in the fluid secretory tissue of the type III acinus (Harris & Kaufman, 1981). Mated partially fed females below the critical weight lost about 75% of their fluid secretory competence by day 4, and it plateaued at this level for at least 15 days (Harris & Kaufman, 1984). At least two lines of evidence indicated that this large decline in fluid secretory rate in partially fed ticks is not due to tissue degeneration. First, it was not accompanied by the appearance of autophagic vacuoles in the type III acini. Secondly, if such ticks are returned to the host after 4 days of removal, and if they reattach and feed for further 2 days, much of the predicted 75% loss by day 4 is not observed (Harris & Kaufman, 1984). The mechanism behind this reversible loss of fluid secretory competence is unknown (reviewed by Kaufman, 1989; Friesen & Kaufman, 2009) .

1.6 Ovary development

1.6.1 Organization of the ovary

The tick ovary is a single, tubular organ, located posterior to the synganglion and towards the ventral surface (Figure 1.7). The wall of the ovary consists of oocytes and interstitial cells prior to the blood meal. There is a longitudinal groove along the ovary that contains oocytes at the earliest stages of development. During oogenesis, oocytes begin to protrude into the surrounding haemolymph and also migrate peripherally away from the longitudinal groove (Diehl *et al.,* 1982b).

1.6.2 Growth of ovary and oocyte

In ixodid ticks, oocytes of various sizes and developmental stages are present at any given time during the gonotrophic cycle. As a consequence the oviposition period lasts for several weeks and a large number of eggs are produced (Lunke, 1991).

Tick oogenesis was first described in detail by Balashov (1972). He divided the process into five distinct stages (known subsequently as "Balashov's stages I-V"). In Balashov's stage I, the primary oocytes are in the period of 'small cytoplasmic growth'; this stage first appears in engorged, female nymphs during the entry to the prophase of the first maturation division. At this stage the volume of the oocyte increases only slightly. Oocytes enter Balashov's stage II after the initiation of the adult blood meal. This stage is called the 'great cytoplasmic growth phase', and is marked by an enlargement of the nucleus and cytoplasm, which results a protrusion of the oocytes into the haemocoel. At the end of stage II, the endoplasmic reticulum and Golgi bodies are well developed and the oocytes contain numerous mitochondria. Balashov's stage III and IV comprise the period of yolk-uptake, at the end of which ovulation occurs. Balashov's stage V is the ovulation stage when the oocytes pass into lumen of the ovary. Peristaltic contractions of the ovary push the resulting eggs through the oviducts and then into the vagina. The eggs are then taken from the genital pore by the mouthparts and placed on the dorsal surface of the capitulum where they come in contact with the everted Géné's organ. Géné's organ secretes egg wax to waterproof the oviposited eggs (Diehl, *et al.*, 1982b), and it secretes an antimicrobial substance to protect the eggs from attack by soil microbes (Arrieta *et al.*, 2006;

Yu *et al.,* 2012). The site of fertilization in hard ticks is still not very well defined, although there are two main hypotheses: the spermatozoa enter the eggs either in the lower third of the oviduct, or deep inside the ovaries prior to oocyte release in the lumen (Kisjewski *et al.,* 2001).

The Baslashov scoring system refers to individual oocytes. As mentioned earlier, even during oviposition, the ovary contains oocytes at all the Balashov's stages. Seixas *et al.,* (2008) proposed an ovarian growth phase (OGP) system for *A. hebraeum*. This system couples the Balashov system to a measure of ovary size. In OGP 1, ovaries are very thin and translucent white with ovoid shaped oocytes, <150 µm long, containing visible nuclei; OGP 1 corresponds to 0-2 days post engorged ticks. In OGP 2 the oocytes are longer (>250 µm) and thicker, with no visible nuclei, and have not yet taken up a significant amount of yolk. This phase corresponds to day 2-5 post engorgement. In OGP 3, many of the oocytes are reddish-brown, indicating the presence of yolk granules. OGP 4 is the pre-ovulation phase, when the ovary is the largest organ in the haemocoel apart from the midgut. The ovary contains many large, yolk-filled oocytes and distinct yolk spheres are easily visible. This phase lasts from around day 6 or 7 post-engorgement to the beginning of oviposition (day 10 or 11 post-engorgement). In OGP 5, appearance of the ovary is similar to that of OGP 4, except that the ovulated oocytes are evident in the lumen of the ovary and the oviducts, and oviposition may have begun.

1.7 Objectives of the thesis

So far, the concept of CW has been established only for *A. hebraeum*. The informal observations of several colleagues suggest some substantial differences between *Dermacentor* spp. (*D. andersoni* and *D. variablis*) and *A. hebraeum*. For example, unlike *A. hebraeum*, *Dermacentor* females attach and feed readily in the absence of males. Moreover, virgin female *Dermacentor* normally feed well beyond 10 times the unfed weight, even though they do not engorge fully and do not detach. In our laboratory, we define engorgement of a mated tick when it increases its weight at least 50 times above the unfed weight and detaches from the host spontaneously.

The major question arises: when a virgin *Dermacentor* female feeds beyond 10-13 times its unfed weight, is it feeding beyond its CW, or is its CW much higher than that of *A. hebraeum*? The objectives of my study were:

1. Establish the feeding stage (measured as fed/unfed weight ratio) at which salivary fluid secretion is maximal. The SGs of unfed ticks secrete very little fluid. Fluid secretory rate increases as feeding progresses, and reaches a plateau. Determining this plateau in *D. andersoni* is essential in order to subsequently determine the point at which loss of fluid secretory competence is a valid measure of SG degeneration.
2. Determine the pattern of fluid secretory loss in normal engorged ticks as a function of days post engorgement.
3. Determine the fed/unfed weight ratio at which partially fed mated ticks resemble the pattern observed in engorged ticks, as a function of days post removal. Determine the same for virgin partially fed ticks.

4. Demonstrate the appearance of autophagic vacuoles in the SGs of engorged and partially fed ticks as an indication of degeneration in both mated and virgin ticks.

5. Determine the fed/unfed weight ratio at which partially fed ticks no longer reattach to a host.

6. Determine the degree of ovary maturation/oviposition for the above groups. Ovary maturation was assessed by: (a) ovary weight as a % of body weight, (b) length of the most mature oocytes and (c) vitellin content as measured by a spectrophotometric assay of ovary homogenates.

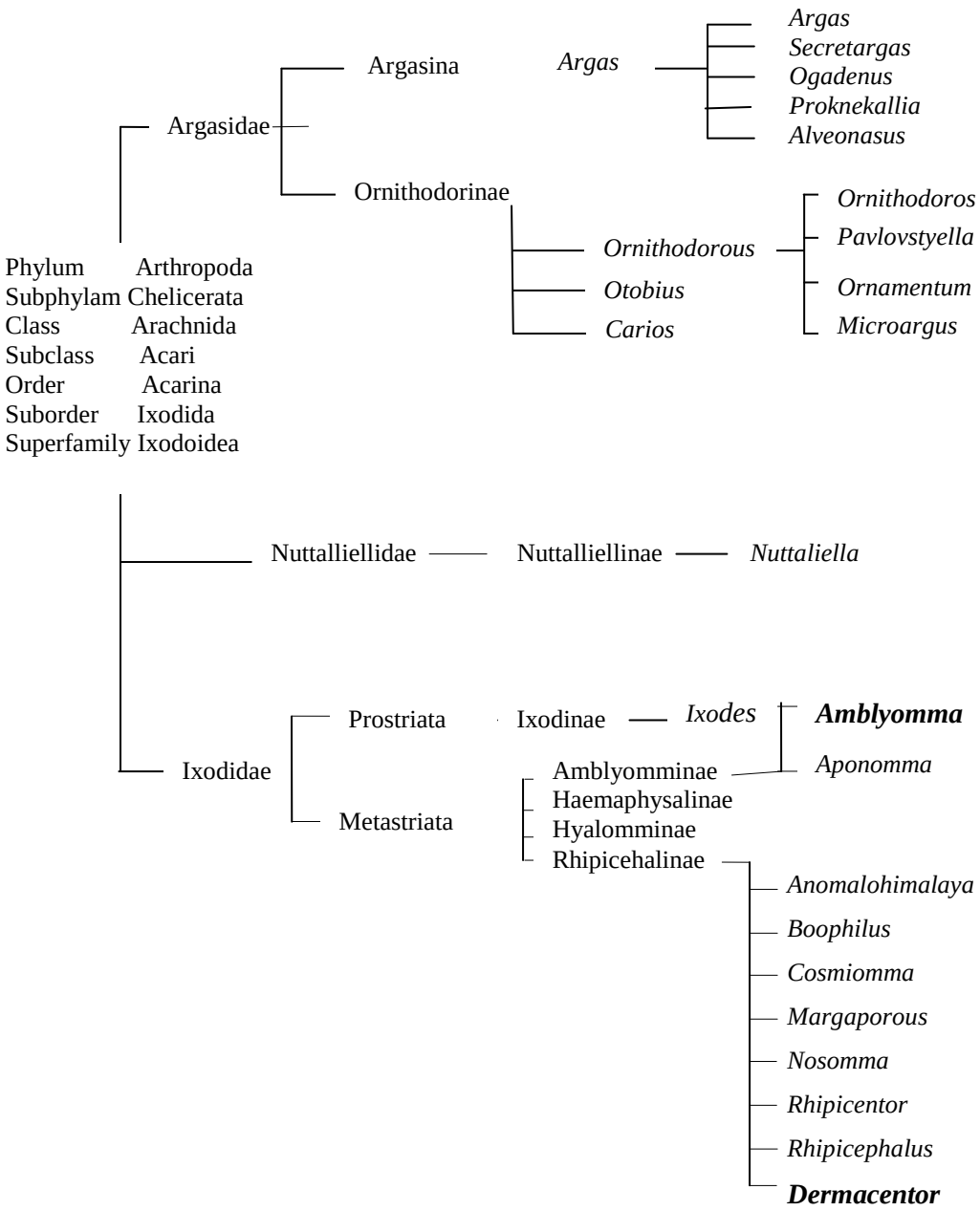

Figure 1.1: The phylogenetic relationships of ticks. This phylogenetic tree was modified from Klompen *et al.*, (1996) and Nava *et al.*, (2009).

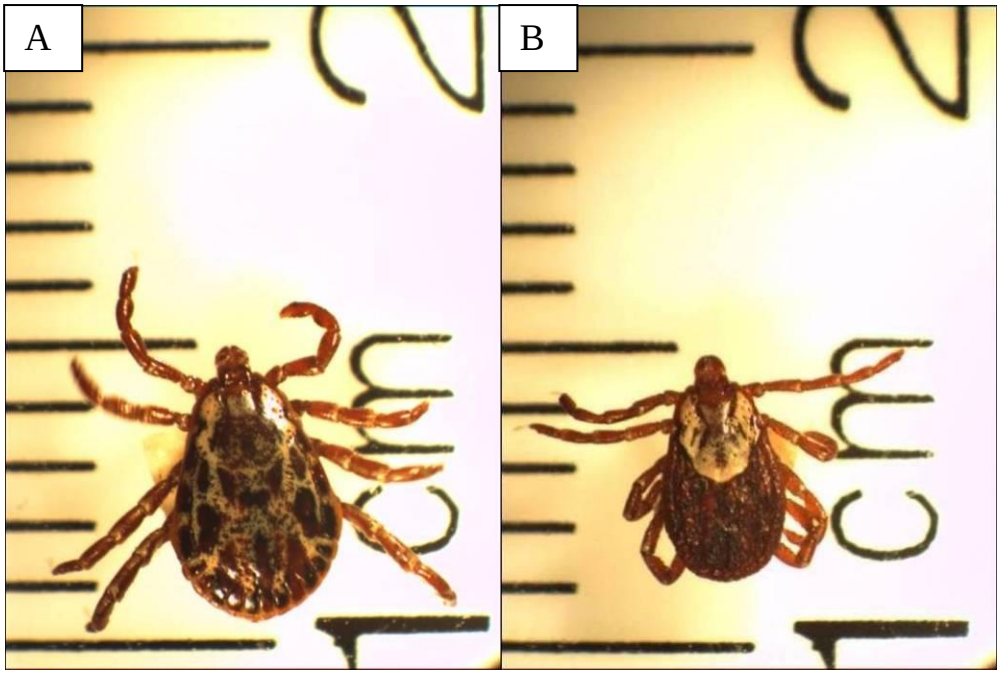

Figure 1.2 Photograph of *D. andersoni* ticks. (A) Unfed male (B) Unfed female. Each horizontal line in the panels represents 1 mm.

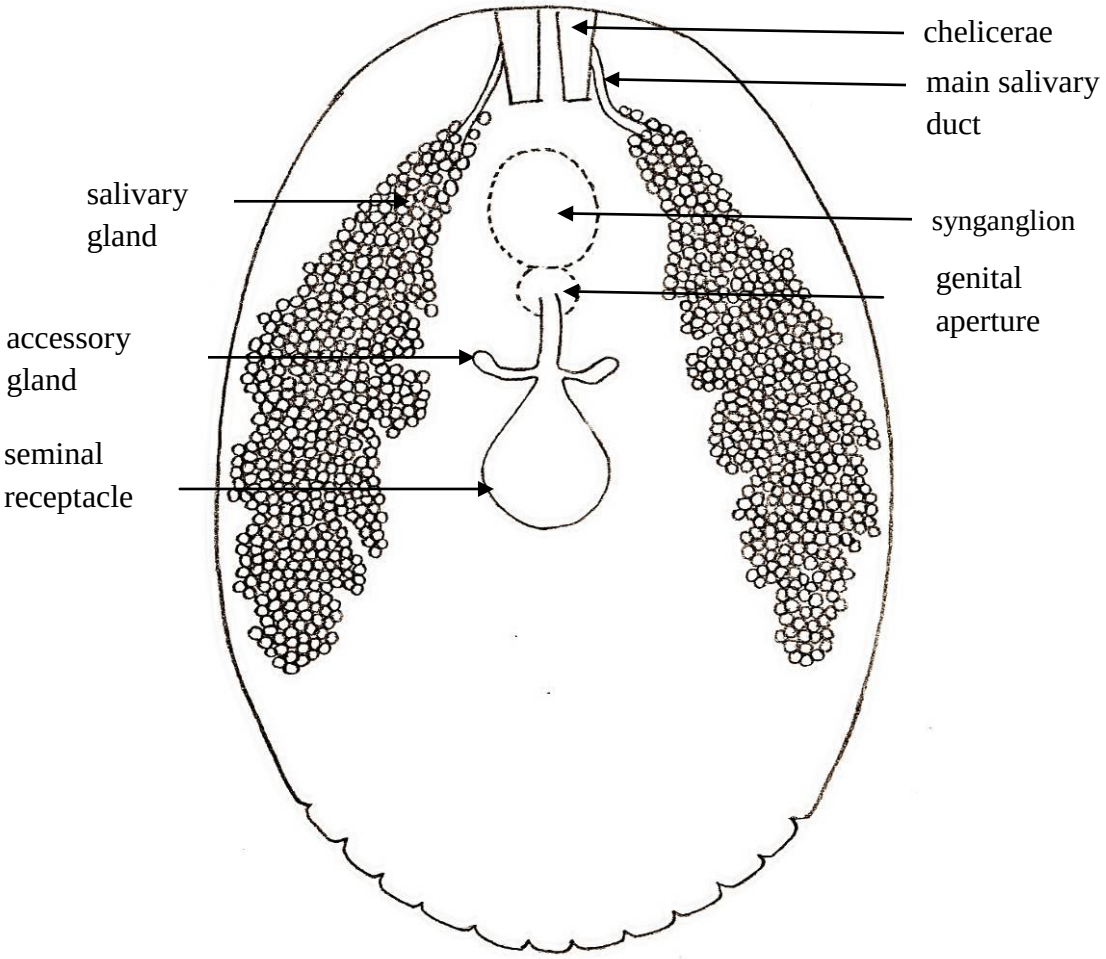

Figure 1.3 General diagram of the SGs of an unfed ixodid female. The gland is made up of numerous acini. The figure was modified from Till (1961), was hand drawn and the image retouched with photo editing software (Photoscape v3.6.1). Other internal organs (ovary, Malpighian tubule *etc.*) from the original image are not shown here.

