Journal of Insect Physiology xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Effect of 20-hydroxyecdysone and haemolymph on oogenesis in the ixodid tick *Amblyomma hebraeum*

Adriana Seixas [a], Kevin J. Friesen [b], W. Reuben Kaufman [b,*]

[a] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, CEP 91501-970, Brazil
[b] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

ARTICLE INFO

Article history:
Received 11 February 2008
Received in revised form 14 May 2008
Accepted 20 May 2008

Keywords:
Ixodid ticks
Amblyomma hebraeum
Dermacentor variabilis
Ecdysteroids
20-Hydroxyecdysone
Vitellogenesis

ABSTRACT

Earlier work from our laboratory indicated that injection of 20-hydroxyecdysone (20E) into non-vitellogenic female *Amblyomma hebraeum* ticks stimulates the synthesis of vitellogenin (Vg), but not its uptake into oocytes [Friesen, K., Kaufman, W.R., 2004. Effects of 20-hydroxyecdysone and other hormones on egg development, and identification of a vitellin-binding protein in the ovary of the tick, *Amblyomma hebraeum*. Journal of Insect Physiology 50, 519–529]. In contrast, Thompson et al. [Thompson, D.M., Khalil, S.M.S., Jeffers, L.A., Ananthapadmanaban, U., Sonenshine, D.E., Mitchell, R.D., Osgood, C.J., Apperson, C.S., Roe, M.R., 2005. In vivo role of 20-hydroxyecdysone in the regulation of the vitellogenin mRNA and egg development in the American dog tick, *Dermacentor variabilis* (Say). Journal of Insect Physiology 51, 1105–1116] demonstrated that injection of 20E into virgin female *Dermacentor variabilis* ticks stimulated both vitellogenesis and Vg uptake into oocytes. In addition to the species difference in the two studies there were substantially different methods for injecting 20E. In our earlier work we injected small partially fed ticks after removing them from the host. Thompson et al. injected the females while they remained attached to the host. So in this study we repeated our earlier experiments on *A. hebraeum* using on-host injection. We also injected 20E into off-host ticks with or without haemolymph collected from engorged ticks (days 2–10 post-engorgement), or from large partially fed mated ticks in the rapid phase of engorgement, to see whether we might detect a 'vitellogenin uptake factor' (VUF) in haemolymph. Off-host injection of 20E (0.45 μg/g body weight (bw)) did not induce ovary development beyond that of vehicle-injected controls. But ticks in this study, receiving 20E plus haemolymph from engorged ticks, showed a significant increase in ovary weight beyond that of 20E alone (1.31 ± 0.05% bw for 20E plus haemolymph and 1.03 ± 0.05% bw; 25 for 20E alone). However, in normal engorged *A. hebraeum*, the ovary exceeds 7% bw at the onset of oviposition. As in our earlier work, in this study 20E stimulated Vg-synthesis (3.9 ± 0.5 mg Vt-equivalents/ml) beyond that occurring in vehicle-injected ticks (0.76 ± 0.14 mg Vt-equivalents/ml), and there was a further increase in ticks injected with 20E plus haemolymph from engorged ticks (8.9 ± 1.0 mg Vt-equivalents/ml). On-host injection of 20E alone (6 μg 20E/g bw) did not produce a statistically significant increase in oocyte length over that of vehicle-injected controls, whereas on-host injection of 20E plus engorged haemolymph resulted in significantly larger oocytes (261 ± 57 μm) compared to vehicle-injected controls (132 ± 11 μm), compared to 20E alone (131 ± 12 μm), or haemolymph alone (124 ± 24 μm). There was a marked stimulation of Vg-synthesis by 31 μg 20E/g bw (6.0 ± 1.5 mg Vt-equivalents/ml) compared to vehicle-injected controls (1.02 ± 33 mg Vt-equivalents/ml). Vt accumulation by ovaries was significantly greater in ticks treated with haemolymph (12 ± 3 μg Vt/mg ovary) or 20E plus haemolymph (56 ± 26 μg Vt/mg ovary) compared to vehicle-injected controls (5.1 ± 1.5 μg Vt/mg ovary). There was also a significant effect of 6 μg 20E/g bw plus engorged haemolymph on ovary weight (1.74 ± 0.29% bw) compared to vehicle-injected ticks (0.95 ± 0.10% bw), but not compared to ticks injected with 20E alone (1.25 ± 0.19% bw). We conclude that at least some of the differences observed between the two laboratories relate to the species difference, and that there is some evidence that the engorged haemolymph of *A. hebraeum* contains a VUF.

1. Introduction

Although the roles of 20-hydroxyecdysone (20E) and juvenile hormone (JH) have been well characterized for vitellogenesis in insects (Raikhel et al., 2005), we know much less about the

* Corresponding author. Tel.: +1 780 492 1279; fax: +1 780 492 9234.
E-mail address: reuben.kaufman@ualberta.ca (W.R. Kaufman).

