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Title: Salivary gland degeneration and vitellogenesis in the ixodid tick Amblyomma hebraeum: Surpassing a critical weight is the prerequisite and detachment from the host is the trigger.

Article Type: Full Length Article

Keywords: ixodid ticks, Amblyomma hebraeum, ecdysteroids, vitellogenesis, ovarian development, critical weight, tick salivary glands.

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Abstract: The normal engorged body weight of female ixodid ticks (Acari: Ixodidae) is about 100X the unfed weight. Virgin female Amblyomma hebraeum normally do not feed beyond 10X the unfed weight. However, about 10-20% of a population of virgins will feed to perhaps 20X the unfed weight, but not much beyond that. In A. hebraeum, when females surpass about 10X the unfed weight, the following changes in physiology occur if they are removed from the host: (a) they will not reattach if given the opportunity, (b) their salivary glands (SGs) will undergo autolysis within 4 days if they are mated or 8 days if they are virgin, and (c) egg maturation and oviposition will occur in due course. Mated or virgin female ticks removed from the host below about 10X the unfed weight do not experience the latter changes (Kaufman and Lomas, 1996, Invertebrate Reproduction and Development 30, 191-198). In 1984 we named this transitional weight, the 'critical weight' (CW). Its absolute value is probably a species-specific characteristic (Kaufman, 2007, Journal of Insect Physiology 53, 264-273). Although mated females tend to engorge within a day of surpassing the CW, virgin females surpassing the CW can remain attached to the host for at

least several weeks more. It is not known whether the physiological changes in the SGs and ovaries listed above occur in those large virgins that remain attached, although we suppose that this would be maladaptive. Instead, we hypothesize in this study that surpassing the CW is only a prerequisite for inducing these changes, and that detachment is the actual trigger. We support our hypothesis by demonstrating that large virgins, remaining attached to a host for 8 days, did not undergo SG degeneration nor complete egg maturation during the attachment period. Those changes occurred only within 8 days following detachment. So some type of sensory information associated with attachment to the host, and still undefined, inhibits expression of the physiological changes hitherto associated merely with surpassing the CW.

Suggested Reviewers: surely not required for a revision? surely not required for a revision?

use last suggested reviewers use last suggested reviewers

why ask for new reviewers? why ask for new reviewers?

Reviewer #1: The authors continue their lab's pioneering research on the physiology of feeding, non-mated ticks. The question they pose has a rather predictable outcome, but it needes to be addressed anyway and was done so in a methodical and detailed manner that gives confidence in the conclusions. As ground breaking as molecular biology can be, it is a refreshing treat to see sound science with good old-fashioned experimental designs and not a primer to be mentioned!

Thank you!

There are however, some points that the authors need to address to improve the paper:

Main concern: the statistical analysis is poorly explained and seems incorrect. Author's cite t-test and say they'll use 1- or 2-tailed as appropriate. I don't know what "as appropriate" means - it is not described elsewhere and I doubt whether it actually is appropriate. Because there are multiple treatments (5 in most cases), t-test should not be used in this repeated manner among several comparisons - this is highly likely to yield Type II errors. Authors should perform ANOVA among the treatments and then assess the differences post hoc if the ANOVA shows a non-homogenous population (e.g. Tukey's, Duncan's, LSD, etc). Looking at the P vlaues this is not likely to change the story in most cases, but as presented this is not a correct treatment of the data.

Response: We chose to use t-tests rather than ANOVA because in this study we are not comparing all combinations of all treatments. Many comparisons would be irrelevant to the study. In each case we are comparing only a given treatment to its specific control. We used 1-tailed t-tests when the hypothesis justified reference to a specific direction of change (either an increase or a decrease in the mean), and 2-tailed when the hypothesis did not predict a specific direction of change. Thus, in Fig 3, ANOVA is not appropriate, in our opinion, because there is no logical reason to compare fluid transport by glands of <CW ticks with those of >CW ticks - - we know from long experience that the glands from larger ticks secrete at a higher rate. For the >CW ticks, the only comparisons that are relevant to the hypothesis are >CW day 8 vs >CW day 0, and >CW day 8+8 vs >CW day 8. Other comparisons are of no interest. We chose 1-tailed tests for the two groups of <CW glands, because previous work showed that the glands of <CW ticks several days off the host secrete less than those just removed (though the reduction is not due to degeneration of the tissue). In comparing >CW glands day 8 vs >CW glands day 0, we used a 2-tailed test, because there was no a priori reason to predict a change in in either direction. But we used a 1-tailed test to compare >CW glands day 8+8 vs >CW glands day 8, because we certainly expected these glands to degenerate 8 days following removal. In both cases, the conclusion wouldn't be affected by changing from one tail to the other. We made similar choices for the other figures.

However, we do not pretend to be experts in the subtleties of statistical tests, so I consulted with a colleague who teaches statistics in our department. He confirmed that our approach is appropriate.

Fig legends: in many cases the legends seem to comment on the results of the data rather than simply explain the figure. The interpretation of the data is in the Results text.

Response: Even though I rather like to have the gist of conclusions in the legends (it saves moving back and forth between main text and figures) we have now removed these comments to abide by tradition.

Fig legend 2: very long and contains the actual data numbers and P values which are alredy in the text.

Response: We have now eliminated these.

Page 5, line 9: it would be useful to the reearch community to state model and manufactuer of the calipers.

Response: Unfortunately, we don't have documentation on this and no manufacturer is mentioned on the calipers themselves.

Pg 8-9: the details for the SDS_PAGE, transfer and immunoblotting could be greatly reduced. These are standard methods and don't need such detail for any one to appreciate the expt or to be able to repeat it.

Response: We have reduced our description of these methods.

Pg 10: line 6: please change sub-title from "SG autolysis" to "SG secretory competenece" or similar.

Response: We have changed it to "salivary fluid secretory competence".

Pg 10, line15-16: delete this sentence - it belongs in the Discussion.

Response: Done.

Pg 12: why the need for a footnote? Include in the textbody.

Response: We have now revised the body of the text to include this information.

Pg 13, lines 12-16: the explanation for the loss of secretory comeptence is very speculative. I don't think this adds anyhting to the paper and could constrain others from believing that other areas could be viable. I suggest the sentence is simply omitted.

Response: Done.

Pg 13: final paragraph: again this speculative in the extreme. I don't agree with the propositions (for example I have often seen ticks detached but still with remnants of cement on mothparts), but that is not the main point. Speculation has its place, but this paragraph is several steps too far! Delete it.

Response: We think it's appropriate to offer at least some suggestions as to what sensory information might be important here, but we have now shortened it, and hope that it is now suitably restrained!

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Reviewer #2: The authors attempt to answers questions about the sensory information associated with attachment of ticks to the host that regulates salivary gland degeneration and ovarian development. The paper is basically well written and provides important information on tick physiology. However, it could be improved by addition of some information especially in the discussion. Therefore, I recommend the manuscript be accepted for publication after some revisions. Suggestions for improvement of the manuscript follow.

1. In the Introduction (Page 4 Line 11), they indicate virgin female ovarian development is complete in virgin females 8 days after being forcibly removed. However, it is unclear what they mean by complete ovarian development. Are the oocytes viable or not, is this species under go parthenogenesis? More explanation is needed to answer these questions.

Response: We now say, "... complete ovarian development and oviposition occur, although the resulting eggs rarely hatch ...". We believe that this is sufficient to indicate that ovarian development is “complete”.

2. In the introduction the authors review literature on ecdysteriods and their importance in salivary gland degeneration and ovarian development but in the discussion they only refer to ecdysteroids as a footnote and in the last sentence. The discussion also needs a more complete explanation of the connection of this study to the regulation by ecdysteroids because without it the reader becomes confused.

Response: We have now made it more explicit at several points in the Discussion that SG degeneration and vitellogenesis are triggered by ecdysteroids.

3. The authors state 3 possible explanations for sensory information and conclude the 3rd explanation is the most likely. However, the explanation they give as to why this is the most likely is unclear and just merely states that sensory receptors have been investigated. They need to make it clearer as to why they make this conclusion.

Response: Actually, we didn’t conclude that, we only “suggested” that the hypothesis is more likely, and we support the suggestion with our discussion on the occurrence of numerous sensory receptors in the mouthparts. However, we have revised the offending sentence as follows: “We suggest that the third possibility may have some merit.” And we continue with a reduced discussion on the sensory receptors as requested by the first reviewer.

4. There are other studies on responses to chemical signals by the mouthparts that are not directly related to feeding but may also provide some other hints to the response if the cement cone provides the key to this sensory information. Paper by Allan et al. in the J. Med Entomol. 1989 indicating the mouthparts respond to fatty acids and Taylor et al. in Exp. Appl. Acarol. ? indicating the sensilla on the mouthparts respond to ecdysteroids to function as sex pheromones for species recognition.

Response: We are aware, of course, of the rich literature on sensory physiology in ticks. However, in accord with the first reviewer, we think it best at this stage to minimize further speculation, at least until there is some direct evidence for our hypothesis about the role of mouthpart receptors.

5. The end of the discussion doesn't include any explanation as to why this study and information presented here is important theoretically or practically so that readers not familiar

with this area of study can understand the importance of this work. It may also be helpful to add a paragraph at the beginning of the introduction to orient the readers to this area of study.

Response: We have now added a sentence at the beginning of the 2nd paragraph of the Introduction to address this point.

6. Other grammatical and typographically errors occur throughout the manuscript so the manuscript should be carefully read and this errors corrected.

Response: We have done our best to locate and correct such errors although, frankly, we haven't found many. Without providing us with specific instances, however, it's difficult to comment further.

7. Sentence on Page 5 Lines 6 and 7 could be improved. Suggested improvement: This study required a method for determining the day on which individual virgin females surpassed the CW without removal from the host for weighing.

Response: We have modified the sentence as follows, based on the spirit of the reviewer's suggestion:

"This study required that we determine the day on which an individual female surpassed the CW, but without removing the tick from the host to weigh it."

8. Authors overuse colons and semicolons. Period is the best punctuation in most scientific writing.

Response: I believe I understand how to use colons and semicolons. But most were optional and we have now removed them.

9. The manuscript needs to be checked carefully for other problems in wording and spacing, i.e. 10-20% not 10-20 %; Figure 1 not Fig. 1 when you begin a sentence; Page 6 line 7 removal and measurement of salivary. not removal and measuring salivary.; end of Page 6 line 9 remove 'which'; Page 6 line 20 refrigerator not refridgerator, etc.

Response: we have attended to these and have made an effort to find and correct others.

10. I see no major problems with the table and figures.

Salivary gland degeneration and vitellogenesis in the ixodid tick *Amblyomma hebraeum*: Surpassing a critical weight is the prerequisite and detachment from the host is the trigger.