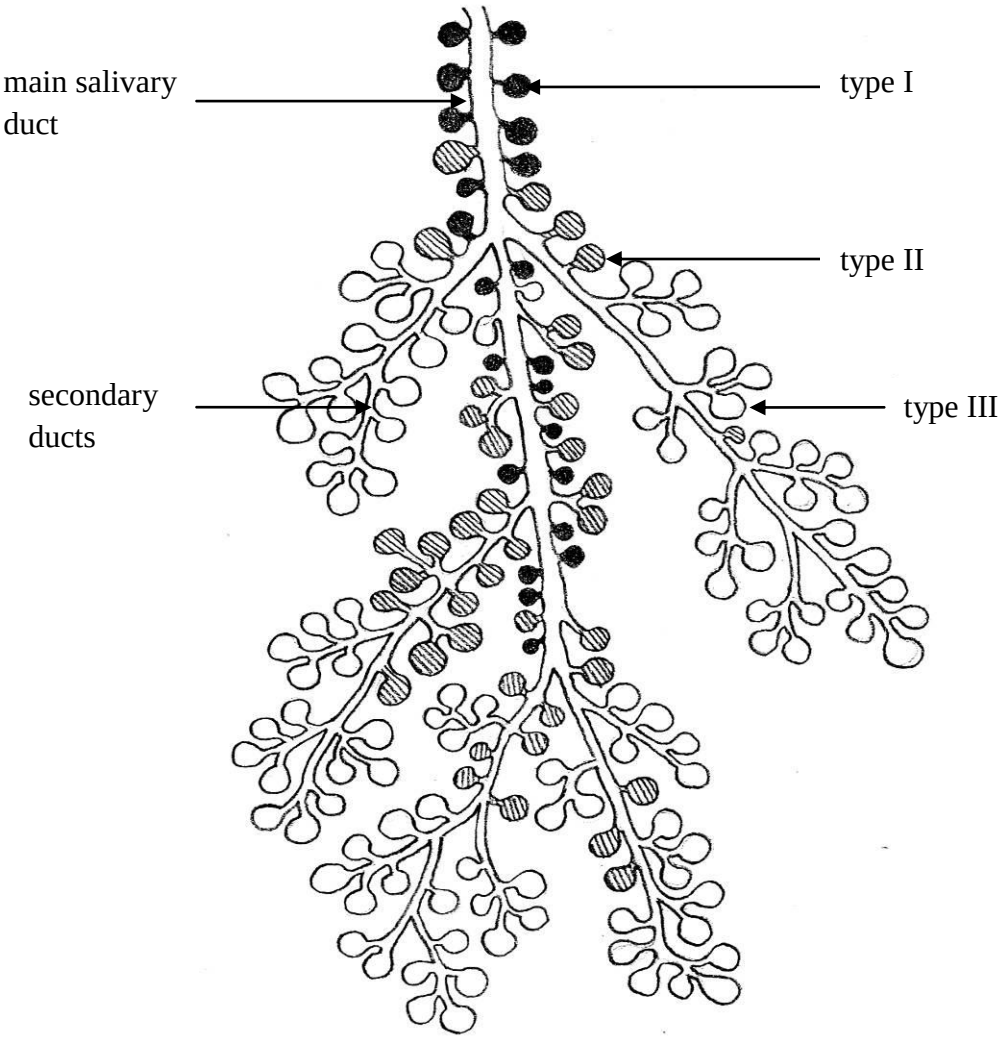

Figure 1.4 Schematic arrangement of the three acinus types of the female ixodid tick SG. The image comes from Binnington, (1978) and has been modified from Kaufman (1989). I redrew the image and retouched it by photo editing software (Photoscape v3.6.1). Type I (filled) acini drain directly to the main salivary duct. Type II (dashed) drain into the main salivary duct and secondary duct and type III (open) drain into the secondary ducts.

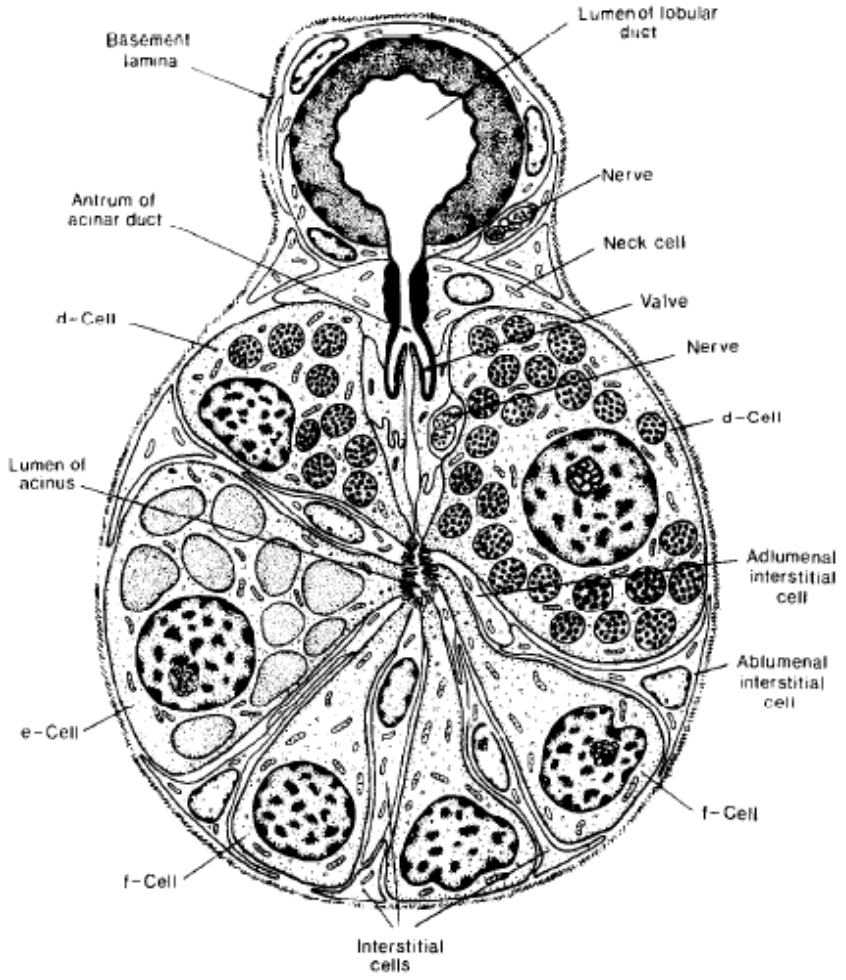

Figure 1.5 Diagramatic representation of the organization of type III acinus from unfed *R. appendiculatus*. The image shows the typical location of the three glandular cell types. The secretory cells are located in the compartments bounded by slender interstitial cells. The interstitial cells are in the alternating position with the glandular cells. The image has been reproduced from Fawcett *et al.*, (1981a) with the kind permission of Elsevier (Oxford, UK), provided by Copyright Clearance Center (CCC).

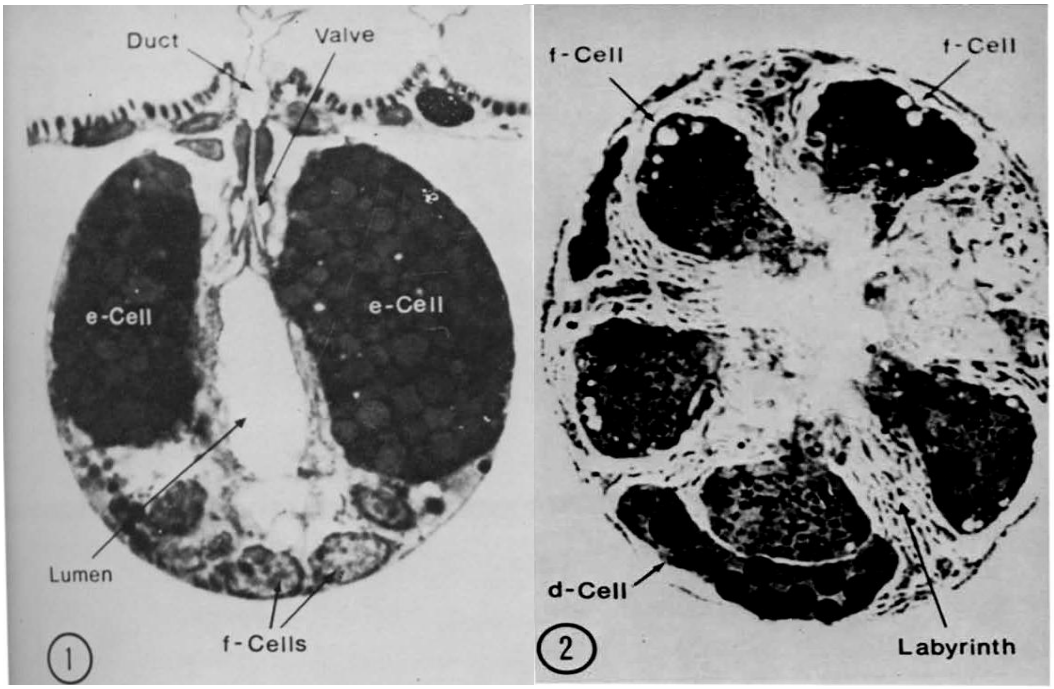

Figure 1.6: Photomicrograph of type III acinus of *R. appendiculatus*, (1) acinus of an unfed tick, showing a pair of e-cells near the valves and a number of f-cells at the fundus. Magnification X 1225 in the original figure. (2) Acinus after 6 days of feeding. Cells d and e appear to be degenerating. The f-cells are enlarged and are separated by pale zones consisting of a fluid secretory labyrinth in the intercellular spaces (comparing plate 1 and 2). The images are reproduced from Fawcett *et al.*, (1981b) with the kind permission of Elsevier (Oxford, UK), provided by Certificate Clearance Center (CCC).

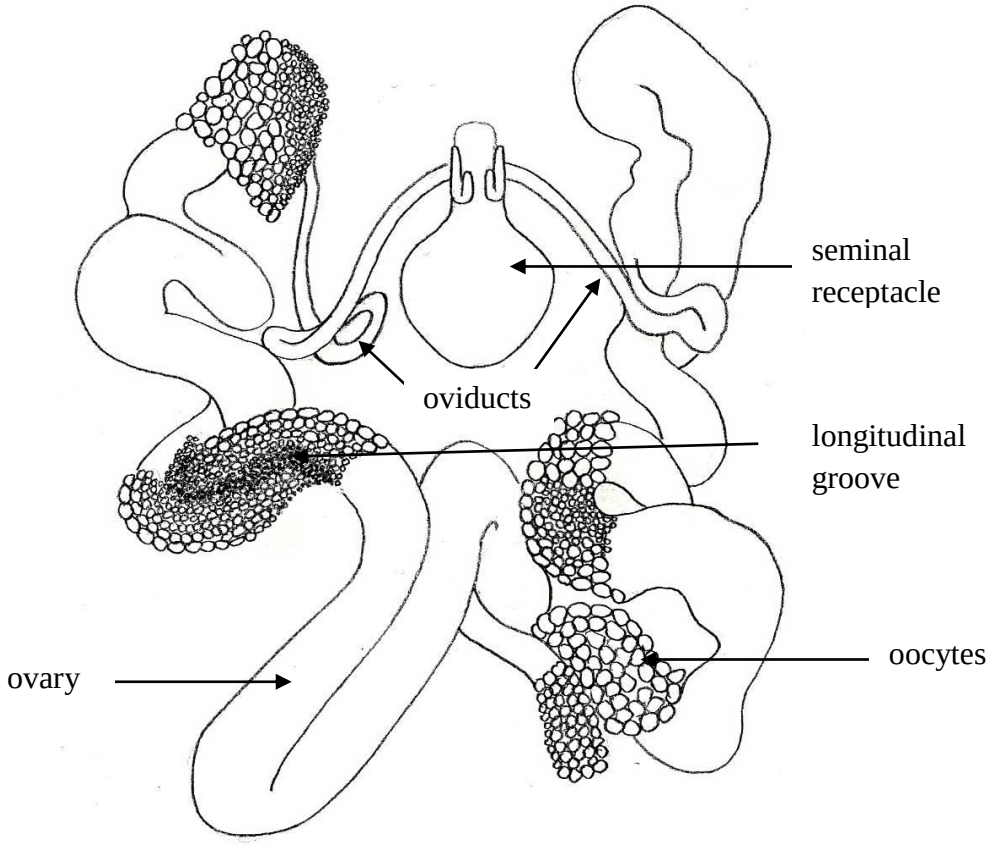

Figure 1.7 Semi-diagrammatic illustration of the ovary of *D. andersoni* (modified from Brinton & Oliver, 1971), hand drawn, image retouched with photo editing software (Photoscape v3.6.1). The ovary is suspended in the haemolymph. Each of the ends of the ovary is connected to an oviduct, which is connected to a common uterus and vagina (not shown in the image, because it underlies the seminal receptacle). A single seminal receptacle is attached to the (not-shown) uterus that stores the spermatophore following copulation.

CHAPTER TWO

Materials and Methods

2.1 Ticks

We used *D. andersoni* ticks from two sources. The first comprised male and female ticks (wild and colony reared) provided by Dr. Tym Lysyk (Lethbridge Research Centre, Agriculture and Agri-food, Canada, Lethbridge, Alberta). The second comprised female ticks provided by Dr. Glen Scoles, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington and Agricultural Research Service, United States Department of Agriculture. Ticks were maintained in darkness, at 7-9°C and held over saturated KNO_3 to maintain a RH of ~93%. The day before putting them on a rabbit, the ticks were transferred to 26°C. The ticks were fed on New Zealand white rabbits (SPF; Charles River, Senneville, Quebec, Canada) as described by Kaufman & Philips (1973). A day before exposing ticks to the rabbit for feeding, a cloth covered foam arena (approximately 12 cm X 8 cm X 2.5 cm) was glued to the shaven back of the rabbit with a latex adhesive (Roberts 8502 Latex, Bramalea, Ontario, Canada). In order to determine the ultimate fed/unfed weight ratio of each experimental tick, prior to feeding, each female was coded using a coloured silk thread tied and glued to a distinct leg and then weighed. Equal numbers of males and females were introduced together to the 'backpack' for experiments requiring mated females; only females were added for experiments requiring virgin females. Ticks were removed from the rabbit at various stages of feeding by grasping them carefully near the mouthparts with curved forceps as required for each

experiment. Once removed from the host, ticks were rinsed with water, dried with tissue paper, weighed and stored individually in a gauze covered plastic vial until dissection. The maximum duration that each rabbit was exposed to ticks for feeding was 21 days (the maximum time that our animal use protocol permits us to feed ticks on a given host). The animal use protocol was approved by the Biosciences Animal Policy and Welfare Committee, University of Alberta.

2.2 Tick groups

Ticks used for experiments were divided into four categories,

i) mated engorged: the ticks which were mated and dropped off sponaneously from the host after feeding to repletion

ii) virgin engorged: the ticks which were virgin (were put on the host without any males) and removed from the host after 21 days,

iii) mated partially fed: the ticks which were mated and removed forcibly from the host during various stages of feeding; and,

iv) virgin partially fed: the ticks which were virgin. Partially fed ticks were further divided into three groups based on fed/unfed weight ratio (4-9X, 13-19X and 19-60X the unfed weight).