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doi:10.1016/j.jinsphys.2008.05.004

hormonal control of vitellogenesis in ticks. Although JH was initially hypothesized to be involved in vitellogenin (Vg)-synthesis (Pound and Oliver, 1979; Connat et al., 1983), more critical studies using gas chromatography/mass spectrometry uncovered no evidence for the occurrence of JH or JH-like molecules in several tick species (Connat, 1987; Neese et al., 2000).

The weight of evidence is now in favor of an ecdysteroid as the vitellogenic hormone in soft ticks (family Argasidae; Ogihara et al., 2007; Horigane et al., 2007). In ixodid ticks, ecdysteroids play a fundamental role in both post-engorgement salivary gland degeneration (Kaufman, 1991; Lomas et al., 1998; Mao and Kaufman, 1998, 1999), and vitellogenesis (Rosell and Coons, 1990; James et al., 1997; Sankhon et al., 1999; Friesen and Kaufman, 2002).

To demonstrate a direct effect of 20E on vitellogenesis, Friesen and Kaufman (2004) used an ELISA to measure haemolymph Vg-concentration of partially fed *Amblyomma hebraeum* following multiple bolus injections of 20E. Although 20E caused a substantial rise in haemolymph Vg-titre, Vg was not taken up by the oocytes. These results suggested that perhaps the oocytes lacked a Vg-receptor at this stage of the feeding cycle. However, Friesen and Kaufman (2004) identified a Vt-binding protein (i.e., a putative Vg-receptor) in the ovaries of small partially fed ticks. Another possibility is that a 'Vg-uptake factor' (VUF), distinct from 20E, may be required for accumulation of yolk in the eggs of *A. hebraeum*.

Thompson et al. (2005) developed a technique to inject virgin female *Dermacentor variabilis* with 20E while the ticks are still attached to the host ('on-host injection'). This technique offers the distinct advantage that tick feeding is not disrupted during hormone injection. Under these conditions, 20E-treated virgin females did not engorge within 4 days, but their oocytes accumulated a substantial amount of Vt, indicating that, in this species, 20E alone is a sufficient signal to trigger both Vg-synthesis and Vg-uptake into the oocytes.

In the present study, we tested the effects of 20E on Vg-uptake in virgin *A. hebraeum* using on-host injection, to determine whether our earlier failure to elicit Vg-uptake by 20E-treatment alone might have been due to the difference in technique (off-host injection vs. on-host injection) rather than the difference in tick species. We also attempted to identify a putative VUF in the haemolymph of engorged females as follows: we injected 20E (to stimulate Vg-synthesis) along with haemolymph from engorged ticks (as a source for a putative VUF), into small, partially fed, off-host ticks.

2. Materials and methods

2.1. Ticks

Our *A. hebraeum* colony was kept in darkness, at 27 °C and >85% humidity. Tick feeding occurred on rabbits as described by Kaufman and Phillips (1973). Depending on the experiment, ticks were allowed to engorge and detach spontaneously, or were forcibly removed from the host below the critical weight (CW) necessary to begin vitellogenesis (Kaufman and Lomas, 1996; Lomas and Kaufman, 1999). In this study, engorged weight ranged from 900 to 3500 mg, and partially fed females (below the CW) ranged from 100 to 220 mg. Ticks were rinsed with water, weighed, and used for off-host injection experiments or stored individually in gauze-covered glass vials until needed for dissection and collection of haemolymph (see Section 2.2.1).

2.2. Experimental protocols

2.2.1. Testing the effects of 20E and haemolymph on partially fed mated off-host ticks

In this experiment, partially fed ticks below the CW were removed from the host and injected with 20E (Sigma), or 20E plus haemolymph, in order to test for a VUF. The vehicle for these off-host experiments was 0.63% ethanol in 1.2% NaCl.

Haemolymph for injection was collected as follows: Ticks were fixed, ventral side down, to disposable Petri dishes with a cyanoacrylate glue, and chilled in a refrigerator for 15 min. The cuticle was slit in various places with a razor blade microscalpel, and the exuding haemolymph was collected in volumetric capillary tubes, immediately diluted 1:2 (v/v) in 1.2% NaCl, frozen on dry ice, and stored at −20 °C until needed for injection. Haemolymph was collected from four stages of the feeding cycle: (1) from non-engorged females forcibly removed from the host during the rapid-phase of feeding (referred to here as ">CW"), (2) from engorged females on days 2 and 3 post-engorgement (samples from these days were pooled together; referred to here as day "2.5"), (3) from engorged females on day 5 post-engorgement and (4) from engorged females on day 10 post-engorgement. 20E was dissolved in 70% ethanol to make a 5 mg/ml solution that was diluted to a working concentration of 45 μg/ml in 1.2% NaCl. One microlitre of the 20E stock solution was combined with 4 or 9 μl of haemolymph, or with the same volume of vehicle, such that the injected concentration of 20E was 0.45 μg/g body weight (bw). This concentration was shown by Friesen and Kaufman (2004) to elicit the maximum vitellogenic response in partially fed females without also leading to marked toxicity. An initial experiment was performed using 4 μl of haemolymph/100 mg bw (total injected volume being 5 μl/100 mg bw), but haemolymph volume was later increased to 9 μl/mg bw (total injected volume being 10 μl/100 mg bw) in an attempt to maximize the dose of a putative VUF. Ultimately, there were no apparent differences between ticks receiving 4 or 9 μl of haemolymph, so the 5 μl/100 mg bw data and 10 μl/100 mg bw data were pooled for statistical analysis. Control ticks were injected with the vehicle. All injected ticks were surface sterilized in 70% ethanol for 1 min, and injected through the camerostomal fold (the articulation between the capitulum and scutum) using a Hamilton® syringe fitted with a 30 g needle. Injections were repeated on days 2 and 5 post-removal. Ticks were kept under colony conditions until their haemolymph and tissues were collected on day 10 (see Section 2.3).