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Abstract

The normal engorged body weight of female ixodid ticks (Acari: Ixodidae) is about 100X the unfed weight. Virgin female *Amblyomma hebraeum* normally do not feed beyond 10X the unfed weight. However, about 10-20% of a population of virgins will feed to perhaps 20X the unfed weight, but not much beyond that. In *A. hebraeum*, when females surpass about 10X the unfed weight, the following changes in physiology occur if they are removed from the host: (a) they will not reattach if given the opportunity, (b) their salivary glands (SGs) will undergo autolysis within 4 days if they are mated or 8 days if they are virgin, and (c) egg maturation and oviposition will occur in due course. Mated or virgin female ticks removed from the host below about 10X the unfed weight do not experience the latter changes (Kaufman and Lomas, 1996, Invertebrate Reproduction and Development 30, 191-198). In 1984 we named this transitional weight, the 'critical weight' (CW). Its absolute value is probably a species-specific characteristic (Kaufman, 2007, Journal of Insect Physiology 53, 264-273). Although mated females tend to engorge within a day of surpassing the CW, virgin females surpassing the CW can remain attached to the host for at least several weeks more. It is not known whether the physiological changes in the SGs and ovaries listed above occur in those large virgins that remain attached, although we suppose that this would be maladaptive. Instead, we hypothesize in this study that surpassing the CW is only a prerequisite for inducing these changes, and that detachment is the actual trigger. We support our hypothesis by demonstrating that large virgins, remaining attached to a host for 8 days, did not undergo SG degeneration nor complete egg maturation during the attachment period. Those changes occurred only within 8 days following detachment. So some type of sensory information associated with attachment to the host, and still undefined, inhibits expression of the physiological changes hitherto associated merely with surpassing the CW.

Keywords: ixodid ticks, *Amblyomma hebraeum*, ecdysteroids, vitellogenesis, ovarian development, critical weight, tick salivary glands.

1. Introduction

The taking of a blood meal in female ixodid ticks is conventionally divided into three phases (Balashov, 1972). (1) During a ‘preparatory phase’ (about 1 day) the female establishes a feeding lesion. (2) During the ‘slow phase’ (7 - 9 days) it grows to about 10X its unfed body weight. (3) During the ‘rapid phase’ (about 1 day) it increases its weight a further

10-fold and detaches from the host. In our laboratory colony of the ixodid tick, *Amblyomma hebraeum* (Koch), engorgement of the female normally occurs within about 10 days of attachment.

It has been known for a long time that the salivary glands (SGs) of ticks undergo substantial development during feeding, and then are resorbed within a number of days post-engorgement (Till, 1961). Haemolymph ecdysteroid titre in *A. hebraeum* rises substantially during the first week post engorgement (Kaufman, 1991; Friesen and Kaufman 2002). This rise in ecdysteroids triggers SG autolysis (Harris and Kaufman, 1984, 1985; Kaufman, 1991; Chang and Kaufman, 2005). Ecdysteroids also stimulate synthesis of the main yolk protein, vitellogenin (Vg) in both ixodid ticks [*A. hebraeum* (Friesen and Kaufman, 2002, 2004) and *Dermacentor variabilis* (Sankhon et al. 1999, Thompson et al. 2007)] and argasid ticks [*Ornithodoros moubata* (Ogihara et al. 2007; Horigane et al., 2007)].

In most ixodid species, mating occurs on the host. Copulation facilitates the female to enter the rapid phase of engorgement and to feed past a 'critical weight' (CW) that is characterized by several behavioral and physiological changes. For example, if a female is removed from the host prior to reaching the CW, it will actively seek a new host in order to complete the blood meal. But if it is removed after exceeding the CW, it will no longer seek a host, nor will it attach to one if given the opportunity. In addition, the SGs of females above the CW will undergo autolysis, and ovarian development will proceed to full maturation. The CW for mated female *A. hebraeum* is approximately 10X the unfed body weight (Harris and Kaufman, 1984; Kaufman and Lomas, 1996), although a more rigorous experimental design has shown that the exact value (between 10-13X the unfed weight) depends on which physiological parameter is being measured (Weiss and Kaufman, 2001).

Under normal circumstances, the female engorges and detaches from the host within 24 h of surpassing the CW. Although most virgin *A. hebraeum* do not feed beyond the CW, perhaps up to 20% do, though rarely do they feed beyond 20X the unfed weight, and they don't engorge to repletion. Virgins heavier than the CW can remain attached to the host for at least several weeks (Kaufman and Lomas, 1996; Lomas and Kaufman, 1999). Moreover, if such large virgins are forcibly removed from the host, their SGs degenerate within 8 days (Lomas and Kaufman 1992a, b) and complete ovarian development and oviposition occur, although the resulting eggs rarely hatch (Kaufman, 1991). The question arises: If large virgins can remain attached to the host for several weeks without engorging to repletion, do their SGs undergo autolysis and does complete ovarian development occur during this time? Because it

would be maladaptive for SG degeneration to occur while a tick is still attached to its host, perhaps SG degeneration and complete ovarian development are triggered by the act of detatchment, and not by merely exceeding the CW? The present study was designed to answer this question. Our results indicate that although surpassing the CW is a prerequisite for SG autolysis and complete ovarian development, detachment from the host constitutes the trigger. Until now the act of detachment has not been appreciated as a crucial part of the signaling pathway to SG degeneration and vitellogenesis in *A. hebraeum*.

2. Materials and Methods

2.1 Ticks and tick feeding

All host animals used in this study were cared for according to the guidelines mandated by the Canadian Council of Animal Care. Our colony of *A. hebraeum* was kept in darkness, at 27°C and at >85% relative humidity. Ticks were fed on rabbits as described by Kaufman and Phillips (1973). Prior to feeding, individual females were weighed and marked by gluing (cyanoacrylate glue) a short length of colored thread to a leg. Female *A. hebraeum* are reluctant to attach in the absence of males. Hence, a number of male ticks were first allowed to attach to the back of the host rabbit. The males were confined within a cloth bag that permitted their attachment to the host but prevented copulation. The next day, unfed females were added to the same rabbit. When females were removed at the appropriate stage for experiments, they were rinsed with water, dried with tissue paper, weighed, and stored individually in gauze-covered glass vials until their tissues were collected (see Section 2.4).

2.2 Estimating tick body weight while they are attached to the host

This study required that we determine the day on which an individual female surpassed the CW, but without removing the tick from the host to weigh it. We did this by measuring body volume of feeding ticks as follows. Using digital calipers, we measured (1) the width of the body at its apparently widest part (approximately at the site of the posterior pair of legs; dimension X), (2) the anterior-to-posterior length of the body along the midline (dimension Y), and (3) the dorso-ventral thickness of the body (dimension Z). For these measurements the calipers could be read to the nearest 0.01 mm. We calculated tick volume from the formula:

$$\text{Volume} = 4/3\pi * 0.5 * (XYZ)$$

This formula approximates the geometry of a fed tick (Patriquin, 1991). In a preliminary trial, we prepared a standard curve as follows for converting volume to weight: Body volume of ticks

of diverse sizes (from about 2X to about 30X the unfed weight) was measured on-host and the ticks were then forcibly removed, washed and weighed to the nearest 0.1 mg. Body volume was re-measured off-host. Off-host measurement was considered to be intuitively more accurate because of greater ease to align the ticks with the calipers. Standard curves were drawn plotting volume as a function of body weight using Microsoft Excel software. Figure 1 shows the standard curves for on-host and off-host volume measurements. The linear standard curves corresponded to each other almost exactly.

2.3 Experimental Procedure

The day on which each tick was deemed from its volume to have surpassed the CW was designated as 'day 0'. Experimental ticks were assigned to one of five groups. Group 1 consisted of ticks above the CW removed from the host on day 0. Henceforth this group is labeled '>CW day 0'. Salivary fluid secretory competence was measured as described in Section 2.5, and ovarian development was determined as described in Section 2.6. Group 2 consisted of ticks above the CW that were allowed to remain attached to the host for an additional 8 days before removal and measurement of salivary fluid secretory competence and ovarian development. Henceforth this group is labeled '>CW day 8'. Group 3 consisted of ticks that were similar to Group 2, but were kept off-host in the colony incubator for an additional 8 days before salivary secretion and ovarian development were measured. Henceforth this group is labeled '>CW day 8+8'. Group 4 was a control group, similar to Group 1, except that these were ticks that had not yet reached their CW. Henceforth this group is labeled '<CW day 0'. Group 5, a second control group of below-CW ticks, were treated similarly to Group 3. Henceforth this group is labeled '<CW day +8'.

2.4 Collection of tissue and haemolymph samples

Haemolymph and ovaries were monitored for the following indices: (a) Vg titre of haemolymph, (b) vitellin (Vt) content of ovary, (c) ovary weight and (d) oocyte length. Ticks were immobilized on small plastic petri dishes with a drop of cyanoacrylate glue, and chilled in a refrigerator for 10 min. Cooling inhibits smooth muscle contraction in the gut and so reduces the likelihood of puncturing the gut while collecting haemolymph samples. A small incision was made in the cuticle, and haemolymph was collected in calibrated glass micropipettes. Haemolymph was diluted 1:4 (v/v) in a phosphate-buffered saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0). Samples were stored at -20^{0}C until assayed for the presence of Vg by SDS-PAGE and immunoblot analysis (see Section 2.7).

After collection of haemolymph, ticks were flooded with a modified Hank's balanced saline (200 mM NaCl, 8.9 mM D-glucose, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 27 µM phenol red, pH 7.2), and the dorsal cuticle was removed using a microscalpel. SGs were excised and set aside for measuring salivary fluid secretory competence (see section 2.5). Ovaries were dissected out, and the length of the long axis of the eight apparently largest ovoid oocytes was measured using a calibrated ocular micrometer fitted to a dissection microscope. The mean value for the eight oocytes was recorded for each tick, and ovary growth and oocyte development were scored according to the system described in Section 2.6. The ovaries were then gently blotted, weighed to the nearest 10 µg, rinsed in PBS and stored whole in micro-centrifuge tubes at –20°C until further analysis for Vt by immunoblotting (see Section 2.7).

2.5 Salivary fluid secretory competence.

We used the technique of Harris and Kaufman (1984) to measure salivary fluid secretory competence. Briefly, the SGs were excised from each tick, and the main duct ligated using strands peeled from surgical silk (Dermalon® 8-0, Davis and Geck). The glands were gently blotted with a small strip of filter paper and the wet weight measured on a microbalance to the nearest 10 µg. The glands were then incubated for 10 min in TC medium 199 (Gibco) rendered approximately isosmotic to tick haemolymph by adding 36 mM NaCl; pH was adjusted to 7.3 with 10 mM MOPS buffer (Sigma). This medium also contained 10 µM dopamine (Sigma) to induce a maximal rate of salivary fluid transport (Harris and Kaufman, 1984). Following incubation, the SGs were blotted, and re-weighed. In this assay, weight increase by the SGs is a direct measure of salivary fluid secretory transport. Loss of fluid transport ability compared to appropriate controls is a quantitative measure of SG degeneration (Harris and Kaufman, 1984).