Ticks within the weight range of 10-13X the unfed weight were not included in the analysis because of their ambiguous size relative to the CW of *A. hebraeum.*

2.3 Assay for salivary fluid secretory competence

The technique used was based on that described by Harris & Kaufman (1984). Prior to dissection, ticks were glued ventral surface down to a plastic Petri dish and cooled in a refrigerator for about 30 min. They were then bathed in modified Hank's saline (per litre: NaCl 11.5 g, D-glucose 1.6 g, KCl 0.4 g, phenol red 0.01g; the pH was adjusted to 7.2) and the SGs and ovary dissected out. These tissues were transferred to TC medium 199 made with Hank's salts (Sigma Chemical Company, St. Louise, Missouri, USA), supplemented with 2.09 g morpholinopropanesulphonic acid (MOPS - used as the buffer) and 2.1 g NaCl (used to make the medium isosmotic to tick haemolymph), in 1 litre milliQ (Millipore) water and pH adjusted to 7.2. The SGs and ovary were then dissected free of extraneous tissue. The main salivary duct was ligated with a fine strand peeled from silk thread. Each gland was transferred to a hydrophobic surface (Parafilm®) and extra-glandular fluid was removed by gentle application of a piece of filter paper. The gland was then weighed to the nearest 10 µg on a 5-place electronic balance (Mettler AE240, Mettler-Toledo AG, Greifensee, Switzerland) and immediately transferred to TC medium 199 containing freshly prepared dopamine (DA; 10 µM). Dopamine is probably the natural neurotransmitter that controls salivary fluid secretion in female ixodid ticks, and 10 µM is a supra-maximal concentration (Kaufman, 1976).

2.4 Ovary maturation assays

While the tissue was bathed in TC medium 199, the lengths of 10 largest oocytes from each tick were measured using a dissecting microscope fitted with an ocular micrometer. Each ovary was gently blotted using filter

paper, and weighed to the nearest 10 µg. The ovary was then homogenized in 1 ml milliQ water, frozen quickly on dry ice, and stored at -20°C in a small microfuge tube. For the spectrophotometric analysis of vitellin content, these ovary homogenates were thawed and diluted with milliQ water to a final volume of 2 ml. After gentle vortexing, the homogenates were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and stored at -20°C. For the final analysis, samples and blank were thawed and the absorbance at 400 nm (specific for the haem moiety of vitellin) and 500 nm (non-specific for haem) were measured on a spectrophotometer (Ultrospec 3300 pro, Biochrom Ltd, Cambridge, England). The difference between the two absorbance readings was recorded as a measure of vitellin content and normalized to ovary weight (expressed as 400 nm Δ 500 nm per gram ovary; Kaufman *et al.*, 1986; Seixas *et al.*, 2008).

2.5 Transmission Electron Microscopy (TEM) of SGs

The technique was based on the optimized protocol used in the Microscopy Unit, Department of Biological Sciences, University of Alberta. Briefly, SGs were dissected out under Hank's saline, large tracheae removed and the glands were fixed at 4 °C for at least 2 h in a mixture containing 2.5% gluteraldehyde, 2% paraformaldehyde, 0.1 M phosphate buffer, the pH being adjusted to 7.3. After fixation, the tissue was washed four times with 0.1 M phosphate buffer (two quick changes followed by two changes 10 min apart). Glands were post fixed with 1% OsO_4 in 0.12 M cacodylate buffer, pH 7.2 for 1-2 h. The glands were washed again with three changes of 0.1 M phosphate buffer over 30-60 min. The glands were then dehydrated through a graded ethanol series (50%, 70%, 90%; 15 min each change), followed by three

changes of absolute ethanol. The glands were incubated overnight in 1:1 ethanol: low viscosity spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA). This was followed by two changes of pure spurr resin, a few hours apart. Glands in resin were transferred to moulds and held at 70°C-80°C for 16-18 h. Tissue blocks were sectioned (70-90 nm thick) using an Ultracut E Reichert-Jung Ultramicrotome. Sections were transferred to copper grids (200 mesh). The sections were stained with 4% uranyl acetate for 25 min and counterstained in lead citrate (per 50 ml solution: 1.33 g lead citrate, 1.76 g sodium citrate in double distilled vacuum filtered H_2O, pH adjusted to 12.0 by 0.01 N NaOH) for 2 min. Grids were examined with transmission electron microscopes (Phillips Morgagni 268 and Phillips 410, operating at 80 kV) and the photomicrographs were taken with a Gatan Orius CCD camera.

2.6 Reattachment assay

Partially fed females (both mated and virgin) were removed (as mentioned earlier) from the host, weighed and placed in individual plastic vials in the colony incubator (26-27 °C, ~95% RH) for ~24 h. Females were returned to the host and given the opportunity to reattach. Reattachment was monitored after several hours in the first day and at 24 h-intervals from the next day onward.

2. 7 Statistics

Unless otherwise stated, all data were reported as mean ± SEM (*N),* using Microsoft Excel software (Microsoft Office 2007). Statistical significance was determined by student's *t*-test using STATA 10.0 (StataCorp, Texas, USA) on a Macintosh Computer. Statistical difference between the

same group (4-9X, 13-19X and 19-60X) on various days was compared to the first day of observation. Asterisks (*) represent the statistical significance among the groups on the same day post-detachment, compared to the 4-9X the unfed weight. Statistical significance is indicated as follows: (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

CHAPTER THREE

Results

3.1 SG degeneration

The fed/unfed weight ratio for engorged *D. andersoni* used in this experiment was in the range of 50-100 [average: mated 87 ± 7 (62); virgin 57 ± 4 (79)]. Mated *D. andersoni* females normally take 6-8 days to engorge fully and detach spontaneously. Virgin *D. andersoni*, on the other hand, do not detach spontaneously, at least not within 3 weeks.

3.1.1 Engorged ticks (mated and virgin females)

There was a continuous 95% decline in salivary fluid secretory competence of the mated ticks from day 0 post engorgement (PE) [4.60 ± 0.3 (20) mg/gland/15min] to day 4 PE [0.23 ± 0.06 (13) mg/gland/15 min] (Figure 3.1). There was no significant change in the fluid secretory competence of the virgin tick SGs from day 0 PE [1.90 ± 0.12 (23) mg/gland/15 min) to day 3 PE [1.73 ± 0.13 (30) mg/gland/15 min]. Rate of salivary fluid secretion of these glands began to decrease substantially after day 3 PE, and they eventually lost 91% of their initial secretory competence by day 8 PE [0.15 ± 0.06 (10) mg/gland/15 min] (Figure 3.1).

3.1.2 Ultrastructural evidence for salivary gland degeneration

Autophagic vacuoles were not observed in the SGs of partially fed females on day 0 PD (Figure 3.2. A, B). Autophagic vacuoles were apparent already on day 0 PE in both mated and virgin tick SGs (Fig 3.3 A, B). In mated ticks, the apparent density of autophagic vacuoles increased progressively through day 4 PE. The situation was the same for virgin ticks,

except that they required 8 days to achieve a similar density of autophagic vacuoles that mated ticks achieved in 4 days (Figure 3.4 to 3.7).

3.1.3 Partially fed ticks (mated and virgin females)

The salivary fluid secretory competence of the mated 4-9X group was 0.97 ± 0.15 (23) mg/gland/15 min on day 0 PD and reduced significantly by day 4 PD [0.53 ± 0.06 (27) mg/gland/15 min; Figure 3.8]. The salivary fluid secretory competence of mated 13-19X group was 3.02 ± 0.2 (17) mg/gland/15 min on day 0 PD and 90% lower by day 4 PD. The salivary fluid secretory competence of the mated 19-60X group was 3.83 ± 0.4 (16) mg/gland/15 min on day 0 PD and was 92% lower by day 4 PD. The difference in salivary fluid secretory competence between mated 13-19X group and 19-60X group was not statistically significant from day 0 PD through day 4 PD (Figure 3.8).

The salivary fluid secretory competence of virgin 4-9X group did not reduce significantly over 8 days post-detachment (Figure 3.9). The salivary fluid secretory competence of the 13-19X group was 1.07 ± 0.14 (14) mg/gland/15 min on day 0 PD and was 50% lower by day 8 PD. The salivary fluid secretory competence of the 19-60X group was 1.75 ± 0.14 (29) mg/gland/15 min on day 0 PD and was 70% lower by day 8 PD.

3.1.4 Ultrastructural evidence for salivary gland degeneration in the partially fed ticks

Autophagic vacuoles were not found in the SGs of partially fed females on day 0 PD (Figure 3.10). Autophagic vacuoles also did not appear in the partially fed ticks 7X (mated) and 8X (virgin) the unfed weight ticks by 4

days PD (Figure 3.11). However, autophagic vacuoles appeared in the 14X (mated tick) and 16X (virgin tick) by 4 days PD (Figure 3.12). Autophagic vacuoles also appeared in 42X the unfed weight tick's SG on day 6 PD (Figure 3.13).

3.2 Ovary Development Assay

3.2.1 Ovary weight of engorged ticks (mated and virgin)

There was a progressive increase in the ovary weight of the mated ticks from day 0 PE to day 4 PE (Figure 3.14). The ovary weight then declined at day 6 PE and more on day 8 PE, probably because oviposition began by day 4 PE and was well underway by day 6 PE. On the other hand, there was no significant increase in ovary weight of the virgin ticks up to day 3 PE ($P > 0.05$). The ovary weight increased significantly on day 4 PE ($P = 0.03$) and remained at this level thereafter up to day 8 PE (Figure 3.14).

3.2.2 Ovary weight of partially fed ticks (mated and virgin)

In the mated 4-9X group; the ovary weight did not change significantly from day 0 PD to day 4 PD ($P > 0.05$) (Figure 3.15). In the mated 13-19X group, there was no significant change from day 0 PD to day 3 PD ($P > 0.05$) though it increased significantly by day 4 PD ($P = 0.001$). The ovary weight increased progressively in the 19-60X the unfed weight group from day 0 PD to day 4 PD ($P \leq 0.000$).

The ovary weight of the virgin partially fed ticks in the 4-9X group also did not change significantly from day 0 PD to day 8 PD ($P > 0.05$) (Figure 3.16). There was also no significant change in the ovary weight of the 13-19X group from day 0 PD through day 8 PD ($P > 0.05$). However, in the

19-60X group the ovary weight increased progressively from day 0 PD to day 4 PD ($P < 0.000$) where it remained up to day 8 PD.

3.2.3 Oocyte length of engorged ticks (mated and virgin)

In the mated ticks oocyte length increased progressively from day 0 PE to day 4 PE and maintained this level up to day 8 PE ($P \leq 0.000$). In the virgin ticks, the oocyte length remained unchanged up to day 3 PE ($P > 0.05$). It increased significantly on day 4 PE (P=0.000) and maintained this level thereafter up to day 8 PE (Figure 3.9). The oocytes of the mated ticks were apparently larger than the virgin ticks during oviposition [mated: 564 ± 28.9 µm (11) on day 4 PE; virgin 457.9 ± 16.9 µm (12) on day 8 PE; $P = 0.0001$ (day 4 PE); $P \leq 0.003$ (day 8 PE)].

3.2.4 Oocyte length of partially fed ticks (mated and virgin)

The oocyte length of the 4-9X mated group did not increase significantly from day 1 PD through day 4 PD ($P > 0.05$; Figure 3.18). In the 13-19X the unfed weight group the oocyte length did not increase until days 3 ($P = 0.02$) and 4 PD ($P \leq 0.000$). There was progressive increase of the oocyte length of the 19-60X group from day 1 PD to day 4 PD ($P \leq 0.000$).

There was no significant growth of the oocyte length of the virgin partially fed 4-9X group from day 0 PD through day 8 PD ($P > 0.05$; Figure 3.19). Oocyte length of the 13-19X group was generally higher than that of the 4-9X group; however, the change in the oocyte length from day 0 PD through day 8 PD was not statistically significant ($P > 0.05$). The oocyte length of the 19-60X group was generally higher than that of the other two groups on day 0

PD but did not increase significantly up to day 3 PD ($P > 0.05$). It increased on day 4 PD ($P = 0.000$) and peaked on day 6 PD.

3.2.5 Ovary vt content in engorged ticks (mated and virgin)

In the mated ticks the amount of vt increased steeply from day 0 PE to day 4 PE ($P = 0.000$; Figure 3.20). Although the ovary vt content of the virgin ticks on day 0 PE was higher ($P = 0.001$) than that of the mated ticks, in virgins it remained unchanged up to day 3 PE after which it increased progressively, peaking by day 6 PE.

3.2.6 Ovary vt content in the partially fed ticks

In the mated partially fed 4-9X group the ovary vt content did not increase significantly through day 4 PD ($P > 0.05$; Figure 3.21). There was also no increase in the ovary vt content of the 13-19X group through day 4 PD ($P > 0.05$). Comparatively higher amount of vt were measured in the 19-60X group on day 3 PD ($P = 0.01$) and on day 4 PD ($P = 0.000$) compared to that of day 1 PD. In the virgin 4-9X group the ovary vt content did not increase significantly the 8-day period ($P > 0.05$). In the 13-19X group the vt content increased slowly by day 6 PD ($P = 0.000$). However, ovary vitellin content of the 19-60X group did not increase until day 4 PD, but rose progressively thereafter through day 8 PD ($P = 0.000$; Figure 3.22).

3.3 Reattachment Assay

There was no clearly defined fed/unfed weight ratio observed for reattachment to the host by both mated and virgin ticks. Among the eight mated partially fed ticks that were put on the host (8-14X the unfed weight), 7

of them attached within 24-48 hours and engorged within 72-96 hours of reattachment. One tick (11.5X the unfed weight) never reattached (Figure 3.23).

Out of the 11 virgin partially fed ticks that were allowed to reattach (20-36X the unfed weight), 7 of them reattached within 24 - 48 hours. Three ticks (two of them were ~25X the unfed weight and one was ~27X the unfed weight) never attached to the host. One tick (~32X the unfed weight) reattached but later on was found dead but still attached. Unfortunately, I did not have a better overlapping of weight ranges between virgin and mated ticks; see a further word on this in the discussion.