2.2.2. Testing the effect of 20E and haemolymph on partially fed virgin on-host ticks

We injected 20E into virgin females on-host, following the method described by Thompson et al. (2005) as closely as possible. This technique avoids potential complications associated with interrupted feeding. A stock solution of 20E was prepared (12.3 mg 20E/ml of 0.1% DMSO and 0.15% ethanol in 1.2% NaCl). This stock solution was further diluted such that each tick received 0.6, 6, 31 or 154 μg/g bw, based on an estimated tick body weight of ~200 mg at the time of injection; the injected volume was 5 μl per tick. The solutions were prepared such that each tick also received 0.05% DMSO plus 0.075% ethanol in 1.2% NaCl as vehicle. Some ticks were also injected with day 2 engorged haemolymph and some with the 20E-solution mixed 1:4 with day 2 engorged haemolymph.

Injections into the tick haemocoel were performed using a Hamilton® syringe fitted with a 30 g needle. The needle was inserted with care at the posterior midline so as not to puncture the midgut. Five microlitres of solution were injected, and the needle held in place for a further minute to minimize leakage of injected

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solution or haemolymph upon withdrawing the needle. Injected ticks were allowed to feed for 6 days following injection, and then removed from the host for data collection.

2.3. Collection of tissue and haemolymph samples

For analysis of haemolymph and ovaries for indices of vitellogenesis (Vg-concentration in haemolymph and ovary, ovary weight and oocyte size), ticks were immobilized as described in Section 2.2.1 above. A small incision was made in the cuticle and haemolymph was collected in a calibrated glass micropipette. Haemolymph used for the ELISA was diluted 1:4 (v/v) in phosphate-buffered-saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0). Samples were stored at −70 °C until assayed for Vg-concentration by an ELISA (see Section 2.5).

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. Salivary glands were excised and set aside for measuring salivary fluid secretory competence (see Section 2.4). 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 compound 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 2.6.2. The ovaries were then gently blotted, weighed to the nearest 10 μg, rinsed in PBS and stored whole in a micro-centrifuge tube at −20 °C until further analysis for Vt by an ELISA or by a spectrophotometric assay (see Sections 2.5 and 2.6.1).

2.4. Salivary fluid secretory competence

Salivary gland degeneration in female ixodid ticks is triggered by an ecdysteroid hormone (Harris and Kaufman, 1985). To confirm the general efficacy of 20E-injections in this study, we measured salivary fluid secretory competence using the technique of Harris and Kaufman (1984). Briefly, salivary glands were excised from each tick, and the main duct ligated with strands peeled from 8 to 0 Dermalon® silk thread (a gift from Davis and Geck Co., Pearl River, New York). The glands were gently blotted with a small strip of filter paper and the wet weight was measured on a microbalance to the nearest 10 μg. The glands were then incubated for 10 min in TC medium 199 (Gibco; supplemented with 36 mM NaCl, 10 mM MOPS (pH 7.3) and 10 μM dopamine; Sigma), blotted, and re-weighed. As demonstrated by Harris and Kaufman (1984), 10 μM dopamine stimulates a maximum rate of salivary fluid secretion, which in this assay is recorded as an increase in wet weight of the gland because of the ligated salivary duct. A lower gain of wet weight, compared to appropriate controls, is a quantitative measure of salivary gland degeneration in this assay.

2.5. Preparation of Vt for the ELISA

Vt was partially purified from the ovaries of day 10 engorged ticks as previously described by Friesen and Kaufman (2002), with minor modifications. Briefly, an ovary homogenate (113 mg ovary, from 13 females, in 2 ml PBS) was centrifuged and the supernatant passed through a gel filtration column (Superose 6B, Pfizer-Pharmacia, ~74 cm × 1.5 cm) at low pressure, and then pooled fractions of interest (50 ml) were concentrated to 1.5 ml using Centriprep (Amicon) centrifuge tubes and passed through a Sephacryl S-300 column (General Electric Healthcare; ~60 cm × 1.6 cm). Because tick Vt contains a haem moiety (Sonenshine, 1991) the fractions containing large amounts of both haem and protein, as determined by spectrophotometry (400 and 280 nm respectively), were analyzed by immunoblot for the presence of Vt by using antibodies raised against the two Vg proteins, Vg 211 and Vg 148 as described by Friesen and Kaufman (2002). Protein concentration of all samples was measured using the Bradford reagent kit (Sigma). Haemolymph and ovary homogenates were assayed for the presence of Vg or Vt, respectively, using the indirect competitive ELISA described by Friesen and Kaufman (2002).