2.6. Whole-mount microscopic determination of ovarian growth phases

The stages of ovarian development were determined as follows: Dissected ovaries were photographed using a Nikon DXM1200 digital camera attached to a dissection microscope. The degree of ovarian development, including the accumulation of yolk by the oocyte, the size of the ovary, and the size and appearance of oocytes were scored according to the ovarian growth phases (OGP) described by Seixas et al. (2008). [OGP 1]: Ovaries are very thin and translucent white in hue. Oocytes are primarily ovoid in shape, the nuclei are visible, and most

of the largest oocytes are in the range of 100 – 150 µm in length. In *A. hebraeum* this phase usually corresponds to day 0 - 2 post-engorgement when ticks are held at 26^{0}C. [OGP 2]: Ovaries are significantly longer and thicker. The largest oocytes are opaque and the nuclei are obscured. The oocytes have grown to an approximate range of 200 – 250 µm in length, but have not yet accumulated Vt. This phase is usually seen between days 2 to 4 or 5 post-engorgement in *A. hebraeum* when held at 26^{0}C. [OGP 3]: Considerable growth of the ovary has occurred, due primarily to oocyte maturation, although earlier stages of oocyte development are also visible, especially along the so-called 'longitudinal groove' of the ovary (Diehl et al. 1982). Many oocytes are now as large as 400 µm and contain small yolk granules, giving them the reddish-brown color characteristic of haem-containing Vt. This phase begins at approximately day 5 post-engorgement in *A. hebraeum* when held at 26^{0}C. [OGP 4]: The ovary is, apart from the midgut, the largest organ in the haemocoel and large, yolk-filled oocytes predominate. The largest oocytes are approximately 500 – 600 µm in length. In *A. hebraeum* this phase begins at approximately day 6 or 7 post-engorgement when held at 26^{0}C. [OGP 5]: The ovary appears similar to that of OGP 4, except that ovulated oocytes are now visible in the lumen of the ovary and the oviducts. Oviposition begins on day 10 or 11 when ticks are held at 26^{0}C.

2.7. Gel electrophoresis and immunoblotting

Proteins from egg and haemolymph samples were separated by SDS-PAGE under conditions described by Friesen and Kaufman (2004). Protein concentration of haemolymph and ovary samples was measured using a Bradford Reagent kit (Sigma). Samples were diluted in electrophoresis sample buffer (60 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.0025% (w/v) bromophenol blue, 2% (w/v) SDS) so that all lanes contained 2 µg of protein. All gels consisted of a 3% stacking gel, and a 7.5% continuous resolving gel. Following electrophoresis (110V for 1 hour), the proteins were transferred to polyvinylidene difluoride membrane (PVDF; BioRad). For immunoblotting, we used antibodies previously developed against two haemolymph Vg polypeptides (Vg211 and Vg148; Friesen and Kaufman, 2002). After colour development, immunoblots were converted to digital format using a benchtop scanner.

2.8 Statistical analysis

Results are reported as mean ± SEM (n). Statistical analysis was done using Microsoft Excel on a Macintosh computer. The statistical difference between selected means was determined by t-tests.

3. Results

3.1. Feeding progress of virgin females (Fig. 2)

Virgin females were allowed to feed without interruption for up to several weeks. Of the 230 females applied to rabbits during this study, 45 eventually fed beyond the CW. >CW day 8 ticks (585 ± 36 mg; 21) were significantly heavier than >CW day 0 ticks (323 ± 17 mg; 10; $p<0.001$) and significantly heavier than >CW day 8+8 ticks (448 ± 46 mg; 14; $p = 0.038$). The weight of <CW day 0 ticks (130 ± 18 mg; 14) was not significantly different from that of <CW day +8 ticks (155 ± 22 mg; 12; $p = 0.373$), but they weighed significantly less than >CW day 0 ticks ($p<0.001$).

3.2. Effect of surpassing the CW and of detachment on salivary fluid secretory competence in virgin females (Fig. 3)

The SGs of >CW day 0 ticks secreted fluid (6.04 ± 0.53 mg/gland/10 min; 16) at a significantly higher rate than <CW day 0 ticks (2.46 ± 0.44 mg/gland/10 min; 15; $p<0.001$). Fluid secretory competence remained high in >CW day 8 ticks (6.09 ± 0.49; 29). However, salivary gland function was markedly reduced in >CW day 8+8 ticks (0.28 ± 0.06 mg/gland/10 min; 25; $p < 0.001$). Likewise, the SGs of <CW day +8 ticks secreted significantly less rapidly (1.19 ± 0.13 mg/gland/10 min; 19) than <CW day 0 ticks ($p = 0.005$), but secreted significantly more rapidly than those of >CW day 8+8 ticks ($p<0.001$).

3.3. Effect of surpassing the CW and of detachment on oocyte development and ovarian growth (Figs. 4 and 5 and Table 1)

The ovaries of <CW day 0 ticks were only at stage OGP1 (Fig. 4A; Table 1). The difference between oocyte length in <CW day +8 ticks (156 ± 25 µm; 11) and <CW day 0 ticks (108 ± 10 µm; 14; Fig. 5A) was marginally not significant ($p = 0.065$). >CW day 0 ticks also had small ovaries, corresponding to OGP 1 (Fig. 4B; Table 1) with average oocyte length being 164 ± 7 µm (7; Fig 5A; Table 1). Oocytes of >CW day 8 ticks were significantly larger than those of >CW day 0 ticks (225 ± 7 µm; 15; $p<0.001$; Fig. 5A; Table 1), and the ovaries had reached stage OGP 2 (Fig. 4C; Table 1). The ovaries of >CW day 8+8 ticks were significantly more advanced in development compared to all other groups (OGP 4; Fig. 4D; Table 1), with average oocyte length being 502 ± 16 µm; 10 ($p<0.001$; Fig. 5A).

There was no significant difference in ovary weight of <CW day +8 ticks (2.54 ± 0.57 mg; 11) compared to that of <CW day 0 ticks (1.81 ± 0.40 mg; 14; p = 0.143; Fig 5B). Ovaries of >CW day 0 ticks weighed significantly more than the ovaries of <CW ticks (5.18 ± 0.55 mg; 10; p<0.001). >CW day 8 ticks were at a more advanced ovarian growth phase (OGP 2) than >CW day 0 ticks (Figs. 4C, 5B; Table 1), and ovarian weight (13.26 ± 1.73 mg; 21) was significantly greater than that of >CW day 0 ticks (p = 0.002; Fig. 5B). However, detachment of ticks above the CW resulted in yet greater ovarian development. These ovaries had developed to OGP 4 (Fig. 4D, 5B; Table 1). Ovulation had begun in some ticks (OGP 5), although oviposition had not yet occurred (oviposition in mated ticks >CW does not begin before day 10 or 11; Friesen and Kaufman, 2002). Ovaries from >CW day 8+8 ticks were significantly heavier (25.8 ± 5.4 mg; 14) than >CW day 8 ticks (p = 0.008; Fig 5B) and the oocytes were much larger (502 ± 16 µm; 10; p<0.001) compared to any other group (Fig 5A; p<0.001).

3.4. Presence of vitellogenin in hemolymph and vitellin in ovaries of virgin ticks

Anti-Vg211/Vg148 detected Vg in the haemolymph of both mated engorged and >CW day 8+8 ticks (Fig. 6). Vg could not be detected in the haemolymph of any of the other virgin tick groups. Likewise, an immunoblot of ovary homogenates from >CW day 8+8 ticks showed a virtually identical banding pattern as that observed for a normal, mated, engorged tick 8 days post engorgement (Fig 7). However, there was virtually no sign of Vt in any of the other virgin groups.

4. Discussion

Numerous physiological changes occur in female ixodid ticks following repletion and detachment from the host. Two that have received considerable attention are SG degeneration and ovarian development. It has long been known that exceeding the CW is a signal for these physiological changes to occur (Kaufman, 1983; Harris and Kaufman, 1984), and the reproductive state of the individual (mated or virgin) influences the rapidity of the process. Thus in the case of normal mated females removed from the host above the CW, SG degeneration is virtually complete within 4 days, whereas it requires 8 days to reach an equivalent stage in weight-matched virgins (Lomas and Kaufman, 1992a, b). It is the ecdysteroids released during the post-engorgement period that triggers both SG degeneration and ovarian development. Although there also occurs a small peak of haemolymph ecdysteroid titre during the rapid phase of engorgement (Mao and Kaufman, 1999), what role,

if any, that it plays in post-engorgement events is not known. In the case of mated ticks, the role of detachment from the host was a question that never arose, because engorgement and detachment normally occur within a day of achieving the CW, i.e., before any unambiguous manifestations of SG degeneration or advanced ovarian development are detected. However, those virgin females that exceed the CW do not detach spontaneously, at least not for a number of weeks (Kaufman and Lomas, 1996). Therefore, the latter authors hypothesized that detachment might be an important component of the control pathway, because the tick would require functioning SGs as long as it remains attached to the host. But at the time there were no relevant data pertaining to the question. It is only here that we have results to support the hypothesis.

Virgin females surpassing the CW did continue to increase in size over the subsequent 8 days (Fig 2). However, during this period, there was no statistically significant change in salivary fluid secretory competence (Fig. 3). Oocyte length and ovary weight increased during this time (Fig. 5), although this was not due to yolk accumulation, the ovaries remaining in OGP 2 (Fig 4). Moreover, this ovarian growth was not accompanied by an increase in haemolymph Vg titre (Fig. 6) or yolk accumulation by the oocytes (Fig. 7). In contrast to the foregoing, SG degeneration, Vg synthesis and advanced ovarian development all occurred within 8 days following forcible detachment from the host. Salivary fluid secretory competence had declined by 95% (Fig. 3), oocyte length had increased 3-fold (502 µm vs 164 µm; Fig. 5A) and ovary weight had increased 2-fold (25.8 mg vs 13.3 mg; Fig. 5B). Finally, No Vg could be detected in the haemolymph (Fig. 6), nor Vt in ovary homogenates (Fig. 7), in large virgins remaining attached to the host. Eight days after detachment, however, the haemolymph and ovarian titres of Vg and Vt, respectively, achieved levels similar to those measured in normal engorged ticks, 8 days post-feeding (Figs. 6 and 7). These results indicate that it is the detachment stimulus that triggers post-engorgement SG degeneration, yolk synthesis, and ovarian growth. These physiological changes are mediated by the increase in haemolymph ecdysteroids that occurs at this time (Lomas and Kaufman, 1992b).