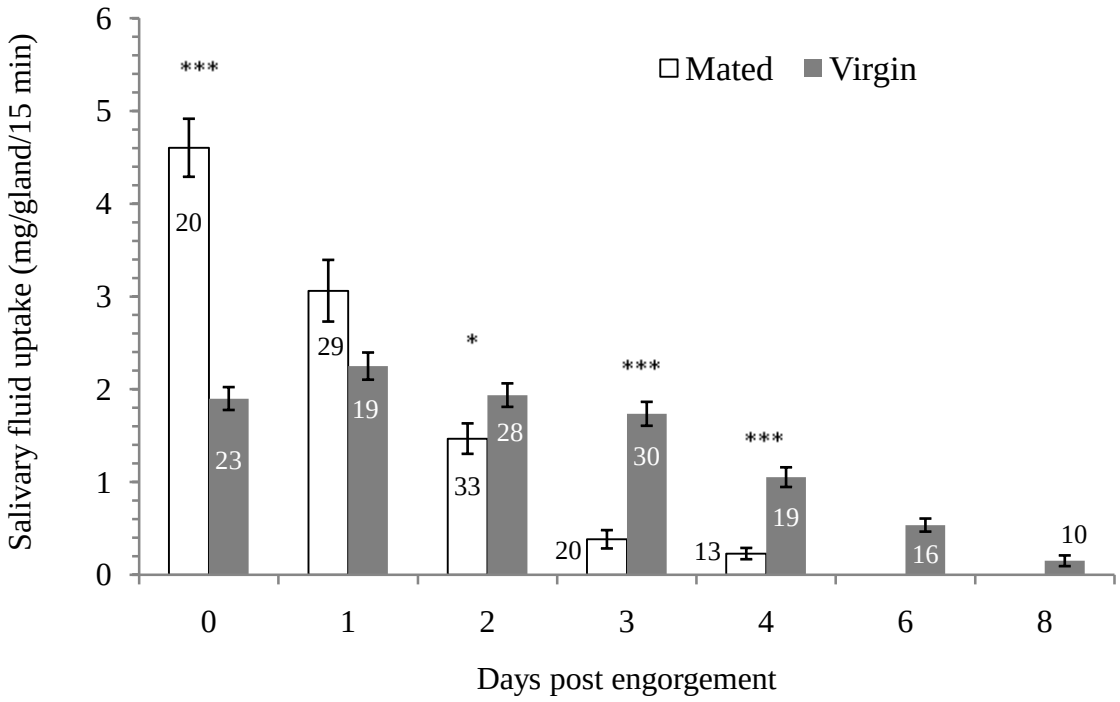

Figure 3.1: Rate of salivary fluid uptake in engorged (mated and virgin) *D. andersoni* females as a function of days post engorgement. Mean, SEM and N are indicated for each bar. Among the mated ticks a significant loss of fluid secretory competence occurred 4 days PE (in all cases $P = 0.000$) in comparison to day 0 PE. Among the virgin ticks a significant loss of fluid secretory competence began only on day 4 PE ($P = 0.000$). Asterisks (*) indicate statistical significance between the mated and virgin ticks on the same day determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

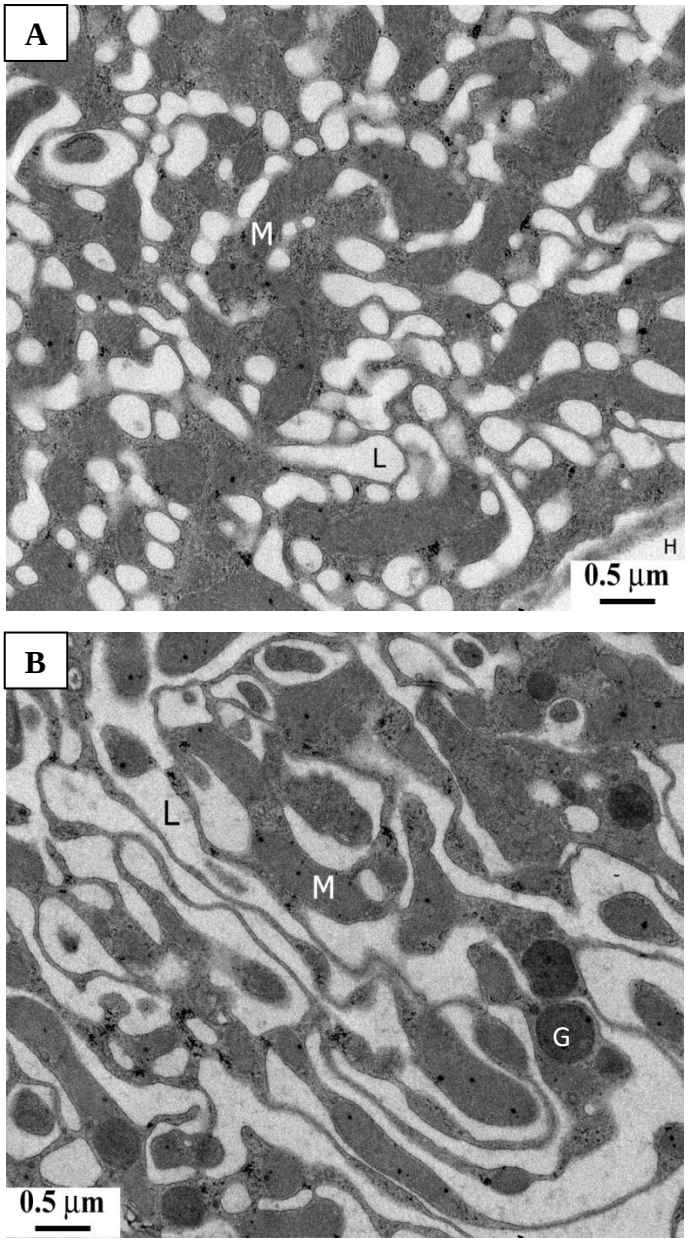

Figure 3.2 Type III acinus of the SG of (A) a partially fed mated female on day 0 post detachment, 7.5X the unfed weight (0 DPD-M) and (B) a partially fed virgin female on day 0 PD, 8X the unfed weight (0 DPD-V). In both mated and virgin ticks there were no apparent autophagic vacuoles in the fluid secretory labyrinth; numerous mitochondria are present. M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph, G- secretory granules.

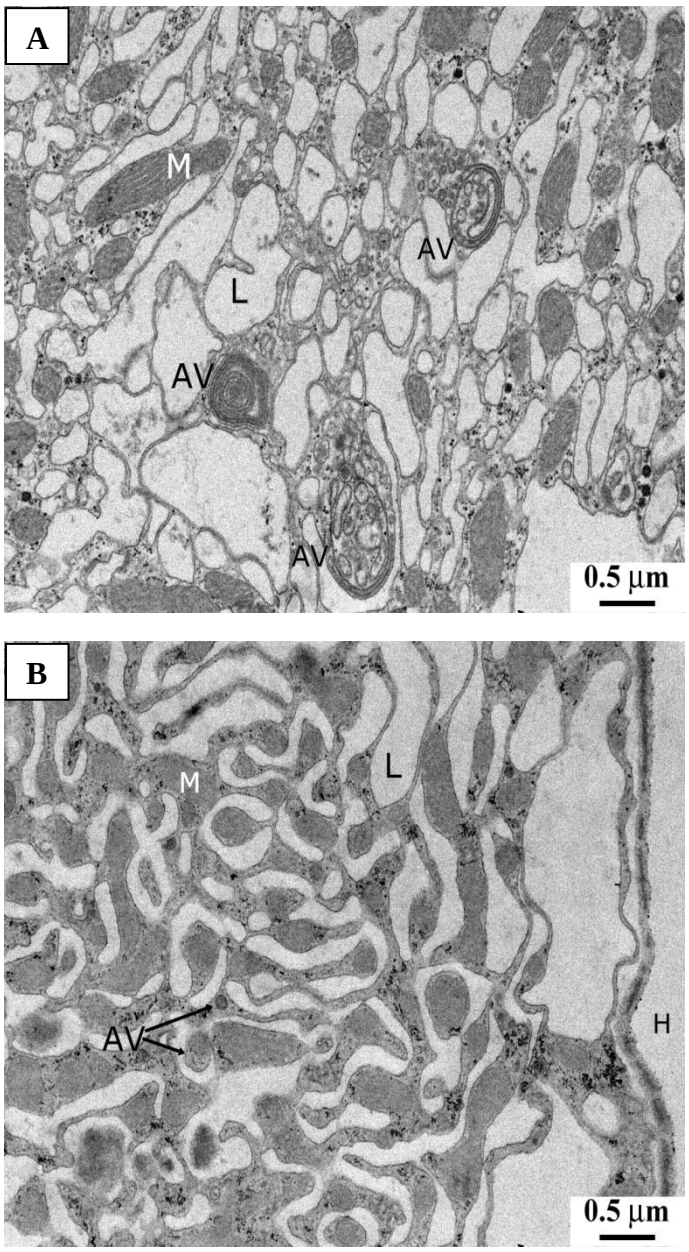

Figure 3.3 Fluid secretory acinus of the type III acinus of (A) a 0 DPE-M female; 93X the unfed weight and (B) a 0 DPE-V female, 81X the unfed weight. In both mated and virgin SGs, autophagic vacuoles are apparent, although they are more prominent in the mated SG. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

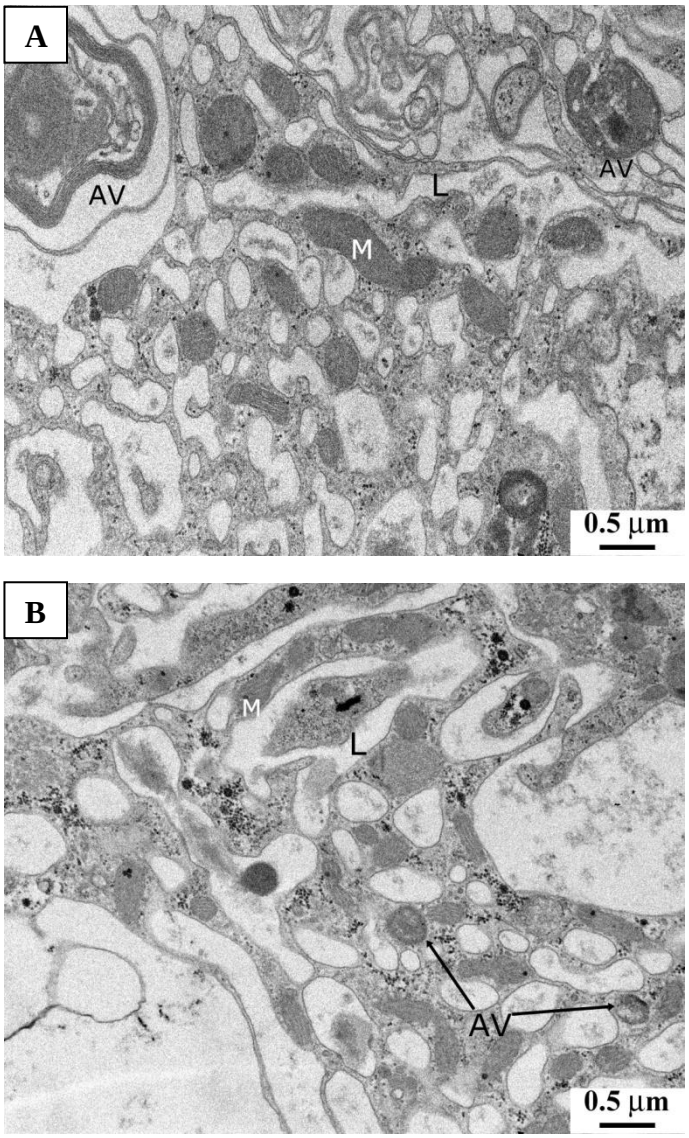

Figure 3.4 Fluid secretory acinus of the type III acinus of (A) a 2 DPE-M female; 96X the unfed weight and (B) a 2 DPE-V female; 102X the unfed weight. In both mated and virgin SGs, autophagic vacuoles are apparent, although they are still more prominent in the mated SG. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

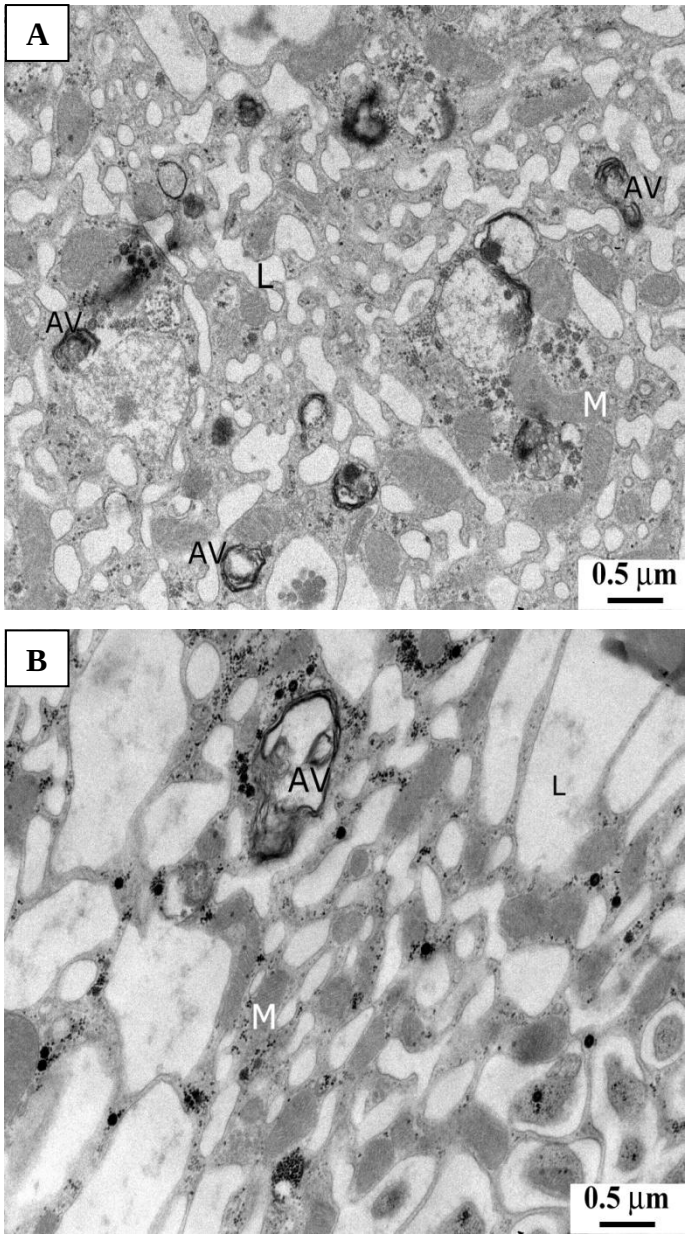

Figure 3.5 Fluid secretory acinus of the type III acinus of (A) a 3 DPE-M, 103X the unfed weight and (B) a 3 DPE-V female, 83X the unfed weight. In both the mated and virgin SGs, autophagic vacuoles are apparent. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

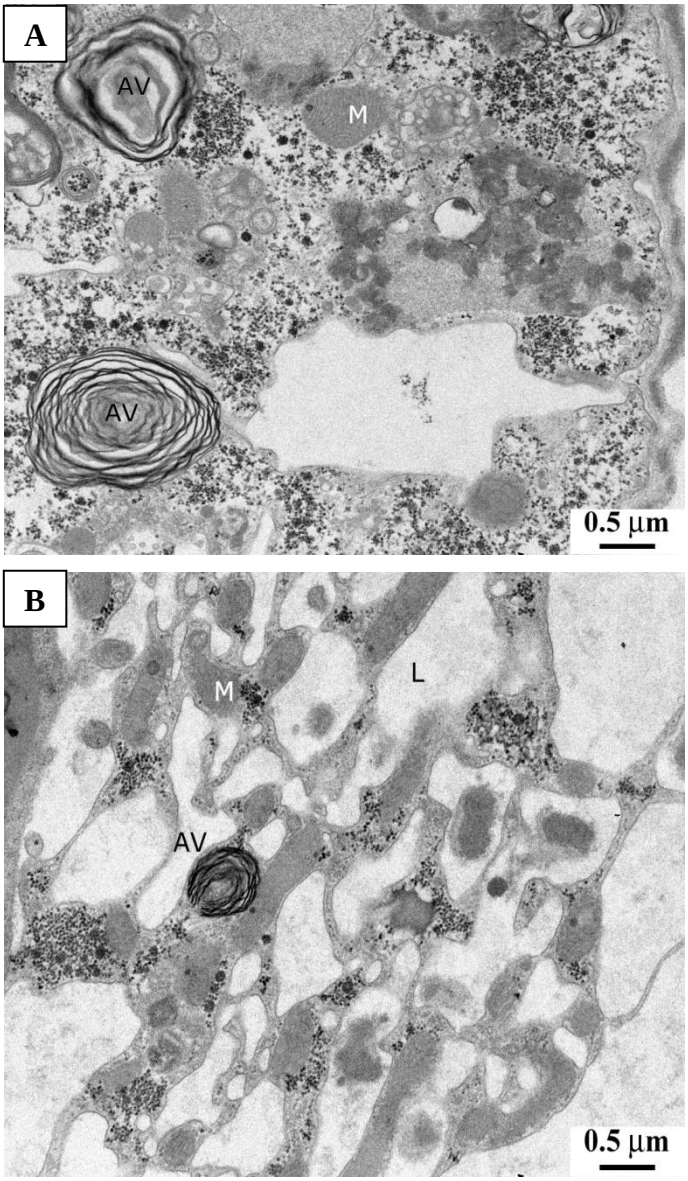

Figure 3.6 Fluid secretory acinus of the type III acinus of (A) a 4 DPE-M female, 80X the unfed weight and (B) a 4 DPE-V female, 86X the unfed weight. In both the mated and virgin SGs, the autophagic vacuoles are well-developed, and there appear to be fewer mitochondria than on earlier days. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