2.6. Other indices of ovarian development

2.6.1. Spectrophotometric assay

Vt-content in ovaries was also estimated using the spectrophotometric method described by Kaufman et al. (1986) with minor modifications. Briefly, the ovaries were homogenized in PBS and centrifuged at 8000 × g for 10 min. Absorbance of the supernatant was measured at 400 nm (near the peak for the haem moiety of Vt) from which was subtracted the absorbance at 500 nm (non-specific to haem). Corrected absorbance was normalized for weight of ovary in the sample.

2.6.2. Determination of ovarian growth phases in whole mounts

To describe the stages of ovarian development in *A. hebraeum*, females were allowed to mate and feed to engorgement. Once detached, ticks were kept in vials under colony conditions until the desired day of dissection, following which the ovaries were photographed using a Nikon DXM1200 digital camera attached to a dissection microscope.

The classic scoring system developed by Balashov (1972) refers only to individual oocytes. Because oocyte development is asynchronous in ticks once yolk uptake begins, one can find all of Balashov oocyte stages throughout the period of ovarian development. The ovarian growth phase (OGP) system referred to in this study, though clearly based on the Balashov system, includes reference to both the degree of oocyte development and the size of the ovary. The phases described here begin on the day of engorgement (Fig. 1). [OGP 1]: Ovaries are very thin and translucent white in hue. Oocytes are primarily ovoid in shape and <150 μm in length with visible nuclei. In *A. hebraeum* this phase usually corresponds to day 0–2 post-engorgement ticks. [OGP 2]: Ovaries have become significantly longer and thicker. At least some oocytes have grown to about 250 μm in length, are opaque with no visible nuclei, but have not yet taken up a significant amount of yolk. This phase is usually seen between days 2 and 5 post-engorgement in *A. hebraeum*. [OGP 3]: Considerable growth of the ovary has occurred, due primarily to oocyte development, although oocytes at all earlier stages of development are also present. Many oocytes are now as large as 400 μm and are reddish-brown, indicating the presence of yolk granules. This phase begins at approximately day 5 post-engorgement in *A. hebraeum*. [OGP 4]: Pre-ovulation; the ovary is, apart from the midgut, the largest organ in the haemocoel and is completely covered with large, yolk-filled oocytes; distinct yolk spheres are visible. In *A. hebraeum* this phase begins at approximately day 6 or 7 post-engorgement and ends at the onset of oviposition on day 10. [OGP 5: ovulation]: 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 and oviposition may have begun.

2.7. Estimating tick body weight of on-host ticks

In order to measure feeding progress following injections, tick body size was measured in ticks remaining attached to the host as

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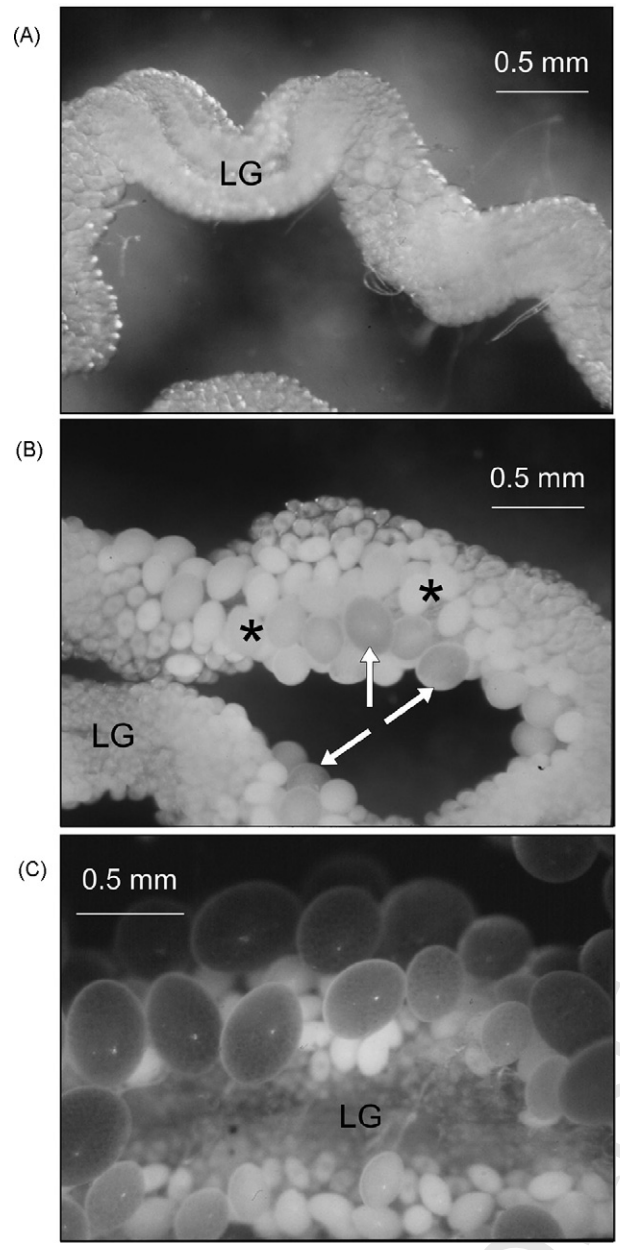