Although SG fluid transport in <CW day +8 ticks was significantly lower than in <CW day 0 ticks (Fig. 3), this is unlikely to reflect SG autolysis. The SGs of ticks removed from the host below the CW are known to lose up to 75% of fluid secretory competence 4 days following removal from the host (Kaufman, 1983; Harris and Kaufman, 1984). However, this loss of fluid secretory competence is not a sign of degeneration because (a) it is reversible if ticks are allowed to reattach and resume feeding, and the SGs of such ticks do not show

autophagic vacuole activity in the group III acinus (Harris and Kaufman, 1984). As discussed by Kaufman (1983), the cause of this reversible loss of fluid secretory competence is not known.

What sensory information associated with attachment might be part of a control pathway for inhibiting the ecdysteroid synthesis/release that triggers SG degeneration and ovarian development? Three possibilities include (1) chemical or tactile stimuli associated with the host or (2) mechanical sensations associated with blood flow in the alimentary canal or (3) mechanical or chemical sensations associated with some other aspect of attachment. The first seems inherently unlikely because these sensations would also occur in unattached ticks. In our opinion the second is also unlikely because for much of the time that virgins remain attached, there is virtually no increase in size (Kaufman and Lomas, 1996). We suggest that the third possibility may have some merit. There are some descriptions in the literature on the sensory receptors associated with the mouthparts. Waladde and Rice (1982) describe the sensory basis of tick feeding behaviour in *Rhipicephalus (Boophilus) microplus*. By histology, transmission electron microscopy and scanning electronic microscopy they identify both chemo- and mechano-receptors on the palps and the cheliceral digits. We are designing experiments to explore the specific attachment stimuli that might attenuate the release of ecdysteroids responsible for post-engorgement events.

Acknowledgements

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Figure Legends

Fig. 1. Calculation of tick body weight from body volume measurements (see Section 2.2). The linear regression curve for off-host ticks ($Y = 1.142X – 10.287$, $R^2 = 0.976$; n = 52) was virtually identical to that for on-host ticks ($Y = 1.106X + 3.32$, $R^2 = 0.960$; n = 79).

Fig. 2. Body weights of virgin female *Ambylomma hebraeum* measured after detachment from the host. Treatment groups in this and subsequent figures are: **<CW day 0**: ticks removed from host below their CW and dissected on the same day; **<CW day +8**: ticks removed from the host below their CW and then kept under colony conditions for an additional 8 days before dissection; **>CW day 0**: ticks removed and dissected on the day that they were deemed, from volume measurements, to have surpassed their CW; **>CW day 8**: ticks removed from the host and dissected 8 days after surpassing their CW; **>CW day 8+8**: ticks removed 8 days after surpassing their CW but kept under colony conditions for an additional 8 days before dissection. All data in this and subsequent figures are reported as mean ± SEM. The number of ticks in each group is indicated above each bar.

Fig. 3. Fluid uptake by isolated SGs from the treatment groups shown in Fig. 2.

Fig. 4. Ovarian growth phases (OGP) in selected ticks from this study. (A) <CW day 0; OGP 1. (B) >CW day 0; OGP 1. (C) >CW day 8; OGP 2. (D) >CW day 8+8; OGP 4. (E) ovary from a mated, engorged female, 8 days post-engorgement; OGP 4/OGP 5.

Fig. 5. The effect of surpassing the CW and of detachment on (A) oocyte length and (B) ovary weight in fed virgin ticks.

Fig. 6. Vg content in haemolymph from the five treatment groups and normal engorged ticks (day 8 post-engorgement). Haemolymph proteins were separated by SDS-PAGE and the Vg proteins identified by immunoblotting as described in Section 2.8. **(Eng.):** haemolymph of a mated, engorged tick (day 8). **(kDa):** molecular weight markers. See legend of Fig. 2 for definitions of other symbols.

Fig. 7. Vt content in ovaries from the five treatment groups and normal engorged ticks (day 8). Ovary homogenates were separated by SDS-PAGE and the Vt proteins identified by immunoblotting as described in Section 2.8. See legend of Fig. 2 for definitions of other symbols.

Figure 1

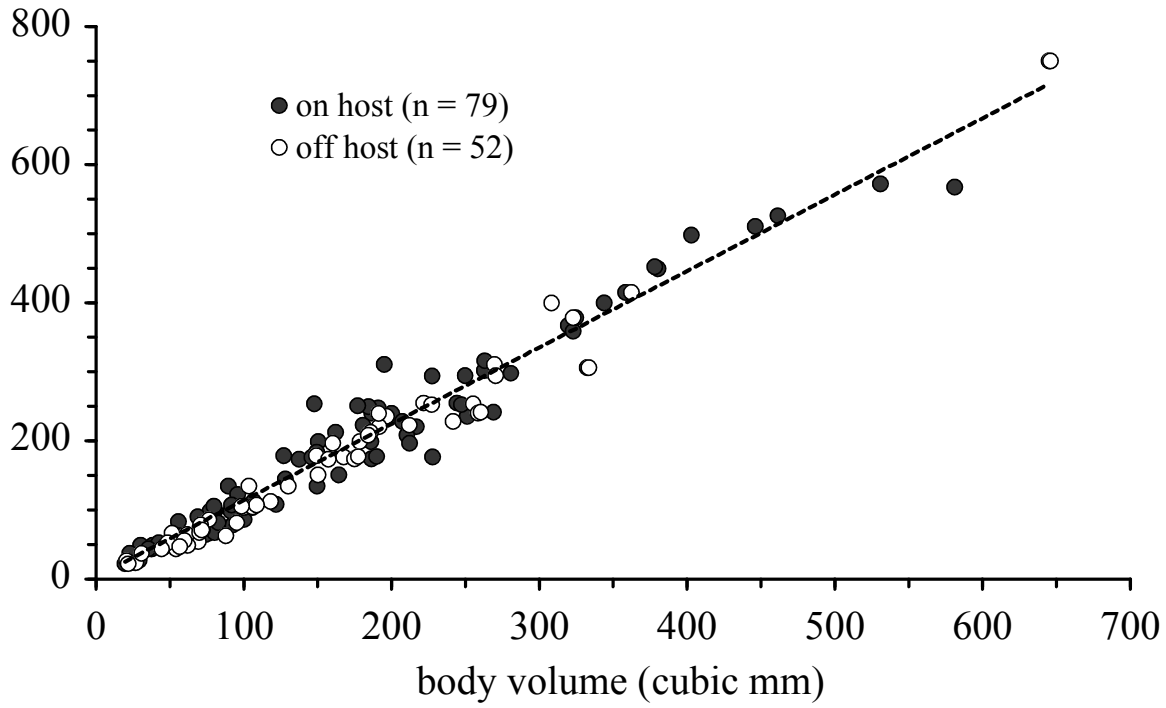

Figure 1.

Figure 5

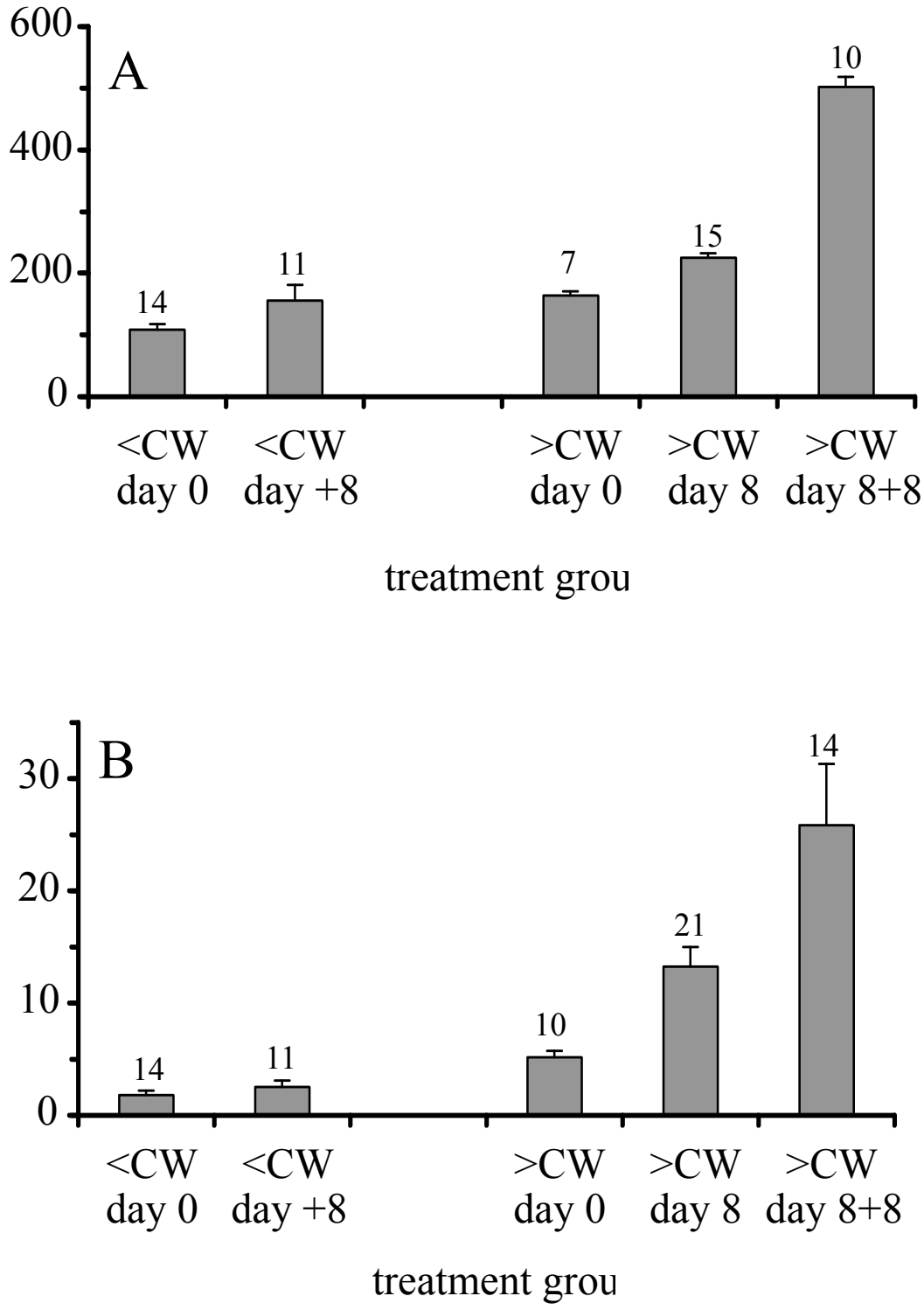

Figure 5.