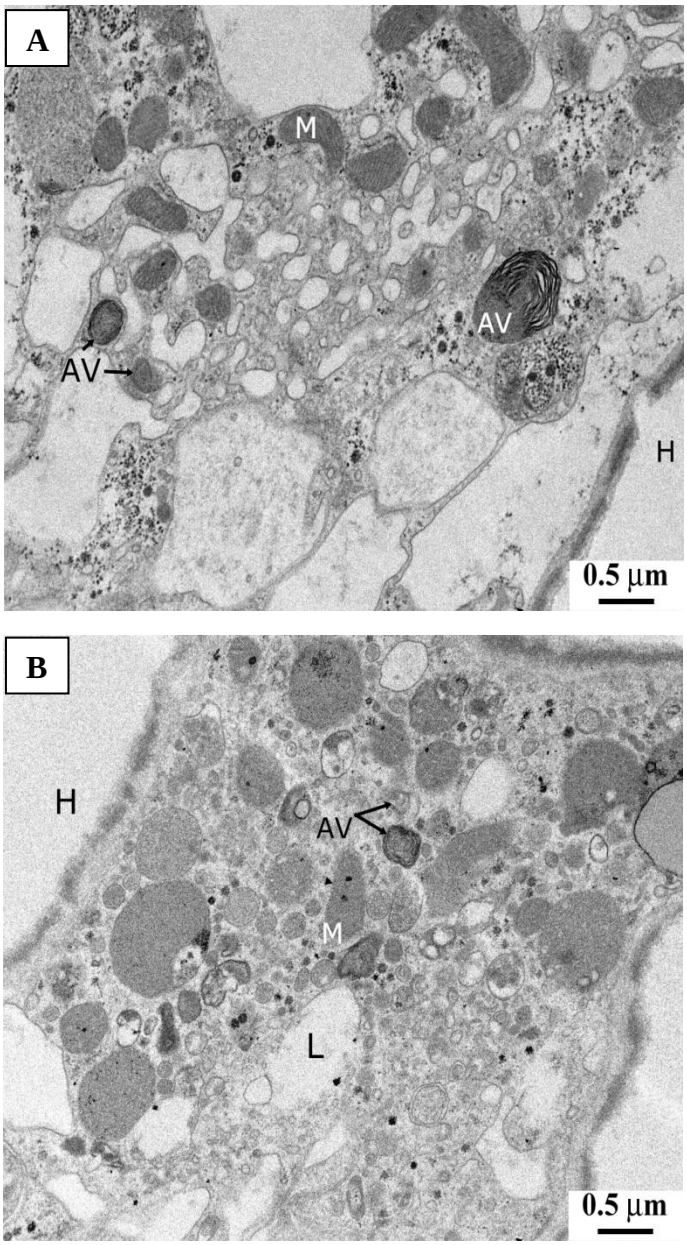

Figure 3.7 Fluid secretory acinus of the type III acinus of (A) a 6 DPE-V female, 84X the unfed weight and (B) an 8 DPE-V female, 134X the unfed weight. The degree of degeneration appears similar in both panels. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

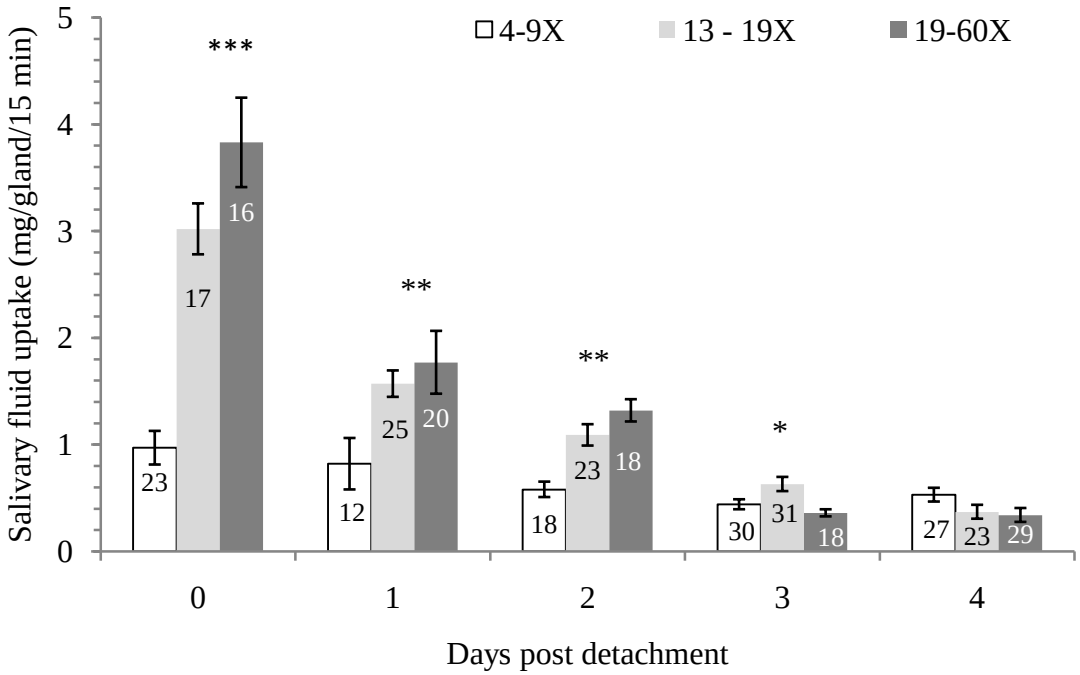

Figure 3.8 Rate of salivary fluid uptake in mated partially fed ticks as a function of days post detachment. Mean, SEM and *N* are indicated for each bar. The apparent loss of fluid secretory competence in the 4-9X group from day 0 PD to day 4 PD was statistically significant ($P = 0.008$). In the 13-19X group fluid secretory competence reduced significantly from day 0 PD through day 4 PD ($P = 0.000$). In the 19-60X group the fluid secretory competence reduced progressively over the first 3 days PD ($P = 0.000$) compared to day 0 PD, but was not significantly lower on day 4 PD than that of day 3PD ($P > 0.05$). Asterisks (*) indicate statistical significance among the groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

In this figure and in figures 3.9, 3.15, 3.16, 3.18, 3.19, 3.21 and 3.22, the data for the 9-13X groups are omitted for the reasons presented in materials and methods section 2.2.

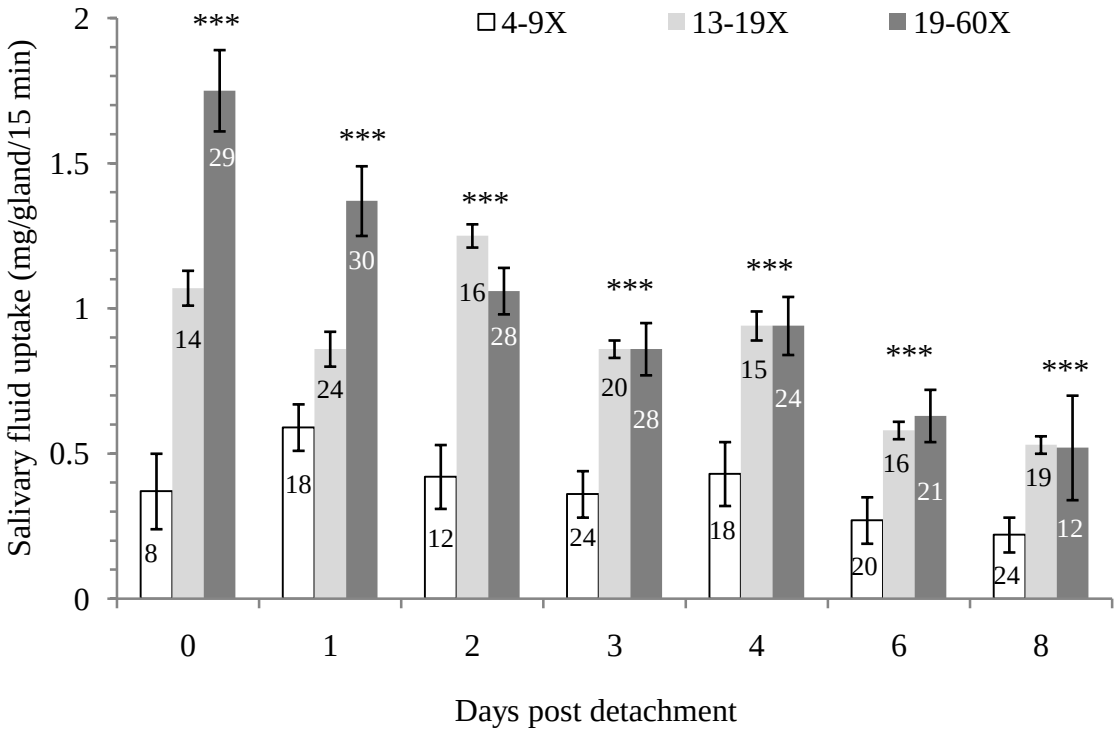

Figure 3.9: Rate of salivary fluid uptake in virgin partially fed ticks as a function of days post detachment. Mean, SEM and *N* are indicated for each group. The rate of fluid secretory competence of the 4-9X group changed significantly only on 8 days PD ($P = 0.02$) in comparison to day 0 PD. The reduction of the fluid secretory competence of the 13-19X group was significant by day 6 PD ($P > 0.000$) compared to day 0 PD. In the 19-60X group the fluid secretory competence reduced significantly over the 8 days PD ($P = 0.000$) compared to day 0 PD. Asterisks (*) indicate statistical significance among the groups on the same day, compared to the 4-9X group, determined by Students *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

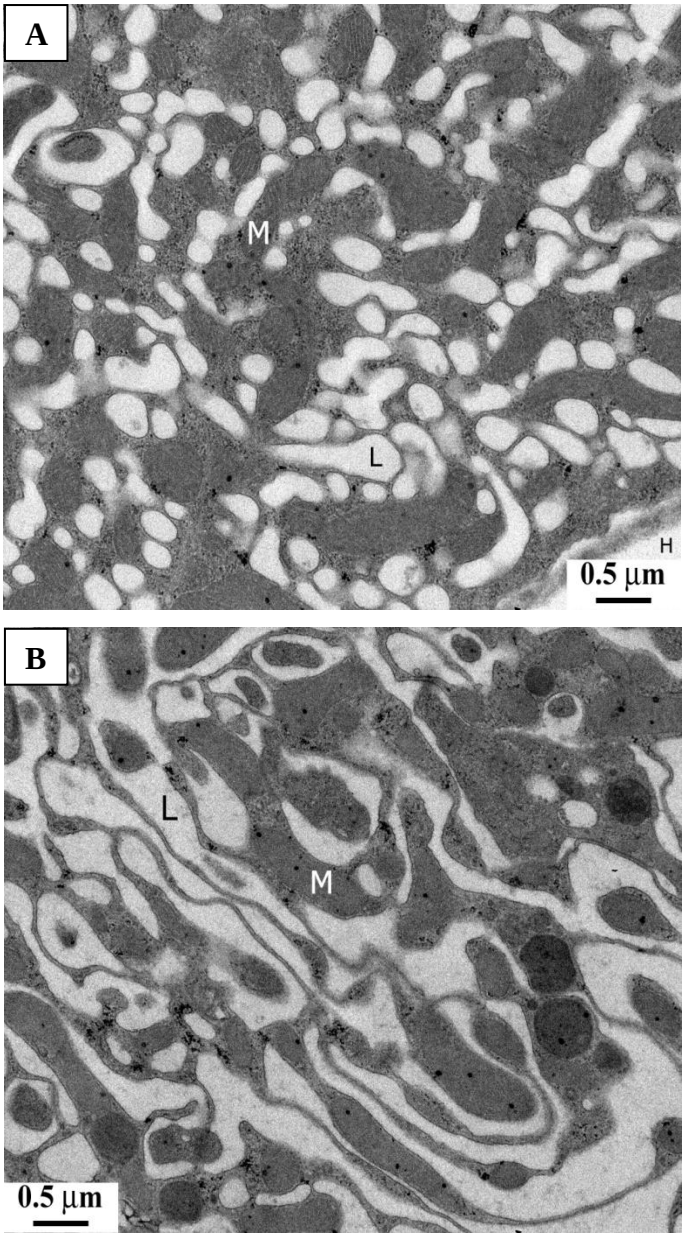

Figure 3.10 Type III acinus of the SG of (A) a partially fed mated tick on day 0 post detachment (0 DPD-M), 7.5X the unfed weight and (B) a partially fed virgin tick on day 0 PD (0 DPD-V), 8X the unfed weight. In both mated and virgin ticks there are no apparent autophagic vacuoles in the fluid secretory labyrinth. M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

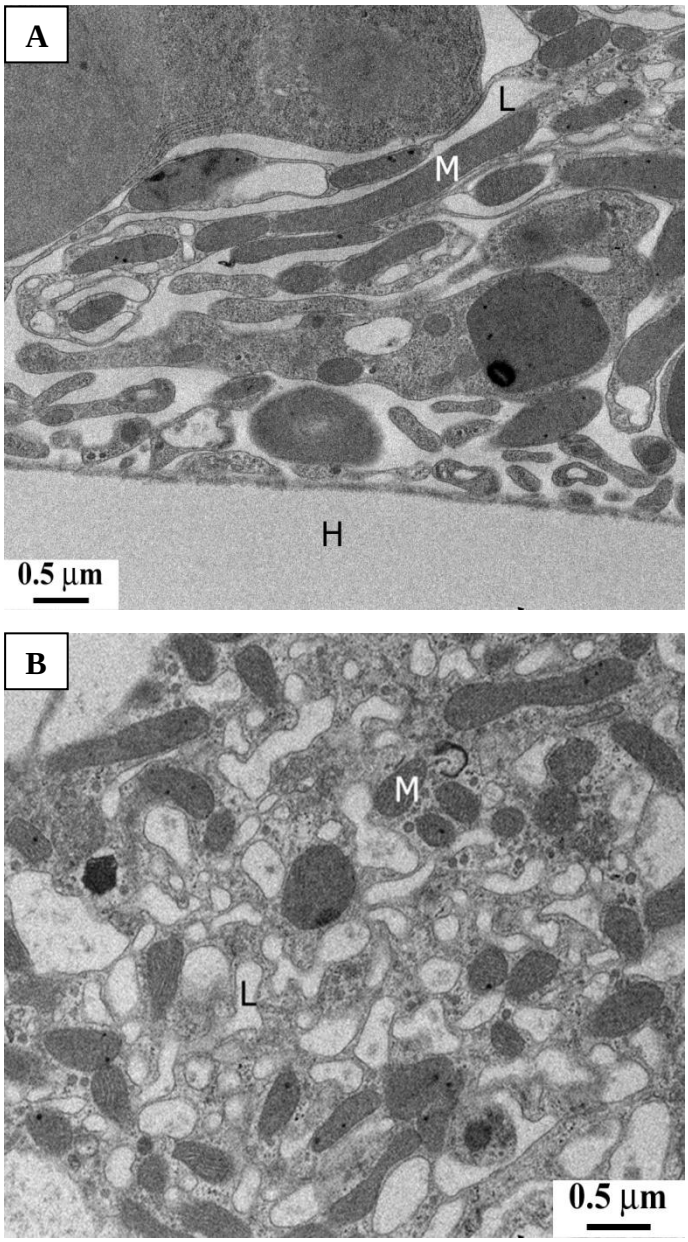

Figure 311 Type III acinus of the SG of (A) a partially fed mated tick on day 4 post detachment 7X the unfed weight (4 DPD-M) and (B) a partially fed virgin tick on day 4 PD 8X the unfed weight (4 DPD-V). In both mated and virgin ticks there are no apparent autophagic vacuoles in the fluid secretory labyrinth. M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