Fig. 1. Ovarian growth phases in *A. hebraeum*. See Section 2.6.2 for the definition of each OGP. Photographs show three of the five stages. (A) OGP 1; oocytes are small with visible nuclei; note the longitudinal groove (LG) which contains the least developed oocytes. (B) Early OGP 3; many oocytes are opaque (asterisks) indicating the period of cytoplasmic growth seen in OGP 2, but some oocytes (arrows) have begun to accumulate Vg, apparent by the darker colour in the figure (characteristic of yolk granules). (C) OGP 4; much of the ovary weight is made up of large, yolk-filled oocytes with distinct yolk spheres, but ovulation has not yet occurred; all earlier Balashov developmental stages of oocytes are still apparent.

follows: using digital electronic calipers, we measured (1) the dorso-ventral thickness of the body and (2) the width of the body at its apparently widest part, approximately at the level of the posterior coxae. All measurements were recorded to the nearest 10 μm. Because ticks tend to crowd very closely together during feeding, it was impractical to record accurate measurements of body length so as to calculate body volume directly. Therefore our measure of tick size was calculated as the product of body width and dorso-ventral thickness ($W \times T$) in mm^2.

Body weight of attached ticks was estimated as follows: Ticks were fed to a wide range of sizes, and their width and dorso-ventral thickness recorded as just described. The ticks were then removed from the host and weighed to the nearest 0.1 mg. A standard curve was drawn plotting $W \times T$ (mm^2) vs. body weight, and body weights of attached ticks (pre-injection) during subsequent experiments were estimated from this standard curve (see Fig. 5A).