Figure 2

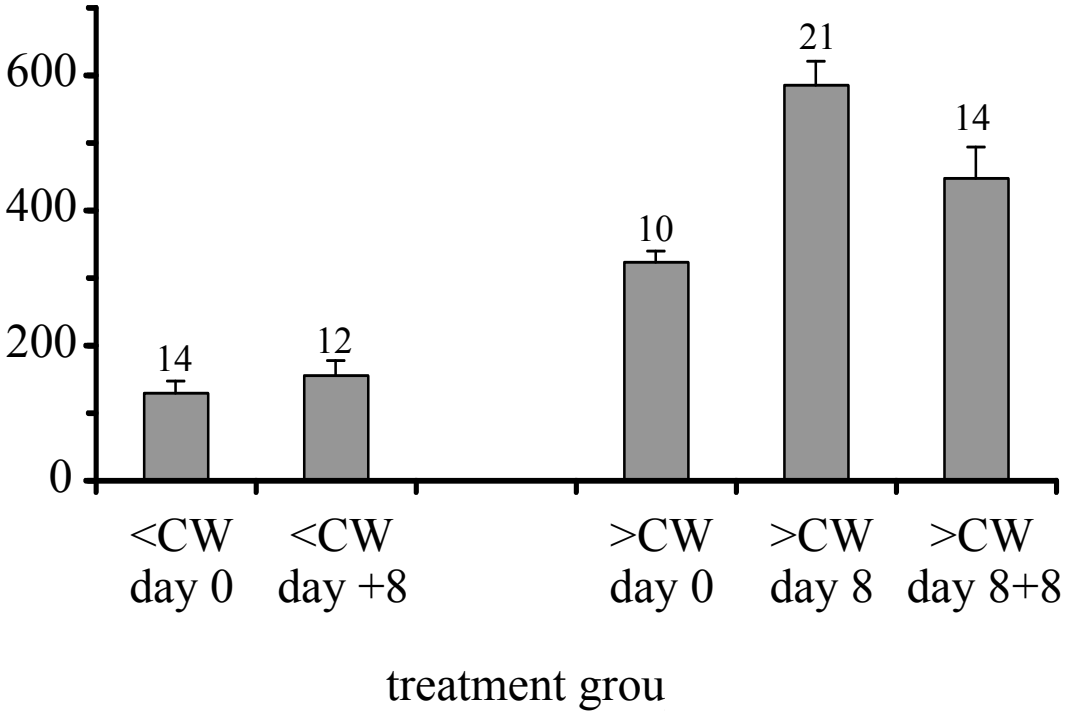

Figure 2.

Figure 3

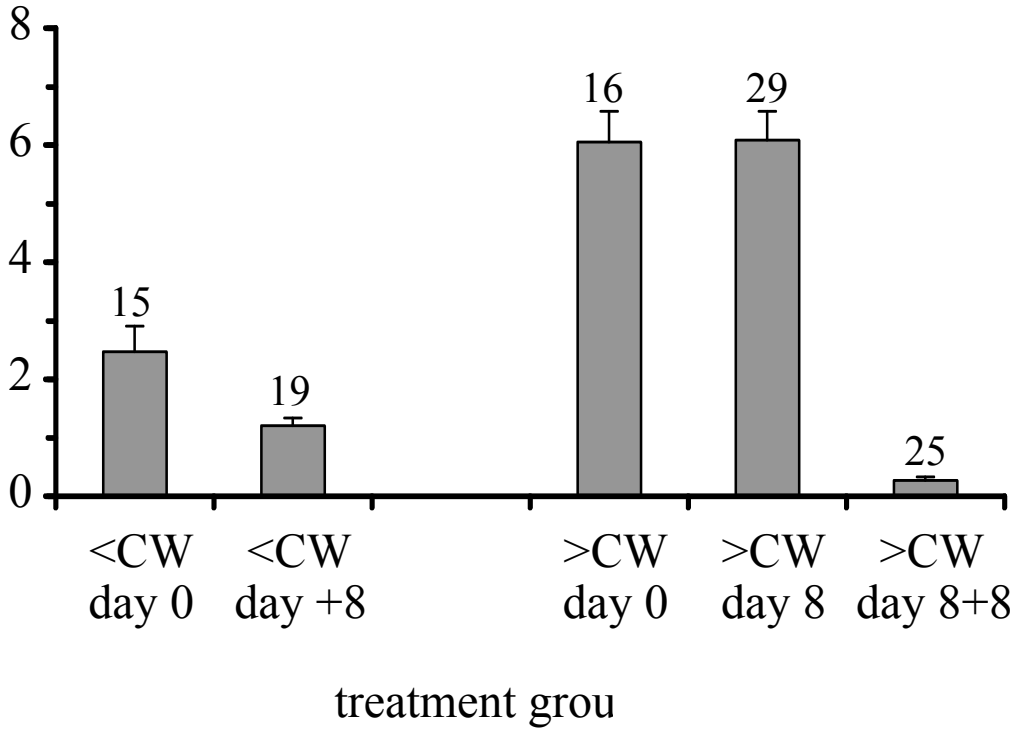

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Figure 4

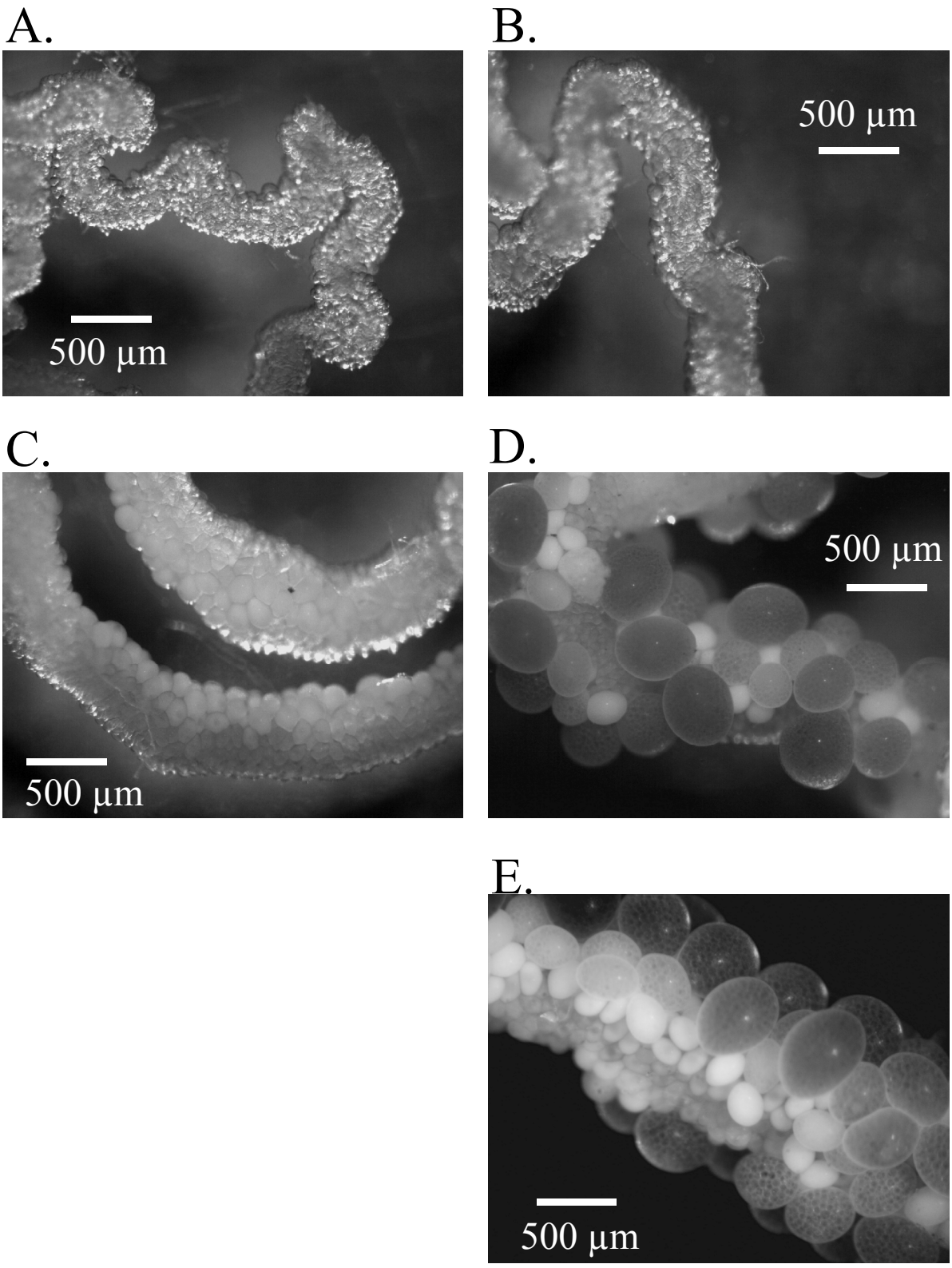

Figure 4.

Figure 6

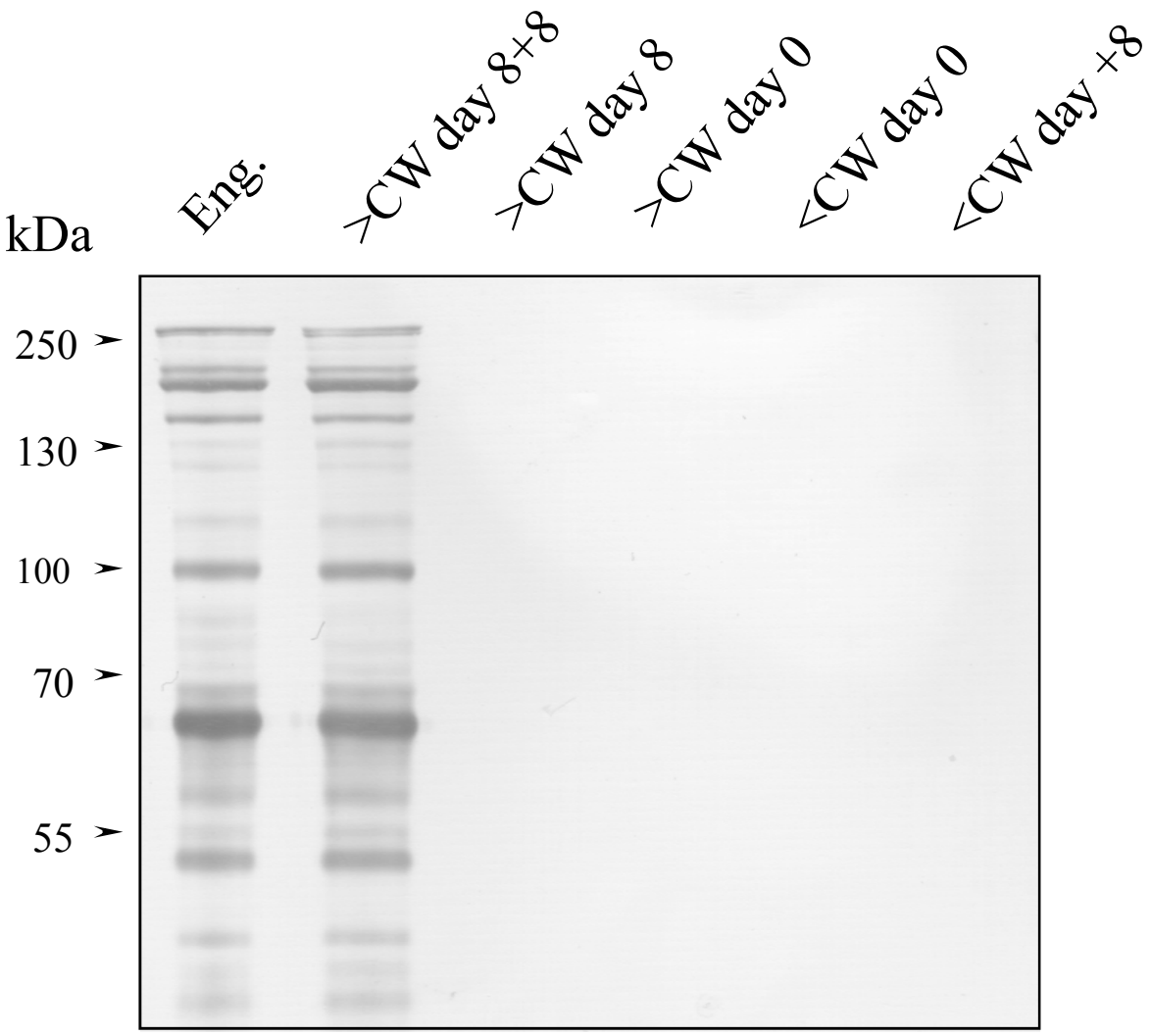

Figure 6.