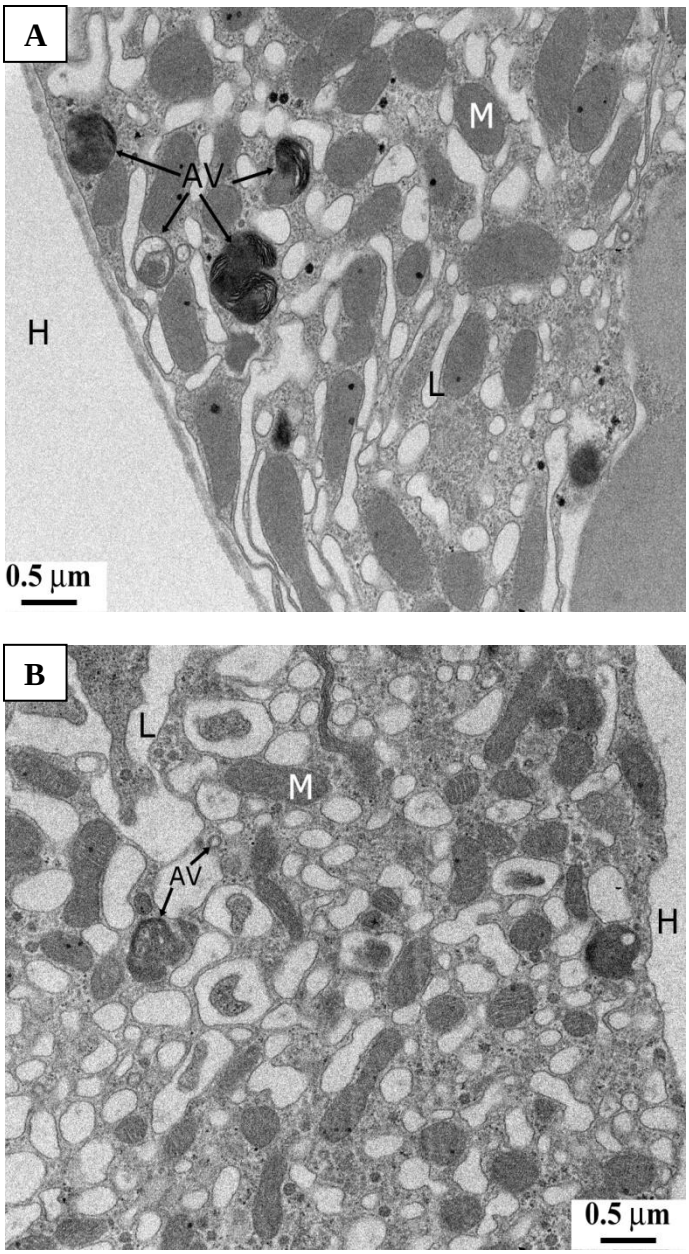

Figure 3.12 Type III acinus of the SG of (A) a partially fed mated tick on day 4 post detachment 14X the unfed weight (4 DPD-M) and (B) a partially fed virgin tick on day 4 PD 16X the unfed weight (4 DPD-V). In both mated and virgin ticks autophagic vacuoles are apparent in the fluid secretory labyrinth. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

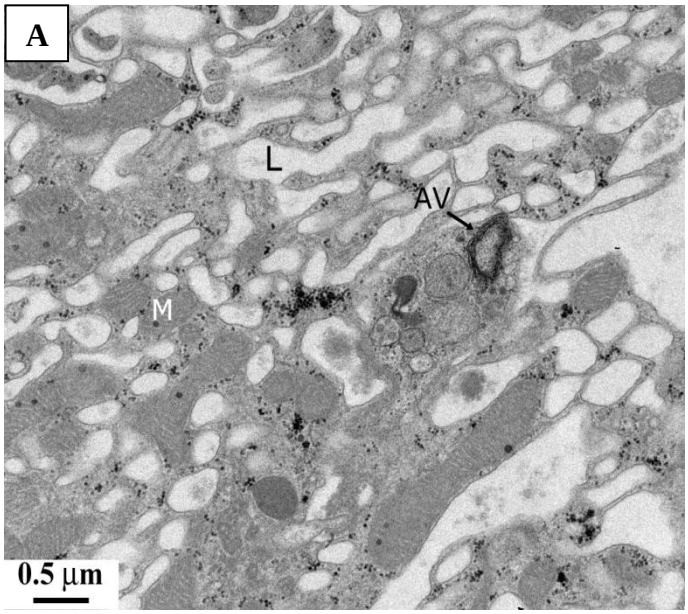

Figure 3.13 Type III acinus of the SG of (A) a partially fed virgin tick on day 6 PD (6 DPD-V, 42X the unfed weight). Autophagic vacuoles are apparent in the fluid secretory labyrinth. AV- autophagic vacuoles; M- mitochondria; L- salivary fluid secretory labyrinth; H- haemolymph.

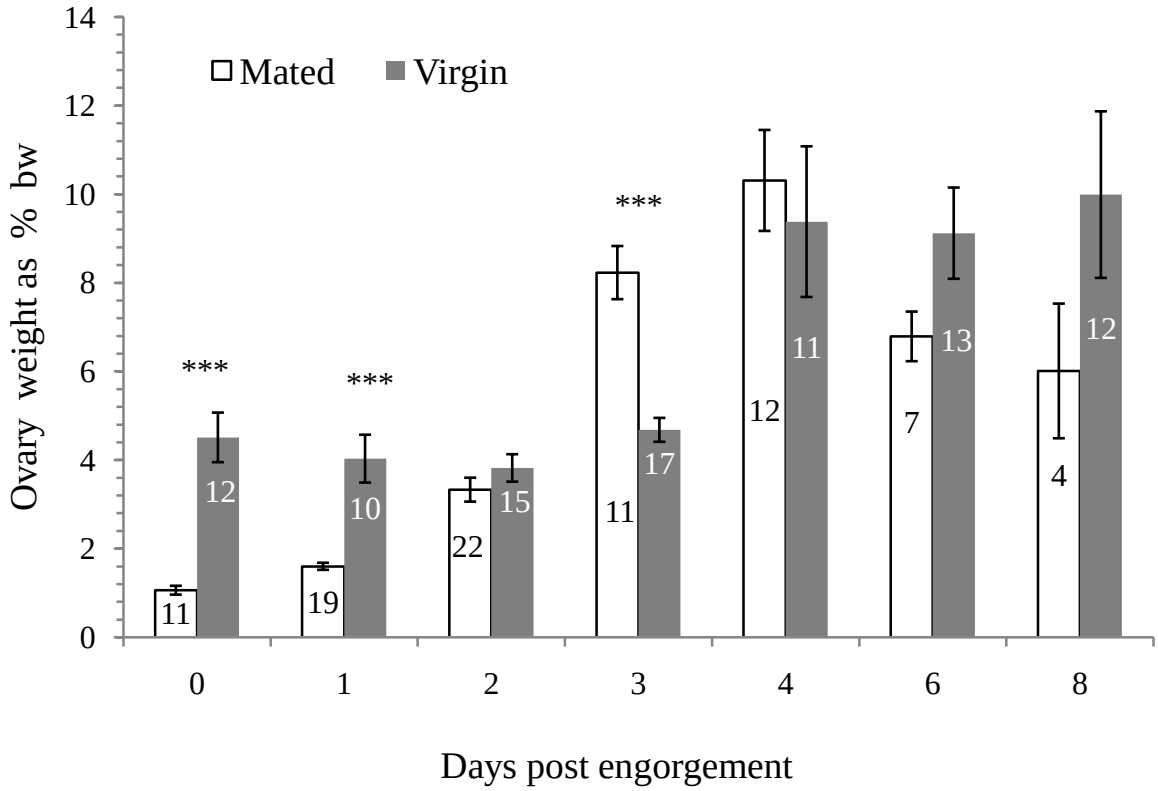

Figure 3.14 Ovary weights of engorged mated and virgin ticks (expressed as % bw) as a function of days post engorgement. Mean, SEM and *N* are indicated for each bar. The increase of the ovary weight of the mated ticks was significant on day 1 PE ($P = 0.000$) compared to day 0 PE. Ovary weight increase among the virgin ticks was significant only by day 4 PE ($P = 0.01$) compared to day 0 PE. Asterisks (*) indicate statistical significance between the mated and virgin ticks on the same day determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

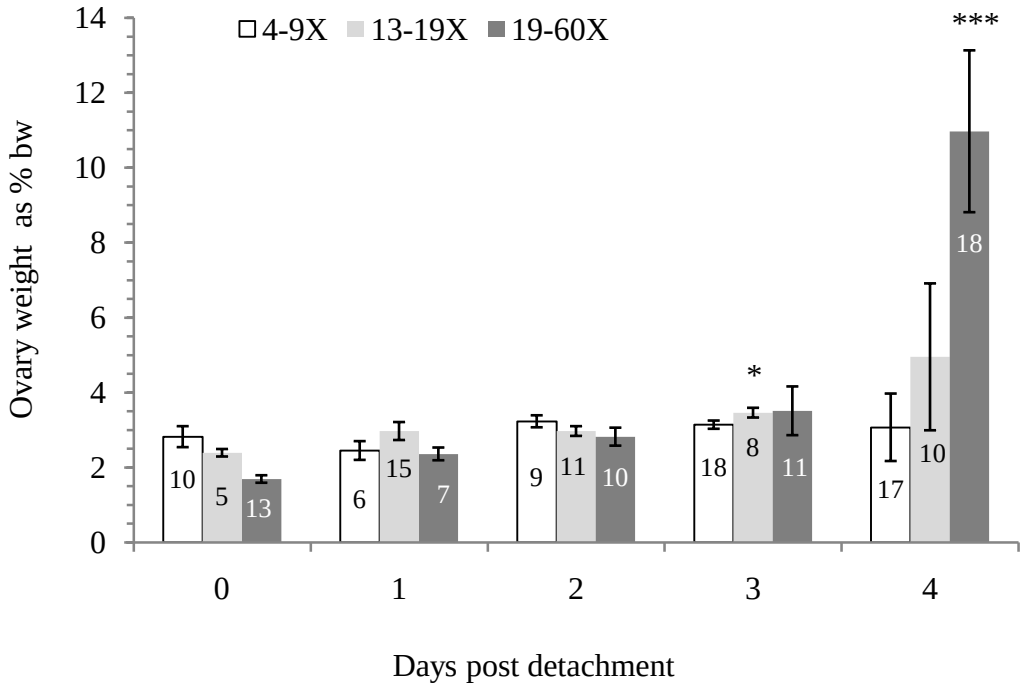

Figure 3.15 Ovary weight of the mated partially fed ticks as a function of days post detachment. Mean, SEM and *N* are indicated for each bar. There was no significant increase in the ovary weight from day 0 PD to day 4 PD in the 4-9X group ($P > 0.05$) compared to day 0 PD. Ovary weight increased significantly by day 3 PD in 13-19X group ($P > 0.01$) compared to day 0 PD. In the 19-60X group, the ovary weight increased progressively over 4 days PD ($P = 0.000$) compared to day 0 PD. Asterisks (*) indicate statistical significance among the three groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

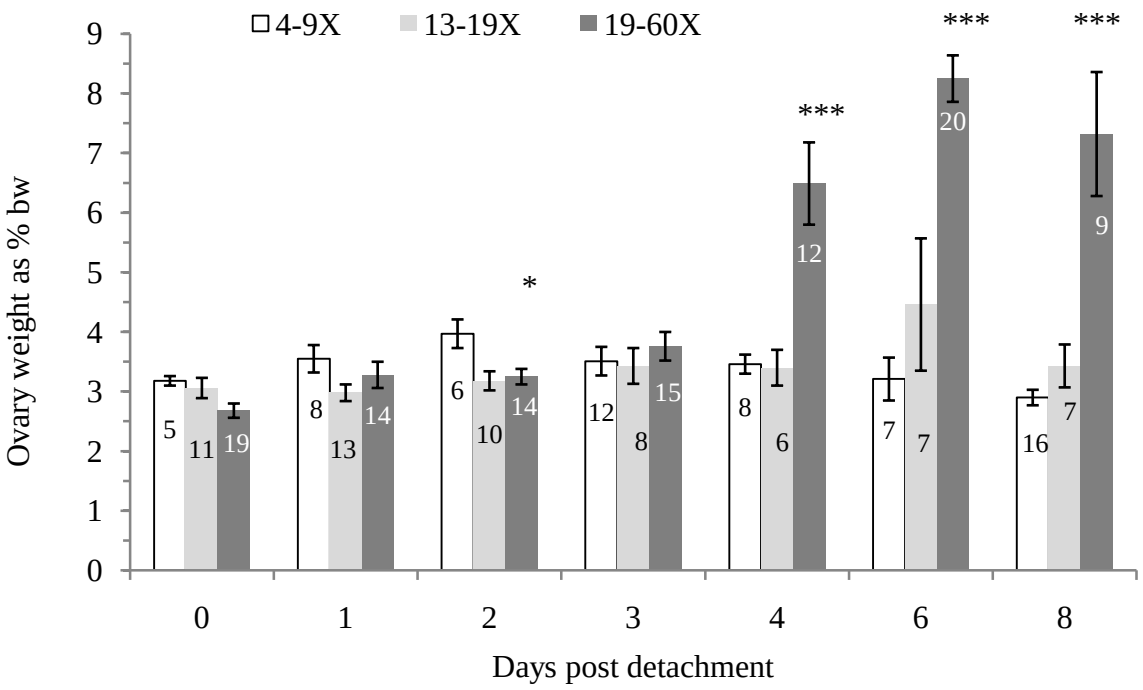

Figure 3.16 Ovary weight of the virgin partially fed ticks as a function of days post detachment. Mean, SEM and *N* are indicated for each bar. The ovary weight of the 4-9X and 13-19X group did not increase significantly from day 0 PD through day 8 PD ($P > 0.05$) compared to day 0 PD of the respective group. In the 19-60X group, the ovary weight increased significantly on day 1 PD ($P = 0.001$; compared to day 0 PD). Asterisks (*) indicate statistical significance among the three groups on the same day, compared to the 4-9X group, Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