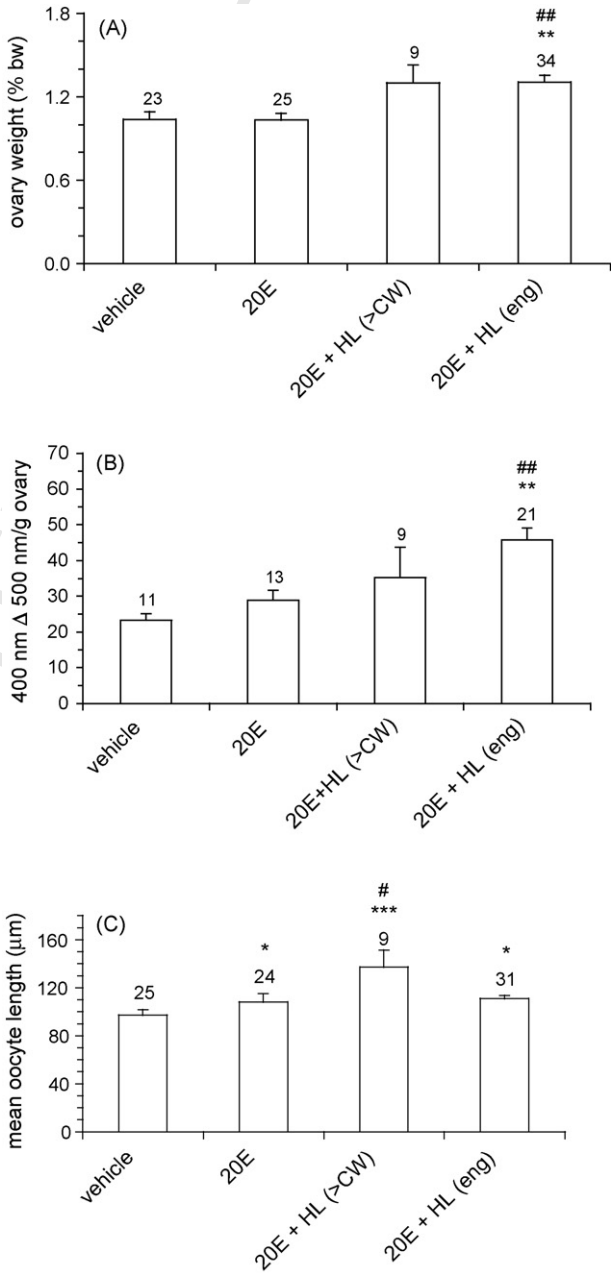

Fig. 2. Effect of 20E and 20E plus haemolymph (abbreviated here and in remaining figures as HL) on off-host partially fed ticks, 10 days post-treatment. (A) ovary weight, (B) Vt-content of ovary as determined by the spectrophotometric assay; the difference in absorbance at 400 and 500 nm, normalized to ovary weight is expressed here as "400 nm Δ 500 nm/g ovary", (C) oocyte length. Females received three injections of 20E alone (0.45 μg/g bw; see Section 2.2.1) or 20E plus haemolymph from the indicated feeding stage (>CW), engorged (days 2.5, 5 and 10 post-engorgement groups pooled). All data in this and subsequent figures are reported as mean ± S.E.M. The number of ticks in each group is indicated above each bar. An asterisk (*) indicates significant difference from the vehicle-injected group; a number sign (#) indicates significant difference from the 20E-injected group. Single symbol represents $0.05 > p > 0.01$; double symbol represents $0.01 > p > 0.001$; triple symbol represents $p < 0.001$.

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2.8. Statistical analysis

Results are reported as mean ± S.E.M. (n). Statistical analysis was done with Stata 10.0 software (StataCorp, College Station, TX, USA). The distribution of the means and variance of the data was tested using the Shapiro–Wilk normality test and Levene's robust equal variance test, respectively. Differences among treatments were then analyzed using a one-way analysis of variance (ANOVA) for parametric data distributions, or the Kruskal–Wallis test for non-parametric data distributions.

3. Results

3.1. Effect of 20E and haemolymph on mated, partially fed off-host ticks

Ticks were removed from the host below the CW, and injected with vehicle, 20E, or 20E plus haemolymph taken from >CW ticks or from ticks at various days post-engorgement (days 2.5, 5, or 10). Because the groups treated with engorged tick haemolymph were not significantly different from each other in any of the measured parameters, the data for engorged haemolymph injected off the host were pooled for statistical analysis.

3.1.1. Effect on ovary weight and oocyte development

Injection of 20E had no significant effect (ANOVA) on ovary weight compared to ticks injected with the vehicle alone ($p > 0.05$; Fig. 2A). The ovaries from females injected with 20E plus haemolymph from engorged ticks weighed significantly more (1.31 ± 0.05% bw; 34) than ticks injected with either vehicle (1.04 ± 0.05; 23; $p = 0.004$) or 20E alone (1.03 ± 0.05; 25; $p = 0.002$). However, the mean ovary weight in ticks injected with 20E plus haemolymph from >CW ticks (1.30 ± 0.13% bw; 9) was not significantly different from those of the vehicle-injected or 20E-injected ticks; this was in spite of the fact that it was virtually identical to that of ticks injected with haemolymph from engorged ticks.

Likewise (Fig. 2B), 20E injections had no statistically significant effect (ANOVA) on ovary Vt-content (28.9 ± 2.8; 400 nm Δ 500 nm/g ovary; 13) when compared with vehicle-injected ticks (23.3 ± 1.8; 400 nm Δ 500 nm/g ovary; 11). But ovaries from ticks injected with 20E plus engorged haemolymph had accumulated significantly more Vt (46.8 ± 3.3; 400 nm Δ 500 nm/g ovary; 21) compared to ovaries from both the vehicle-injected ticks ($p = 0.001$) and 20E-injected ticks ($p = 0.009$). Treatment of ticks with 20E plus >CW haemolymph (35.2 ± 8.5; 400 nm Δ 500 nm/g ovary; 9) had no statistically significant effect on Vt-content relative to vehicle-injected controls or 20E-injected ticks (Fig. 2B).

Injection of 0.45 μg 20E/g bw, or 20E plus haemolymph (either from >CW or engorged ticks) caused a significant increase (Kruskal–Wallis) in oocyte size compared to vehicle-injected controls: (97 ± 4 μm; 25 for controls, 108 ± 7 μm; 24 for 20E, $p = 0.023$, 137 ± 14 μm; 9 for 20E plus >CW haemolymph, $p = 0.00009$, and 111 ± 3 μm; 31 for 20E plus engorged haemolymph, $p = 0.025$; Fig. 2C). In addition, ticks injected with 20E plus >CW haemolymph also had significantly larger oocytes compared to ticks injected with 20E alone (Kruskal–Wallis, $p = 0.012$).

3.1.2. Effect of 20E and haemolymph on Vg-synthesis by small partially fed mated ticks

Vg-concentration was measured in haemolymph collected 5 days following the last bolus injection (i.e., day 10 following tick removal from the host). Haemolymph Vg-concentration increased in partially fed females treated with 20E (3.9 ± 0.5 mg/ml; 21) compared to vehicle-injected ticks (0.76 ± 0.14 mg/ml; 21; Kruskal–Wallis; $p = 0.00007$; Fig. 3). Ticks injected with 20E plus haemolymph also had significantly higher haemolymph Vg-concentrations than the vehicle-injected ticks with values of 4.4 ± 0.7; 8 for 20E plus >CW haemolymph, and 8.9 ± 1.0 mg/ml; 34 for 20E plus engorged haemolymph; $p < 0.0004$. Injection of haemolymph from engorged ticks (but not from >CW ticks) also significantly potentiated the effect of 20E alone ($p = 0.0008$).