Figure 7

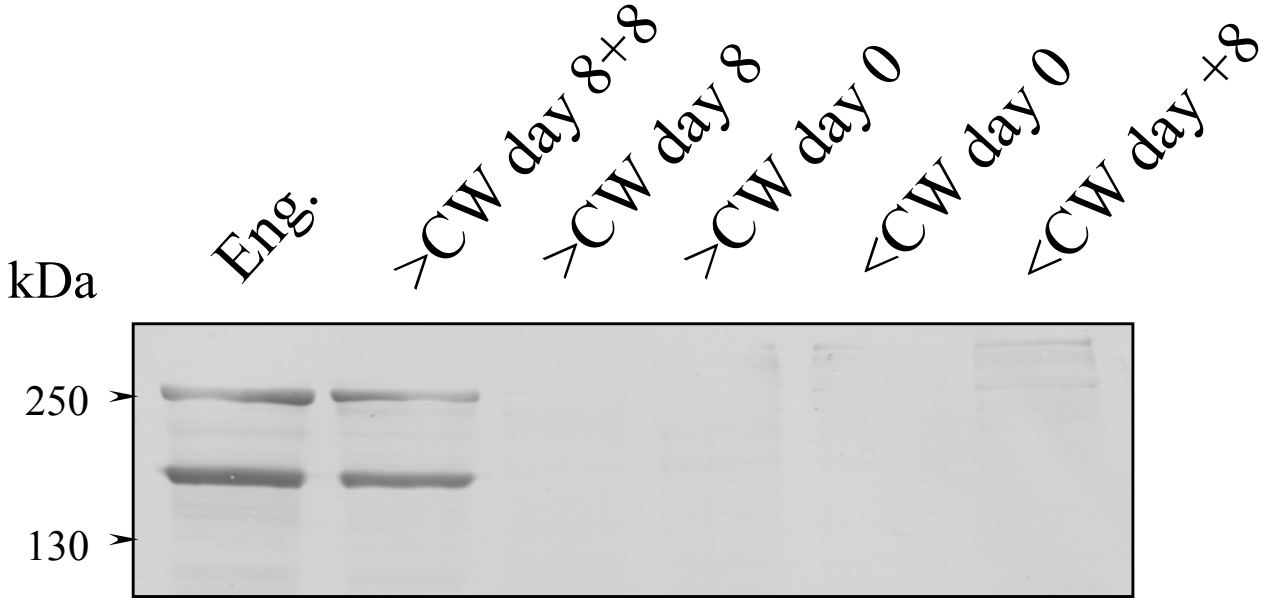

Figure 7.

Table(s)

Table 1. Ovarian development in virgin females detached below or above the critical weight.

treatment group[1] (N)	Mean oocyte length[2] (µm; see Fig. 5A)	Ovary Growth Phase[1] (number of ticks and % at each stage)				
		1	2	3	4	5
<CW day 0 (14)	108 ± 10	14 (100%)	0	0	0	0
<CW day +8 (11)	156 ± 25	8 (73%)	3 (27%)	0	0	0
>CW day 0 (7)	164 ± 7	7 (100%)	0	0	0	0
>CW day 8 (15)	225 ± 7	1 (7%)	14 (93%)	0	0	0
>CW day 8+8 (10)	502 ± 16	0	0	1 (9%)	7 (64%)	2 (18%)

[1] See Materials and Methods for definitions.

[2] In each tick, the length of eight of the apparently largest oocytes were measured (see Materials and Methods).

Salivary gland degeneration and vitellogenesis in the ixodid tick *Amblyomma hebraeum*: Surpassing a critical weight is the prerequisite and detachment from the host is the trigger.

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Abstract

The normal engorged body weight of female ixodid ticks (Acari: Ixodidae) is about 100X the unfed weight. Virgin female *Amblyomma hebraeum* normally do not feed beyond 10X the unfed weight. About 10-20 % of a population of virgins will feed to perhaps 20X the unfed weight, but not much beyond that. In *A. hebraeum*, when females surpass about 10X the unfed weight, the following changes in physiology occur if they are removed from the host: (a) they will not reattach if given the opportunity, (b) their salivary glands (SGs) will undergo autolysis within 4 days if they are mated or 8 days if they are virgin, and (c) egg maturation and oviposition will occur in due course. Female ticks removed from the host below about 10X the unfed weight do not experience the latter changes (Kaufman and Lomas, 1996, Invertebrate Reproduction and Development 30, 191-198). In 1984 we named this transitional weight, the 'critical weight' (CW); its absolute value is probably a species-specific characteristic (Kaufman, 2007, Journal of Insect Physiology 53, 264-273). Although mated females tend to engorge within a day of surpassing the CW, virgin females surpassing the CW can remain attached to the host for at least several weeks more. It is not known whether the physiological changes in the SGs and ovaries listed above occur in those large virgins that remain attached, although we suppose that this would be maladaptive. Instead, we hypothesize in this study that surpassing the CW is only a prerequisite for inducing these changes, and that detachment is the actual trigger. We support our hypothesis by demonstrating that large virgins, remaining attached to a host for 8 days, did not undergo SG degeneration nor complete egg maturation during the attachment period. Those changes occurred only within 8 days following detachment. So some type of sensory information associated with attachment to the host, and still undefined, inhibits expression of the physiological changes hitherto associated merely with surpassing the CW.

Keywords: ixodid ticks, *Amblyomma hebraeum*, ecdysteroids, vitellogenesis, ovarian development, critical weight, tick salivary glands.

1. Introduction

In our laboratory colony of the ixodid tick, *Amblyomma hebraeum* (Koch), engorgement of the female normally occurs within about 10 days of attachment, at which point it weighs up to 100X its unfed body weight. The taking of a blood meal in female ixodid ticks is conventionally divided into three phases (Balashov, 1972). (1) During a ‘preparatory phase’

(about 1 day) the female establishes a feeding lesion. (2) During the 'slow phase' (7 - 9 days) it grows to about 10X its unfed body weight. (3) During the 'rapid phase' (about 1 day) it increases its weight a further 10-fold and detaches from the host.

Haemolymph ecdysteroid titre in *A. hebraeum* rises substantially during the first week post engorgement (Kaufman, 1991; Friesen and Kaufman 2002). This rise in ecdysteroids triggers salivary gland (SG) autolysis (Harris and Kaufman, 1984; Kaufman, 1991; Chang and Kaufman, 2005). Ecdysteroids also stimulate synthesis of the main yolk protein, vitellogenin (Vg) in both ixodid ticks [*A. hebraeum* (Friesen and Kaufman, 2002, 2004) and *Dermacentor variabilis* (Sankhon et al. 1999, Thompson et al. 2007)] and argasid ticks [*Ornithodoros moubata* (Ogihara et al. 2007; Horigane et al., 2007)].

In most ixodid species, mating occurs on the host. Copulation facilitates the female to enter the rapid phase of engorgement and to feed past a 'critical weight' (CW) that is characterized by several behavioral and physiological changes. For example, if a female is removed from the host prior to reaching the CW, it will actively seek a new host in order to complete the blood meal. But if it is removed after exceeding the CW, it will no longer seek a host, nor will it attach to one if given the opportunity. In addition, the SGs of females above the CW will undergo autolysis, and ovarian development will proceed to full maturation. The CW for mated female *A. hebraeum* is approximately 10X the unfed body weight (Harris and Kaufman, 1984; Kaufman and Lomas, 1996), although a more rigorous experimental design has shown that the exact value (between 10-13X the unfed weight) depends on which physiological parameter is being measured (Weiss and Kaufman, 2001).

Under normal circumstances, the female engorges and detaches from the host within 24 h of surpassing the CW. Although most virgin *A. hebraeum* do not feed beyond the CW, perhaps up to 20% do, though rarely do they feed beyond 20X the unfed weight, and they don't engorge to repletion. Virgins heavier than the CW can remain attached to the host for at least several weeks (Kaufman and Lomas, 1996; Lomas and Kaufman, 1999). Moreover, if such large virgins are forcibly removed from the host, their SGs degenerate within 8 days (Lomas and Kaufman 1992a, b) and complete ovarian development occurs (Kaufman, 1991). The question arises: If large virgins can remain attached to the host for several weeks without engorging to repletion, do their SGs undergo autolysis and does complete ovarian development occur during this time? Because that would be maladaptive, perhaps SG degeneration and complete ovarian development are inhibited in large virgins by virtue of remaining attached to the host? The present study was designed to answer this question. Our

results indicate that although surpassing the CW is a prerequisite for SG autolysis and complete ovarian development, detachment from the host constitutes the trigger.

2. Materials and Methods

2.1 Ticks and tick feeding

All host animals used in this study were cared for according to the guidelines mandated by the Canadian Council of Animal Care. Our colony of *A. hebraeum* was kept in darkness, at 27°C and at >85% relative humidity. Ticks were fed on rabbits as described by Kaufman and Phillips (1973). Prior to feeding, individual females were weighed and marked by gluing (cyanoacrylate glue) a short length of colored thread to a leg. Female *A. hebraeum* are reluctant to attach in the absence of males. Hence, a number of male ticks were first allowed to attach to the back of the host rabbit. The males were confined within a cloth bag that permitted their attachment to the host but prevented copulation. The next day, unfed females were added to the same rabbit. When females were removed at the appropriate stage for experiments, they were rinsed with water, dried with tissue paper, weighed, and stored individually in gauze-covered glass vials until their tissues were collected (see Section 2.4).