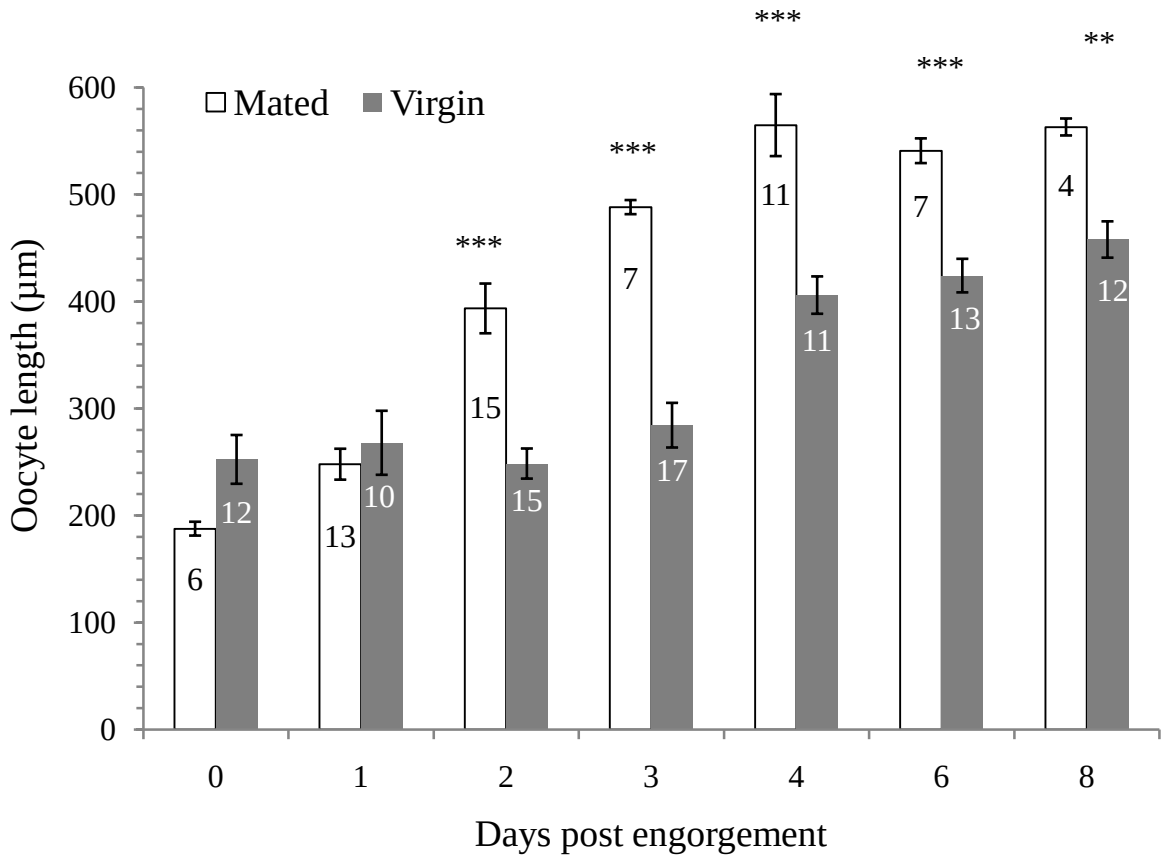

Figure 3.17 Oocyte length of mated and virgin engorged ticks as a function of days post engorgement. Mean, SEM and *N* are indicated for each bar. In the mated ticks a significant increase in the oocyte length was observed on day 1 PE ($P = 0.01$) compared to day 0 PE. In the virgin ticks oocyte length increased significantly only by day 4 PE ($P = 0.000$) compared to day 0 PE. Asterisks (*) indicate statistical significance between the mated and virgin ticks on the same day determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

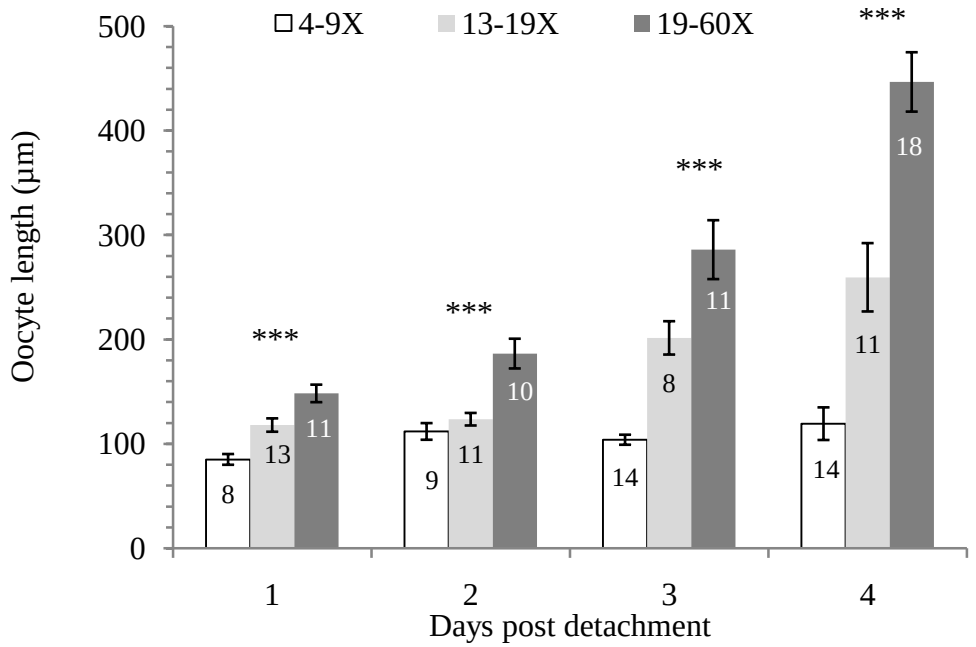

Figure 3.18: Oocyte length of the mated partially fed ticks as a function of days post detachment. Mean, SEM and *N* are indicated for each bar. Oocyte length in the 4-9X group did not change significantly through 4 days PD ($P > 0.05$). Oocyte length increased significantly in the 13-19X group on days 3 PD ($P = 0.000$) and 4 days PD ($P = 0.000$) compared to day 0 PD. In the 19-60X group there was a progressive increase in oocyte length through day 4 PD ($P = 0.000$). Asterisks (*) indicate statistical significance among the three groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

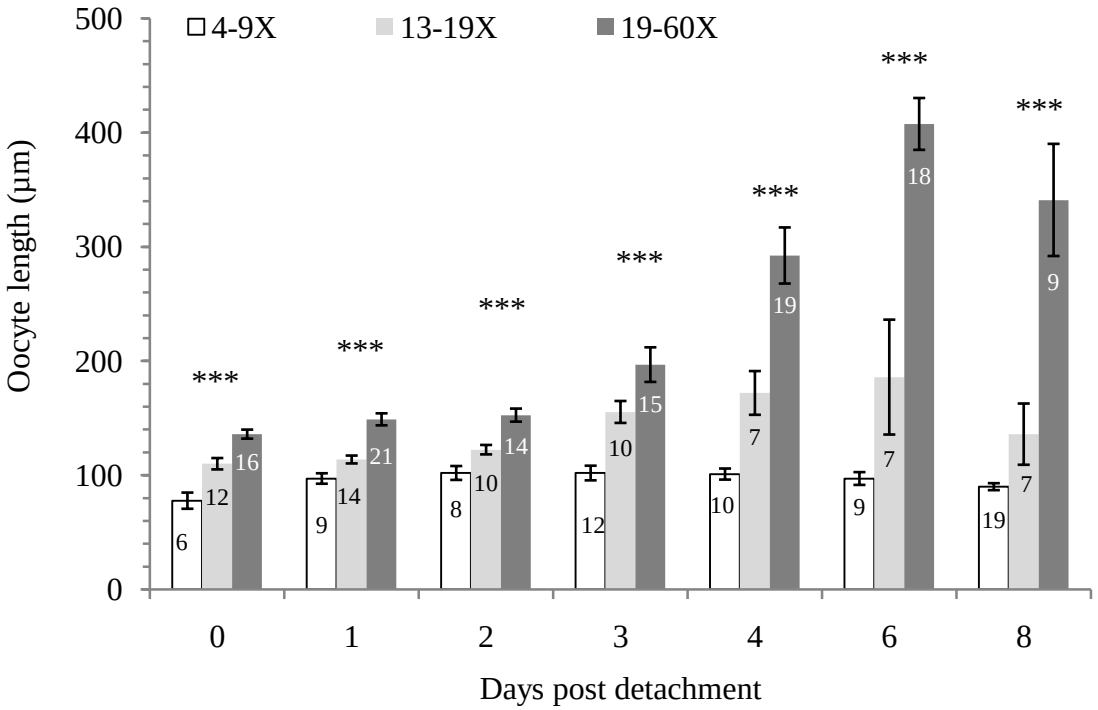

Figure 3.19: Oocyte length of the virgin partially fed ticks as a function of days post detachment. Mean, SEM and *N* are given for each bar. There was no significant change in the oocyte length of the 4-9X and 13-19X groups over 8 days PD ($P > 0.05$) compared to that of day 0 PD of the respective group. Oocyte length increase was significant by day 4 PD in the 19-60X the group ($P = 0.009$; compared to day 3 PD) and on day 6 PD ($P = 0.000$; compared to day 4 PD). Asterisks (*) indicate statistical significance among the three groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

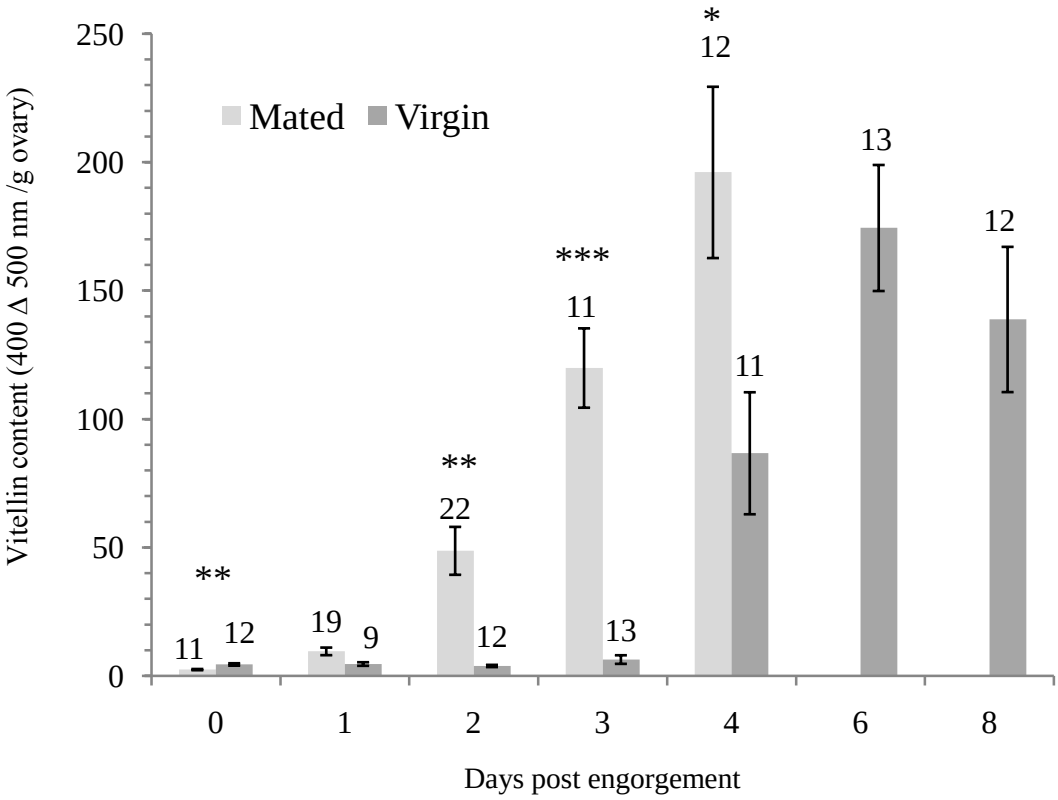

Figure 3.20: Ovary vt content in engorged (mated and virgin) ticks as a function of days post engorgement. Vitellin content is expressed as the difference in absorbance between 400 and 500 nm per g ovary. Mean, SEM and *N* are indicated for each bar. The increase of the vt content in the mated ticks was significant from day 1 PE (*P*= 0.001, compared to day 0 PE). Ovary vt content increased significantly in the virgin ticks from day 4 PE (*P* = 0.002 compared to day 0 PE). Asterisks (*) indicate statistical significance between the mated and virgin ticks on the same day determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

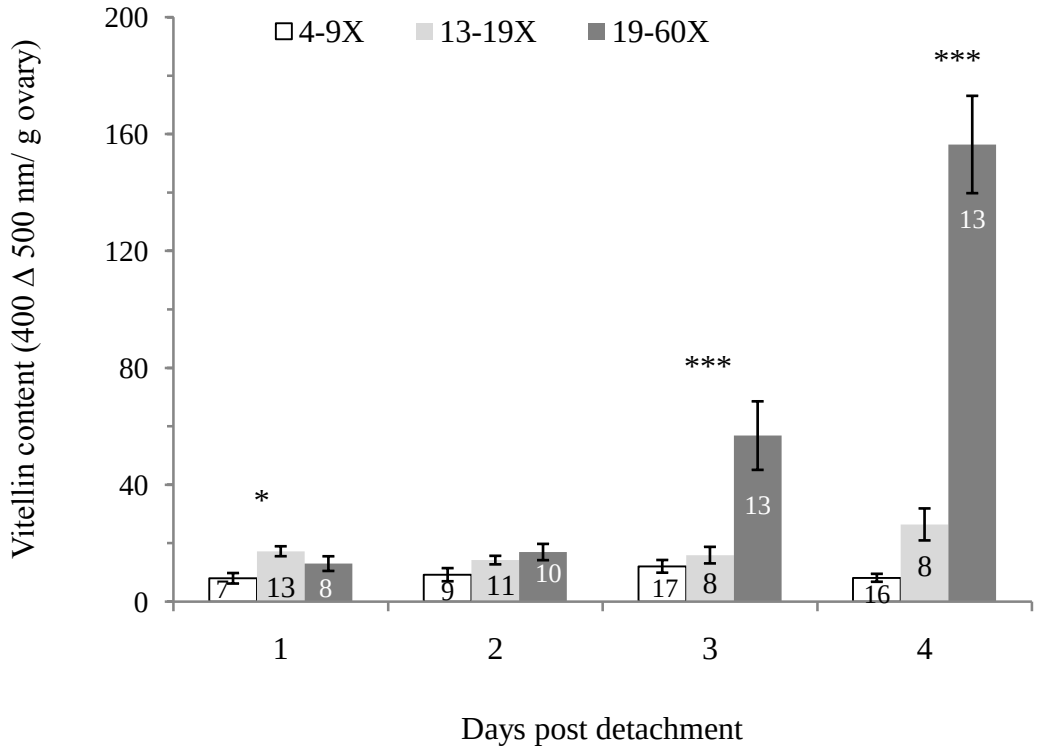

Figure 3.21: Ovary vt content in the mated partially fed ticks as a function of days post detachment. Vitellin content is expressed as the difference in absorbance between 400 and 500 nm per g ovary. Mean, SEM and *N* are indicated for each bar. There was no significant change in the ovary vt content of the 4-9X and 13-19X groups through day 4 PD ($P > 0.05$) compared to day 1 PD. In the 19-60X group, increase of the ovary vt content was significant by day 3 PD ($P = 0.01$, compared to day 1 PD). Asterisks (*) indicate statistical significance among the groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