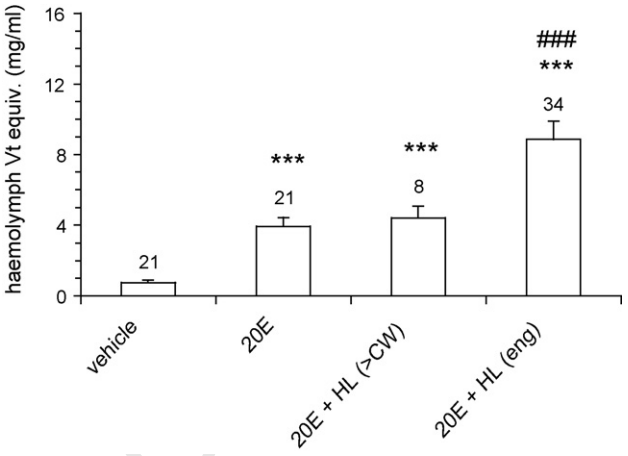

Fig. 3. Effect of 20E and 20E plus haemolymph on haemolymph Vg-concentration (measured as Vt-equivalents by ELISA) in off-host partially fed ticks. 20E alone caused a marked increase in Vg-concentration compared to the vehicle ($p < 0.001$). Although the effect of 20E was not significantly enhanced by >CW haemolymph, injection of engorged tick haemolymph (data of days 2.5, 5 and 10 pooled) resulted in significantly higher Vg-concentrations than did 20E alone.

3.1.3. Effect of 20E and haemolymph on salivary fluid secretion

Salivary fluid secretory competence was greatly reduced (Kruskal–Wallis) in ticks treated with 20E or 20E plus haemolymph compared to vehicle-injected controls (Fig. 4, $p < 0.0004$; 18–51). Salivary glands from vehicle-injected ticks secreted an average of 1.34 ± 0.11 mg/gland/10 min; 43, compared to 0.46 ± 0.08 mg/gland/10 min; 18, for glands from ticks injected with 20E plus >CW haemolymph (the group which elicited the highest fluid secretory rate among all of the 20E-treated groups). The latter group also secreted fluid at a significantly higher rate than both the 20E-alone injected ticks (0.26 ± 0.04, 50, $p = 0.026$) and the 20E plus engorged haemolymph group (0.25 ± 0.03, 51, $p = 0.027$).

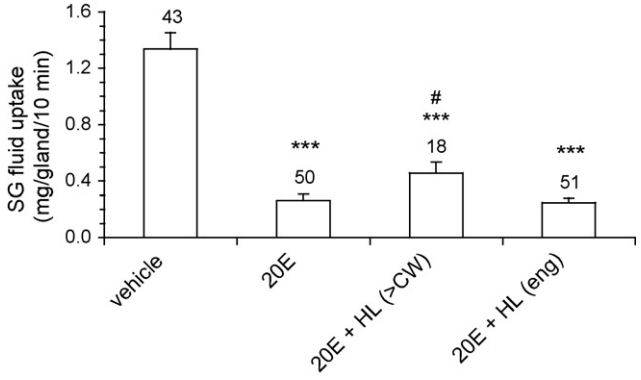

Fig. 4. Effect of 20E and 20E plus haemolymph on salivary gland (SG) fluid uptake by isolated salivary glands from off-host partially fed ticks, 10 days post-treatment. Females received three injections of 20E alone (0.45 μg/g bw) or 20E plus haemolymph from the indicated feeding stage [>CW, engorged (days 2.5, 5 and 10 pooled)]. All treatment groups resulted in a marked inhibition of fluid secretory competence compared to the vehicle-injected controls.

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3.2. Effect of 20E injections on partially fed virgin on-host females

3.2.1. Effects of 20E and 20E plus haemolymph on body size

Partially fed virgin females were injected with several doses of 20E, day 2 engorged haemolymph, or 20E (6 and 31 μg/g bw) plus day 2 engorged haemolymph, while still attached to the host (see Section 2.2.2). A linear relationship was observed between tick bw and $W \times T$ (Fig. 5A). For Fig. 5B, we used the formula derived from Fig. 5A to calculate the approximate pre-injected bw of the ticks. Only the 6 μg 20E/g bw plus day 2 haemolymph and the 31 μg 20E/g bw plus day 2 haemolymph were significantly different from each other (466 ± 64 mg; 5, and 202 ± 50 mg; 8, respectively; ANOVA, $p = 0.038$). An increase in body weight at 6 days post-injection was observed in all treatment groups except those treated with 31 μg/g bw 20E plus day 2 engorged haemolymph, whose bw decreased from 202 ± 32 mg; 8, to 177 ± 50 mg; 8. Only ticks treated with 6 μg/g bw 20E plus day 2 engorged haemolymph (685 ± 115 mg; 5) were significantly heavier than vehicle-injected ticks (303 ± 53 mg; 16, Kruskal–Wallis, $p = 0.0061$). The 6 μg/g bw 20E plus day 2 engorged haemolymph group (685 ± 115 mg; 5) was also significantly heavier than ticks treated with 6 μg/g bw of 20E alone (277 ± 44 mg; 12, Kruskal–Wallis, $p = 0.0064$). The highest dose (154 μg 20E/g bw) was clearly toxic. Four of the eight treated ticks had died within 6 days of injection, one of these having detached spontaneously on the 5th day, the other three remaining attached to the host until removed on the 6th day. One of the ticks detached 1 day after injection and three of them detached 4 days later. The four ticks remaining alive for 6 days were dissected; they had accumulated large amounts of guanine (the nitrogenous waste product in ticks) in the Malpighian tubules and rectal sac, and the hypodermis had fallen away from the overlying cuticle.