2.2 Estimating tick body weight while they are attached to the host

For this study we required a method for determining the day on which individual virgin females had surpassed the CW, but without removing them from the host and weighing them. We did this by measuring body volume of feeding ticks as follows. Using digital calipers, we measured (1) the width of the body at its apparently widest part: approximately at the site of the posterior pair of legs (dimension X), (2) the anterior-to-posterior length of the body along the midline (dimension Y), and (3) the dorso-ventral thickness of the body (dimension Z). For these measurements the calipers could be read to the nearest 0.01 mm. We calculated tick volume from the formula:

$$\text{Volume} = 4/3\pi * 0.5 * (XYZ)$$

This formula approximates the geometry of a fed tick (Patriquin, 1991). In a preliminary trial, we prepared a standard curve as follows for converting volume to weight: Body volume of ticks of diverse sizes (from about 2X to about 30X the unfed weight) was measured on-host and the ticks were then forcibly removed, washed and weighed to the nearest 0.1 mg. Body volume was re-measured off-host. (Off-host measurement was considered to be intuitively more accurate because of greater ease to align the ticks with the calipers.) Standard curves were

drawn plotting volume as a function of body weight using Microsoft Excel software. Fig. 1 shows the standard curves for on-host and off-host volume measurements. The linear standard curves corresponded to each other almost exactly.

2.3 Experimental Procedure

The day on which each tick was deemed from its volume to have surpassed the CW was designated as 'day 0'. Experimental ticks were assigned to one of five groups. Group 1 consisted of ticks above the CW removed from the host on day 0; henceforth this group is labeled '>CW day 0'. Salivary fluid secretory competence was measured as described in Section 2.5, and ovarian development was determined as described in Section 2.6. Group 2 consisted of ticks above the CW that were allowed to remain attached to the host for an additional 8 days before removal and measuring salivary fluid secretory competence and ovarian development; henceforth this group is labeled '>CW day 8'. Group 3 consisted of ticks that were similar to Group 2, but which were kept off-host in the colony incubator for an additional 8 days before salivary secretion and ovarian development were measured; henceforth this group is labeled '>CW day 8+8'. Group 4 was a control group, similar to Group 1, except that these were ticks that had not yet reached their CW; henceforth this group is labeled '<CW day 0'. Group 5, a second control group of below-CW ticks, were treated similarly to Group 3; henceforth this group is labeled '<CW day +8'.

2.4 Collection of tissue and haemolymph samples

Haemolymph and ovaries were monitored for the following indices: (a) Vg titre of haemolymph, (b) Vt content of ovary, (c) ovary weight and (d) oocyte length. Ticks were immobilized on small plastic petri dishes with a drop of cyanoacrylate glue, and chilled in a refridgerator for 10 min. Cooling inhibits smooth muscle contraction in the gut and so reduces the likelihood of puncturing the gut while collecting haemolymph samples. A small incision was made in the cuticle, and haemolymph was collected in calibrated glass micropipettes. Haemolymph was diluted 1:4 (v/v) in a phosphate-buffered saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0). Samples were stored at -20^{0}C until assayed for the presence of Vg by SDS-PAGE and immunoblot analysis (see Section 2.7).

After collection of haemolymph, ticks were flooded with a modified Hank's balanced saline (200 mM NaCl, 8.9 mM D-glucose, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 27 µM phenol red, pH 7.2), and the dorsal cuticle was

removed using a microscalpel. SGs were excised and set aside for measuring salivary fluid secretory competence (see section 2.5). Ovaries were dissected out, and the length of the long axis of the eight apparently largest ovoid oocytes was measured using a calibrated ocular micrometer fitted to a dissection microscope. The mean value for the eight oocytes was recorded for each tick, and ovary growth and oocyte development were scored according to the system described in Section 2.6. The ovaries were then gently blotted, weighed to the nearest 10 µg, rinsed in PBS and stored whole in micro-centrifuge tubes at –20°C until further analysis for Vt by immunoblotting (see Section 2.7).

2.5 Salivary fluid secretory competence.

We used the technique of Harris and Kaufman (1984) to measure salivary fluid secretory competence. Briefly, the SGs were excised from each tick, and the main duct ligated using strands peeled from surgical silk (Dermalon® 8-0, Davis and Geck). The glands were gently blotted with a small strip of filter paper and the wet weight measured on a microbalance to the nearest 10 µg. The glands were then incubated for 10 min in TC medium 199 (Gibco) rendered approximately isosmotic to tick haemolymph by adding 36 mM NaCl; pH was adjusted to 7.3 with 10 mM MOPS buffer (Sigma). This medium also contained 10 µM dopamine (Sigma) to induce a maximal rate of salivary fluid transport (Harris and Kaufman, 1984). Following incubation, the glands were blotted, and re-weighed. In this assay, weight increase by the glands is a direct measure of salivary fluid secretory transport. Loss of fluid transport ability compared to appropriate controls is a quantitative measure of SG degeneration (Harris and Kaufman, 1984).

2.6. Whole-mount microscopic determination of ovarian growth phases

The stages of ovarian development were determined as follows: Dissected ovaries were photographed using a Nikon DXM1200 digital camera attached to a dissection microscope. The degree of ovarian development, including the accumulation of yolk by the oocyte, the size of the ovary, and the size and appearance of oocytes were scored according to the ovarian growth phases (OGP) described by Seixas et al. (2008). [OGP 1]: Ovaries are very thin and translucent white in hue. Oocytes are primarily ovoid in shape, the nuclei are visible, and most of the largest oocytes are in the range of 100 – 150 µm in length. In *A. hebraeum* this phase usually corresponds to day 0 - 2 post-engorgement when ticks are held at 26^0C. [OGP 2]: Ovaries are significantly longer and thicker. The largest oocytes are opaque and the nuclei are

obscured. The oocytes have grown to an approximate range of 200 – 250 µm in length, but have not yet undergone Vg uptake. This phase is usually seen between days 2 to 4 or 5 post-engorgement in *A. hebraeum* when held at 26^{0}C. [OGP 3]: Considerable growth of the ovary has occurred, due primarily to oocyte maturation, although earlier stages of oocyte development are also visible, especially along the so-called 'longitudinal groove' of the ovary (Diehl et al. 1982). Many oocytes are now as large as 400 µm and contain small yolk granules, giving them the reddish-brown color characteristic of haem-containing Vg. This phase begins at approximately day 5 post-engorgement in *A. hebraeum* when held at 26^{0}C. [OGP 4]: The ovary is, apart from the midgut, the largest organ in the haemocoel and large, yolk-filled oocytes predominate. The largest oocytes are approximately 500 – 600 µm in length. In *A. hebraeum* this phase begins at approximately day 6 or 7 post-engorgement when held at 26^{0}C. [OGP 5]: The ovary appears similar to that of OGP 4, except that ovulated oocytes are now visible in the lumen of the ovary and the oviducts; oviposition begins on day 10 or 11 when ticks are held at 26^{0}C.

2.7. Gel electrophoresis and immunoblotting

Proteins from egg and haemolymph samples were separated by SDS-PAGE (Laemlli, 1970). Protein concentration of haemolymph and ovary samples was measured using a Bradford Reagent kit (Sigma). Samples were diluted in electrophoresis sample buffer (60 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.0025% (w/v) bromophenol blue, 2% (w/v) SDS) so that all lanes contained 2 µg of protein. All gels consisted of a 3% stacking gel, and a 7.5% continuous resolving gel. Electrophoresis was performed at constant voltage (110 V) until the dye front reached the bottom of the gels. Gels were then transferred to polyvinylidene difluoride membrane (PVDF; BioRad) for 1 h at 0.3-0.5 mA for immunoblotting. For antibody treatments, we developed antibodies against two haemolymph polypeptides previously determined to be Vg (Vg211 and Vg148; Friesen and Kaufman, 2002). After the transfer, membranes were blocked overnight in blocking buffer [5% skim milk powder, 2% bovine serum albumin (BSA), in Tris-buffered saline containing Tween 20 (TTBS)]. TTBS had the following composition: 60 mM Tris-HCl, 0.3 M NaCl, in 0.04% (v/v) Tween 20, pH adjusted to 7.5. The next day, the membranes were placed in blocking buffer containing a mixture of anti-Vg211 (1:500) and anti-Vg148 (1:500) for 1h. Membranes were then washed three times for 5 min with TTBS and placed in secondary antibody (alkaline phosphatase (AP)-conjugated, goat anti-rabbit IgG; BioRad) diluted 1:2000 in TTBS containing 2% BSA (w/v). One hour later, the membranes were again washed three times for 5 min in TTBS and then stained for

approximately 20 min using an AP-conjugate substrate kit (BioRad). Immunoblots were converted to digital format using a benchtop scanner.

2.8 Statistical analysis

Results are reported as mean ± SEM (n). Statistical analysis was done using Microsoft Excel on a Macintosh computer. Statistical difference between selected means was determined by t-tests, 1-tailed or 2-tailed as appropriate.

3. Results

3.1. Feeding progress of virgin females (Fig. 2)

Virgin females were allowed to feed without interruption for up to several weeks. Of 230 females applied to rabbits, 45 (20 %) fed beyond the CW. >CW day 8 ticks (585 ± 36 mg; 21) were significantly heavier than >CW day 0 ticks (323 ± 17 mg; 10; $p < 0.001$) and significantly heavier than >CW day 8+8 ticks (448 ± 46 mg; 14; $p = 0.038$). The weight of <CW day 0 ticks (130 ± 18 mg; 14) was not significantly different from that of <CW day +8 ticks (155 ± 22 mg; 12; $p = 0.373$), but they weighed significantly less than >CW day 0 ticks ($p < 0.001$).

3.2. Effect of surpassing the CW and of detachment on SG autolysis in virgin females (Fig. 3)

The SGs of >CW day 0 ticks secreted fluid (6.04 ± 0.53 mg/gland/10 min; 16) at a significantly higher rate than <CW day 0 ticks (2.46 ± 0.44 mg/gland/10 min; 15; $p < 0.001$). Fluid secretory competence remained high in >CW day 8 ticks (6.09 ± 0.49; 29). However, salivary gland function was markedly reduced in >CW day 8+8 ticks (0.28 ± 0.06 mg/gland/10 min; 25; $p < 0.001$). Likewise, the salivary glands of <CW day +8 ticks secreted significantly less (1.19 ± 0.13 mg/gland/10 min; 19) than <CW day 0 ticks ($p = 0.005$), but secreted more fluid than those of >CW day 8+8 ticks ($p < 0.001$). However, the reduction in fluid secretory competence experienced by <CW day +8 ticks is unlikely to be the result of degeneration (see Discussion).