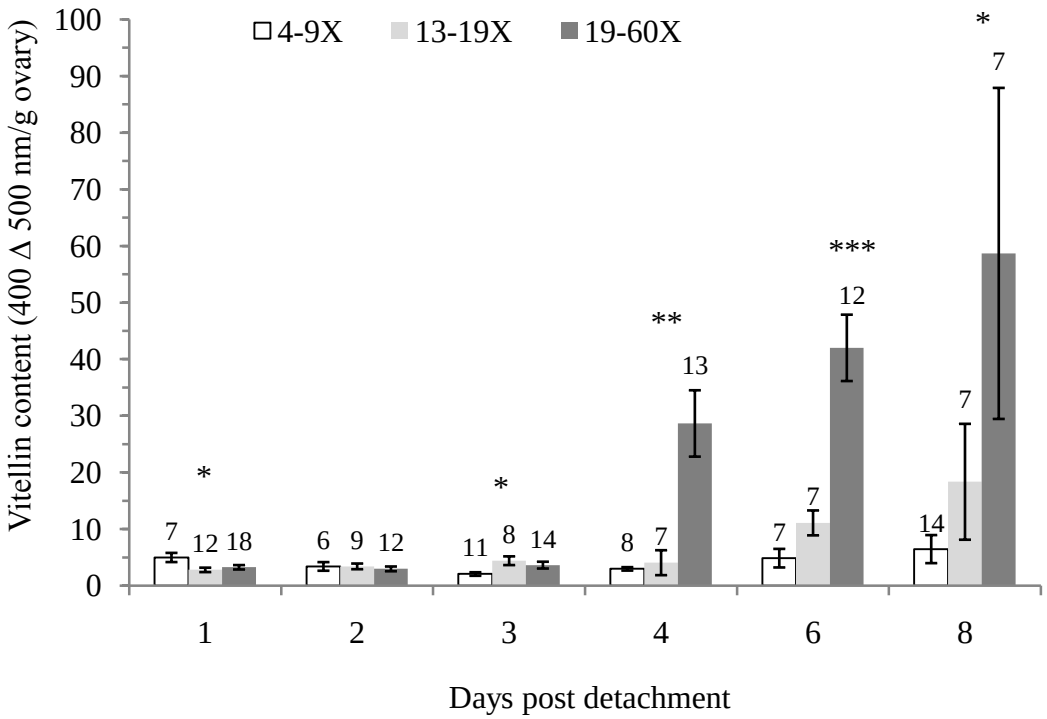

Figure 3.22: Ovary vt content in the virgin partially fed ticks as a function of days post detachment. Vt content is expressed as the difference in absorbance between 400 and 500 nm per g ovary. Mean, SEM and *N* are indicated for each bar. Ovary vt content of the 4-9X group did not change significantly by day 8 PD ($P > 0.05$). In the 13-19X group ovary vt content increased significantly by day 6 PD (0.000; compared to day 1 PD) and 19-60X groups ovary vt content increased significantly by day 4 PD ($P = 0.000$, compared to day 1 PD). Asterisks (*) indicate statistical significance among the groups on the same day, compared to the 4-9X group, determined by Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

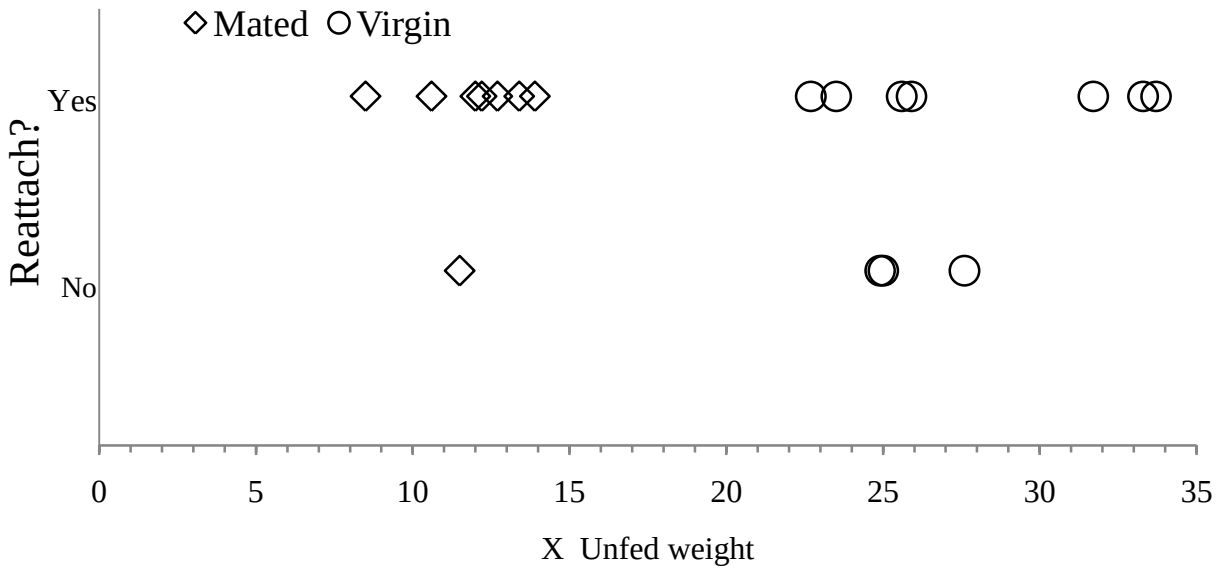

Figure 3.23: Reattachment of partially fed ticks. Most of the mated ticks attached to the host up to 14X the unfed weight and most of the virgin ticks attached to the host having 20-35X the unfed weight. One mated (11.5X the unfed weight) and three virgin (two values are super-imposed at ~25X unfed weight and one ~27X the unfed weight) never attached to the host.

CHAPTER FOUR

Discussion

The concept of CW was first developed by Harris & Kaufman (1984), who demonstrated in mated *A. hebraeum* that the following physiological and behavioural changes occurred at ~10X the unfed weight when a mated female tick is removed from the host; (1) haemolymph ES titre increases dramatically, (2) the tick does not reattach if given opportunity (3) the SGs degenerate within 4 days of detachment, and (4) the ovary develops to a mature stage. It was this transition weight that they named the CW.

Because mating is necessary for ticks to feed to repletion (Snow, 1969; Pappas & Oliver, 1971), Harris & Kaufman (1984) went on to test whether mating has a direct or indirect relationship to SG degeneration. They removed the seminal receptacle from engorged females on the day of engorgement. These ticks did not show any sign of SG degeneration 4 days later (by which time SGs had degenerated in the sham operated control ticks). However, SG degeneration was restored in these ticks if the seminal receptacle was reimplanted immediately, or by injecting an homogenate of male reproductive tract; injecting an homogenate of male SG did not restore SG degeneration. Thus they concluded that something from the male reproductive tract specifically is necessary for SG degeneration to proceed. They named it "male factor' (MF) and distinguished it from TSGDF as follows: (1) MF can be found in the haemolymph of small partially fed mated females (< 10X the unfed weight), whereas SG degeneration does not occur in such females (Harris & Kaufman, 1984; Lomas & Kaufman, 1992b), (2) MF is a protein

(Lomas & Kaufman, 1992a) and TSGDF is an ES (Harris & Kaufman, 1985, Kaufman, 1991); and (3) the amount of ES detected in the extracts of male gonad is physiologically insignificant (Lomas & Kaufman, 1992b).

Harris & Kaufman, (1984) did not measure the fed/unfed weight ratio of all their ticks; consequently the value they determined for the CW was only approximate. Weiss & Kaufman (2001), on the other hand, did record the fed/unfed weight ratio of all their experimental ticks, and thus demonstrated that the CW varies slightly depending on which parameter was measured: for whether or not the tick would reattach to the host it was 9X, for increase in haemolymph ES titre it was 10X, for SG degeneration it was 10X and for ovary development it was 12-13X. Why there should be a slight, but significant, difference in CW among some of these parameters has not been established. But a possible hypothesis is that, although the release of ES into the haemolymph may occur at 10X, the ES threshold for stimulating yolk uptake by the ovary may well be higher than is the threshold for stimulating SG degeneration, which in turn may be higher than the threshold for inhibiting re-attachment.

Because until now the CW has been determined only for *A. hebraeum*, and because there appeared to be significant differences in the feeding behaviour of *D. andersoni* and *D. variabilis* compared to *A. hebraeum*, it became important to conduct this comparative study. So, is the CW for *D. andersoni* significantly higher than that for *A. hebraeum*, or is it just that virgin *D. andersoni* readily feeds beyond its CW? In this thesis, I have established the following major points:

1. In the mated partially fed ticks, salivary fluid secretion was maximal at 19-60X the unfed weight on the day of detachment. This finding is consistent with that of Kaufman (1976) who showed that the maximum salivary fluid secretion occurred at 100 mg body weight and then plateaued up to 250 mg (unfed weight being 5-7 mg). I observed a similar trend for virgin partially fed ticks.

2. In the engorged ticks (both mated and virgin) salivary fluid secretion was maximal on the day of engorgement. However, in the mated ticks, the SGs began to lose their fluid secretory competence earlier, and the rate of loss was more rapid than in the virgins.

3. Under our colony conditions, complete ovary maturation and oviposition occurred much more rapidly in *D. andersoni* (~4 days in mated and 6-8 days PE in virgin ticks) than it did in *A. hebraeum* (~10-12 days for mated ticks; Friesen & Kaufman, 2002).

4. Virgin *D. andersoni* ticks readily attach to the host without the presence of a male and they feed up to 100X the unfed weight, whereas, unfed virgin *A. hebraeum* ticks delay attachment by some days in the absence of attached males, and normally do not feed beyond their critical weight (Kaufman & Lomas, 1996).

5. The CW of *D. andersoni* is in the range of 10-13X the unfed weight, similar to what was observed in *A. hebraeum* by Weiss & Kaufman (2001) as measured by SG degeneration and ovarian development.

The SGs of virgin *A. hebraeum* ticks degenerated when exposed to exogenous ES from mated ticks (Lomas & Kaufman, 1992b). Thus, MF is not

necessary for the up-regulation of the ES receptor in the SGs. But, injection of male gonad into virgin ticks above the CW caused the haemolymph ES level to rise by 77% over 4 days period. Thus they demonstrated that male factor acts by hastening the appearance of ES in the haemolymph. It is the earlier appearance of ES in the haemolymph that explains why the SGs of mated ticks degenerate faster than those of virgin ticks.

Mated and virgin partially fed *D. andersoni* 4-9X the unfed weight ticks lost 40-45% of their salivary fluid secretory competence by day 4 post detachment (Figure 3.8 & 3.9). The equivalent number in mated *A. americanum* was ~50% (Lindsay & Kaufman, 1988) and in mated *A. hebraeum* ~75% (Harris & Kaufman, 1984). Partial loss of fluid secretory competence over 4 days by such ticks was not accompanied by appearance of autophagic vacuoles, thus explaining why this loss of fluid secretory competence was not considered to reflect "degeneration". Moreover, if such small partially fed ticks are returned to the host 4 days after removal, fluid secretory competence recovers significantly (Harris & Kaufman, 1984). In *D. andersoni*, autophagic vacuoles also were not apparent in the salivary glands of the 4-9X group after 4 days post detachment (Figure 3.11 C). What might cause a significant loss of fluid secretory competence, but which is not degeneration of the tissue? The reversible loss of the SG function in ticks below the CW is still unknown (Friesen & Kaufman, 2009), although Kaufman (1986) suggested that it could be due to uncoupling of the DA receptor from the adenylate cyclase system that is part of the fluid secretory

mechanism, and/or it could be a ‘down-regulation’ of the DA receptor itself (Lindsay & Kaufman, 1988).

Kaufman (1976) measured the salivary fluid secretion in a different way from that of the current study with *D. andersoni*. According to his study the maximum rate of salivary fluid secretion was 3.3 mg/15 min for the large partially fed ticks (100 mg to 250 mg bw) in oppose to ~3.8 mg/gland/15 min of the ticks of same fed/unfed weight ratio range of this study. Although the measurement method of fluid secretion rate was different between the studies, a similar increasing trend of salivary fluid secretion as a function of body weight was observed in both the studies.

The rate of salivary fluid secretion in the mated engorged and partially fed ticks was always higher (~50%; Figure 3.3 & 3.4) than that of the virgin engorged and partially fed ticks in a similar weight range. This observation differs from that of Harris & Kaufman (1984) who showed in *A. hebraeum* that both mated and virgin partially fed ticks secrete salivary fluid at an identical rate up to day 8 post removal. What accounts for this difference between the two species is not known.

Like *A. hebraeum* and *A. americanum* females (Harris & Kaufman, 1984; Lindsay & Kaufman, 1988), all the mated *D. andersoni* ticks above 13X the unfed weight lost 90% of their fluid secretory competence and developed autophagic vacuoles by day 4 PD. The percentage loss was significantly less in the same group of virgin *D. andersoni* females on day 4 PD (45% less). However, the appearance of autophagic vacuoles by day 4 and day 6 PD suggest that the SGs of virgins have started to degenerate by this time. Taking

these into consideration and according to the definition of CW based on SG degeneration, like *A. hebraeum* the CW of *D. andersoni* is somewhere in the range of 10-13X the unfed weight.

Ovarian development and oviposition in *D. andersoni* proceeds much quicker (4-5 days) than in *A. hebraeum* (10-12 days) under the same temperature and humidity conditions. Ovarian development in *D. variabilis* is similar to that in *D. andersoni* under the same conditions (Thompson *et al.*, 2005). Kaufman (1991) showed that in mated *A. hebraeum* 20-HE titre in the haemolymph increased synchronously with ovary weight after engorgement. Again in partially-fed *A. hebraeum* 20-HE stimulates vitellogenesis in the presence of MF (Friesen & Kaufman, 2002, 2004). So, ovarian development in the mated engorged ticks can be attributed to the rise of the haemolymph ES titre. However, how does one account for the ovarian development being slower by 4 days in the virgin compared to the mated engorged ticks? The simplest hypothesis is that the absence of MF. In the absence of MF virgins proceed slower than the mated ticks and ecdysteroid is secreted later than earlier (Sonenshine, 1991), which causes a delayed development of the ovary.

In all the parameters of ovary development measured, ovary development did not occur until the ticks achieved 13X the unfed weight. Therefore, according to the definition of CW, the CW of *D. andersoni* is in the range of 10-13X the unfed weight as it is in *A. hebraeum*.

Despite having ~4 - 5 times larger body size in both the unfed and engorged state, the length of the oocytes prior to oviposition is almost similar in mated *A. hebraeum* and mated *D. andersoni* (*A. hebraeum*: ~560 µm; *D.*

andersoni: ~565 µm; Lunke & Kaufman, 1993, Friesen *et. al.,* 2003). This suggests that the egg mass laid by *A. hebraeum* would be several times larger than that of *D. andersoni* and this is the case: ~16,000 eggs for *A. hebraeum* (Alexander Smith, personal communication, 2012) and ~6000 eggs for *D. andersoni*. However, why the oocyte length of the virgin *D. andersoni* ticks should be smaller than that of the mated ticks (Figure 3.9) is unknown.

In the wild *A. hebraeum* ticks normally takes ~3 years to complete its life cycle (Norval, 1977) whereas it is more rapid (1-2 years depending on the geographical location) in *D. andersoni* (James *et. al.,* 2006). Our colony incubator is adjusted to be optimal for *A. hebraeum* (27 °C), but this is a significantly higher average temperature that *D. andersoni* would experience in the wild. This may account for the onset of oviposition being earlier in *D. andersoni* than in *A. hebraeum* in this study.

In a reattachment experiment, Weiss & Kaufman (2001) allowed partially fed *A. hebraeum* ticks to reattach after 5, 7 or 10 days off host. The fed/unfed weight ratio at which the ticks reattached varied among the three groups (12.5X, 10.5X and 8.8X the unfed weight for 5, 7 and 10 days off host respectively). Therefore they suggested that, CW based on reattachment to the host is a less well defined measurement. In my study, after being kept off the host for 24 h, both mated and virgin partially fed *D. andersoni* ticks reattached above the CW weight shown for *A. hebraeum.* The biggest mated tick was only 14X the unfed weight, not much above the CW. However, the virgin ticks that reattached were much larger (Figure 3.15). Unfortunately I did not have enough ticks left in the colony at the end of my project to better define

the cut-off in mated ticks, but in any case I would not have been able to test it beyond just a few days post detachment, because oviposition begins in above CW *D. andersoni* within 4 days off the host.

Conclusion

I have established that the CW of *D. andersoni* does not differ significantly from that of *A. hebraeum*. Female *D. andersoni* ticks switch from a 'feeding strategy' to a 'reproductive strategy' once they surpass about 10-13X their unfed weight and are removed from the host. Virgin *D. andersoni* females regularly feed to ~60X (and occasionally to 100X) the unfed weight, unlike virgin *A. hebraeum* females that rarely feed much beyond 10X the unfed weight.

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