3.3. Effect of surpassing the CW and of detachment on oocyte development and ovarian growth (Figs. 4 and 5 and Table 1)

The ovaries of <CW day 0 ticks were only at stage OGP1 (Fig. 4A; Table 1). The difference between oocyte length in <CW day +8 ticks (156 ± 25 µm; 11) and <CW day 0 ticks (108 ± 10 µm; 14; Fig. 5A) was marginally not significant ($p = 0.065$). >CW day 0 ticks also had small ovaries, corresponding to OGP 1 (Fig. 4B; Table 1) with average oocyte length being

164 ± 7 µm (7; Fig 5A). Oocytes of >CW day 8 ticks were significantly larger than those of >CW day 0 ticks (225 ± 7 µm; 15; $p < 0.001$; Fig. 5A), and the ovaries had reached stage OGP 2 (Fig. 4C; Table 1). The ovaries of >CW day 8+8 ticks were significantly more advanced in development compared to all other groups (OGP 4; Fig. 4D; Table 1), with average oocyte length being 502 ± 16 µm; 10 ($p < 0.001$; Fig. 5A).

There was no significant difference in ovary weight of <CW day +8 ticks (2.54 ± 0.57 mg; 11) compared to that of <CW day 0 ticks (1.81 ± 0.40 mg; 14; $p = 0.143$; Fig 5B). Ovaries of >CW day 0 ticks weighed significantly more than the ovaries of below CW ticks (5.18 ± 0.55 mg; 10; $p < 0.001$). >CW day 8 ticks were at a more advanced ovarian growth phase (OGP 2) than >CW day 0 ticks (Fig. 4C, 4B and Table 1), and ovarian weight (13.26 ± 1.73 mg; 21) was significantly greater than that of >CW day 0 ticks ($p = 0.002$; Fig. 5B). However, detachment of ticks above the CW resulted in yet greater ovarian development. These ovaries had developed to OGP 4 (Fig. 4D; Table 1). Ovulation had begun in some ticks (OGP 5), although oviposition had not yet occurred, because oviposition in mated ticks above the CW does not begin before day 10 or 11 (Friesen and Kaufman, 2002). Ovaries from >CW day 8+8 ticks were significantly heavier (25.8 ± 5.4 mg; 14) than >CW day 8 ticks ($p = 0.008$; Fig 5B) and the oocytes were much larger (502 ± 16 µm; 10; $p < 0.001$) compared to any other group (Fig 5A; $p < 0.001$).

3.4. Presence of vitellogenin in hemolymph and vitellin in ovaries of virgin ticks

Anti-Vg211/Vg148 detected Vg in the haemolymph of both mated engorged and >CW day 8+8 ticks (Fig. 6). Vg could not be detected in the haemolymph of any of the other virgin tick groups. Likewise, an immunoblot of ovary homogenates from >CW day 8+8 ticks showed a virtually identical banding pattern as that observed for a normal, mated, engorged tick 8 days post engorgement (Fig 7). However, there was virtually no sign of Vt in any of the other virgin groups.

4. Discussion

Numerous physiological changes occur in female ixodid ticks following repletion and detachment from the host. Two that have received considerable attention are SG degeneration and ovarian development. It has long been known that exceeding the CW is a signal for these physiological changes to occur (Kaufman, 1983; Harris and Kaufman, 1984), and the reproductive state of the individual (mated or virgin) influences the rapidity of the process. Thus in the case of normal mated females removed from the host above the CW, SG

degeneration is virtually complete within 4 days, whereas it requires 8 days to reach an equivalent stage in weight-matched virgins (Lomas and Kaufman, 1992a, b). It is the ecdysteroids released during the post-engorgement period that triggers both SG degeneration and ovarian development*. In the case of mated ticks, the role of detachment from the host was a question that never arose, because engorgement and detachment normally occur within a day of achieving the CW, i.e., before any unambiguous manifestations of SG degeneration or advanced ovarian development are detected. However, those virgin females that exceed the CW do not detach spontaneously, at least not for a number of weeks (Kaufman and Lomas, 1996). Therefore, the latter authors hypothesized that detachment might be an important component of the control pathway, because the tick would require functioning SGs as long as it remains attached. But at the time there were no relevant data pertaining to the question. It is only here that we have results to support the hypothesis.

Virgin females surpassing the CW did continue to increase in size over the subsequent 8 days (Fig 2). However, during this period, there was no statistically significant change in salivary fluid secretory competence (Fig. 3). Oocyte length and ovary weight increased during this time (Fig. 5), although this was not due to yolk accumulation, the ovaries remaining in OGP 2 (Fig 4). Moreover, this ovarian growth was not accompanied by an increase in haemolymph Vg titre (Fig. 6) or yolk accumulation by the oocytes (Fig. 7). In contrast to the foregoing, SG degeneration, Vg synthesis and advanced ovarian development all occurred within 8 days following forcible detachment from the host. Salivary fluid secretory competence had declined by 95% (Fig. 3), oocyte length had increased 3-fold (502 µm vs 164 µm; Fig. 5A) and ovary weight had increased 2-fold (25.8 mg vs 13.3 mg; Fig. 5B). Finally, No Vg could be detected in the haemolymph (Fig. 6), nor Vt in ovary homogenates (Fig. 7), in large virgins remaining attached to the host. Eight days after detachment, however, the haemolymph and ovarian titres of Vg and Vt, respectively, achieved levels similar to those measured in normal engorged ticks, 8 days post-feeding (Figs. 6 and 7).

Although SG fluid transport in <CW day +8 ticks was significantly lower than in <CW day 0 ticks (Fig. 2), this is unlikely to reflect SG autolysis. The SGs of ticks removed from the host below the CW are known to lose up to 75% of fluid secretory competence 4 days following removal from the host (Kaufman, 1983; Harris and Kaufman, 1984). However, this loss of fluid secretory competence is not a sign of degeneration because (a) it is reversible if

* There occurs a small peak of haemolymph ecdysteroid titre during the rapid phase of engorgement (Mao and Kaufman, 1999), but its physiological significance has not yet been adequately explored.

ticks are allowed to reattach and resume feeding, and the SGs of such ticks do not show autophagic vacuole activity in the group III acinus (Harris and Kaufman, 1984). The cause of this reversible loss of fluid secretory competence is not known, although it might be the result of 'uncoupling' of the dopamine receptor from the adenylate cyclase, a reduction in catalytic activity of adenylate cyclase, or a reduction in the number of dopamine receptors in the tissue (Kaufman, 1983).

What sensory information associated with attachment might be part of a control pathway for the ecdysteroid synthesis/release that triggers SG degeneration and ovarian development? Three possibilities include (1) chemical or tactile stimuli associated with the host or (2) mechanical sensations associated with blood flow in the alimentary canal or (3) mechanical (or chemical) sensations associated with some other aspect of attachment — perhaps contact with the cement cone enveloping the mouthparts. The first seems inherently unlikely because these sensations would also occur in unattached ticks. In our opinion the second is also unlikely because for much of the time that virgins remain attached, there is virtually no increase in size (Kaufman and Lomas, 1996). We suggest that the third possibility is the most promising. There are some descriptions in the literature on the sensory receptors associated with the mouthparts. Broadly speaking, ticks possess chemosensilla, mechanosensilla, photosensilla and thermosensilla, and usually these can be identified by morphological criteria (Sonenshine, 1991). Waladde and Rice (1982) describe the sensory basis of tick feeding behaviour in *Rhipicephalus (Boophilus) microplus*. By histology, transmission electron microscopy and scanning electronic microscopy they identify both chemo- and mechano-receptors (the latter including proprioceptors) on the palps and the cheliceral digits. We are designing experiments to explore the specific attachment stimuli that attenuate the release of ecdysteroids responsible for post-engorgement events.

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Figure Legends

Fig. 1. Calculation of tick body weight from body volume measurements (see Section 2.2). The linear regression curve for off-host ticks ($Y = 1.142X – 10.287$, $R^2 = 0.976$; n = 52) was virtually identical to that for on-host ticks ($Y = 1.106X + 3.32$, $R^2 = 0.960$; n = 79).

Fig. 2. Body weights of virgin female *Ambylomma hebraeum* measured after detachment from the host. Treatment groups in this and subsequent figures are: **<CW day 0**: ticks removed from host below their CW and dissected on the same day; **<CW day +8**: ticks removed below their CW and then kept under colony conditions for an additional 8 days before dissection; **>CW day 0**: ticks removed and dissected on the day that they were deemed, from volume measurements, to have surpassed their CW; **>CW day 8**: ticks removed from the host and dissected 8 days after surpassing their CW; **>CW day 8+8**: ticks removed 8 days after surpassing their CW but kept under colony conditions for an additional 8 days before dissection. All data in this and subsequent figures are reported as mean ± SEM. The number of ticks in each group is indicated above each bar. The weight of >CW day 8 ticks (585 mg) was significantly heavier than >CW day 0 ticks (323 mg; $p < 0.001$). The small reduction in weight of >CW day 8+8 (448 mg) over >CW day 8 ticks was also statistically significant ($p = 0.025$).

Fig. 3. Fluid uptake by isolated SGs from the treatment groups shown in Fig. 2. Ticks that were kept under colony conditions for 8 days after detachment had significantly reduced fluid uptake compared to ticks dissected on the day they were removed from the host, irrespective of whether they had surpassed their CW (>CW day 8+8; $p < 0.001$) or not (<CW day 8; $p = 0.005$).

Fig. 4. Ovarian growth phases (OGP) in selected ticks from this study. (A) <CW day 0; OGP 1. (B) >CW day 0; OGP 1. (C) >CW day 8; OGP 2. (D) >CW day 8+8; OGP 4. (E) ovary from a mated, engorged female, 8 days post-engorgement; OGP 4/OGP 5.

Fig. 5. The effect of surpassing the CW and of detachment on (A) oocyte length and (B) ovary weight in fed virgin ticks. Ticks that had fed beyond the CW and kept under colony conditions for 8 days (>CW day 8+8) had significantly larger oocytes and heavier ovaries than ticks from any other group.

Fig. 6. Vg content in haemolymph from the five treatment groups and normal engorged ticks (day 8 post-engorgement). Haemolymph proteins were separated by SDS-PAGE and the Vg proteins identified by immunoblotting as described in Section 2.8. Only virgin ticks

feeding beyond the CW and kept off the host for 8 days contained a detectable amount of Vg in the haemolymph. (Eng.): haemolymph of a mated, engorged tick (day 8). (kDa): molecular weight markers. See legend of Fig. 2 for definitions of other symbols.

Fig. 7. Vt content in ovaries from the five treatment groups and normal engorged ticks (day 8). Ovary homogenates were separated by SDS-PAGE and the Vt proteins identified by immunoblotting as described in Section 2.8. Only virgin ticks feeding beyond the CW and kept off the host for 8 days contained a detectable amount of Vt in the ovaries. See legend of Fig. 2 for definitions of other symbols.