3.2.2. Effect of 20E and 20E plus haemolymph on ovary development

Six days after injection of 20E, the ovaries of on-host virgin ticks were not significantly heavier (ANOVA) than the vehicle-injected controls (1.25 ± 0.19% bw; 11, for the 31 μg/g bw dose and 0.95 ± 0.10% of bw; 14, for vehicle-injected ticks; Fig. 6A). Only ticks injected with 6 μg/g bw 20E plus day 2 haemolymph were significantly heavier than the vehicle-injected controls (1.74 ± 0.29% bw; 5; $p = 0.042$; Fig. 6A). However, the ticks treated with the highest dose of 20E (154 μg/g bw) had the smallest ovaries among all the treatment groups (0.80 ± 0.10% bw; 4), which might be a reflection of the toxicity of very high doses of 20E mentioned above.

Injection of 20E did not cause a statistically significant increase in oocyte length compared to vehicle-injected controls (Fig. 6B). However, ticks treated with 6 μg 20E/g bw plus day 2 haemolymph also had significantly larger oocytes (261 ± 57 μm, 5) than vehicle-injected controls (132 ± 11 μm, 15; $p = 0.041$), as well as both 6 μg 20E/g bw (131 ± 12 μm, 13; $p = 0.047$) or haemolymph alone (124 ± 24 μm, 11; $p = 0.035$; Fig. 6B).

Egg development was also scored (Table 1) according to the OGP system described in Section 2.6.2. By 6 days following

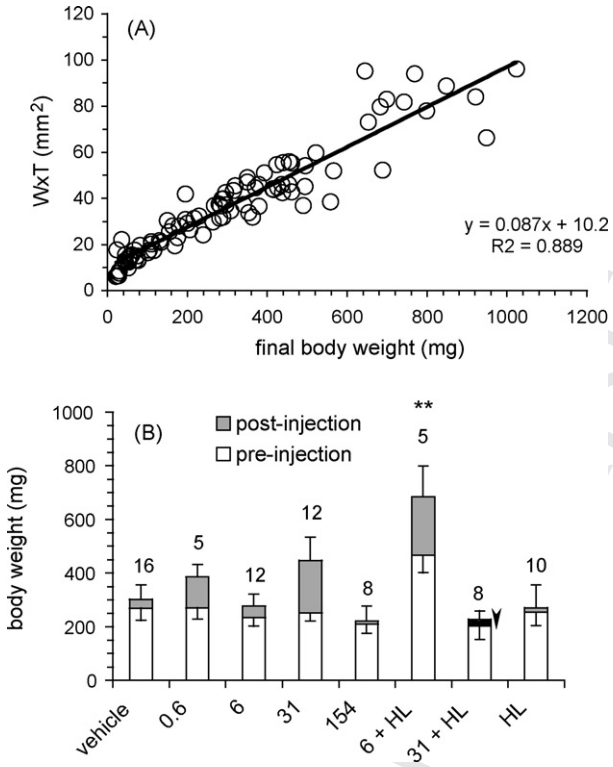

Fig. 5. Effect of 20E and haemolymph on feeding success by virgin females while still attached to the host. Ticks were treated with the indicated doses of 20E (0.6, 6, 31, or 154 μg/g bw) or 20E plus day 2 haemolymph, or vehicle (see Section 2.2.1). As indicated, some ticks were injected with day 2 haemolymph alone (HL). (A) Standard curve generated using measured body weights and product of body width and dorso-ventral thickness ($W \times T$) upon removal of the ticks from the host 6 days post-injection (see Section 2.7). The linear regression formula was calculated by Microsoft Excel software. (B) Pre-injection and post-injection body weights of ticks following treatment. Pre-injection weights in (B) were calculated from the standard curve shown in (A). Post-injection weights were recorded directly on a microbalance. It was not practical to mark individual ticks for this experiment, and thus not possible to rigorously control the pre-injection weights. Hatched bar with arrow (31 + HL) indicates a weight loss from pre-injection weight to the weight recorded at tick removal.

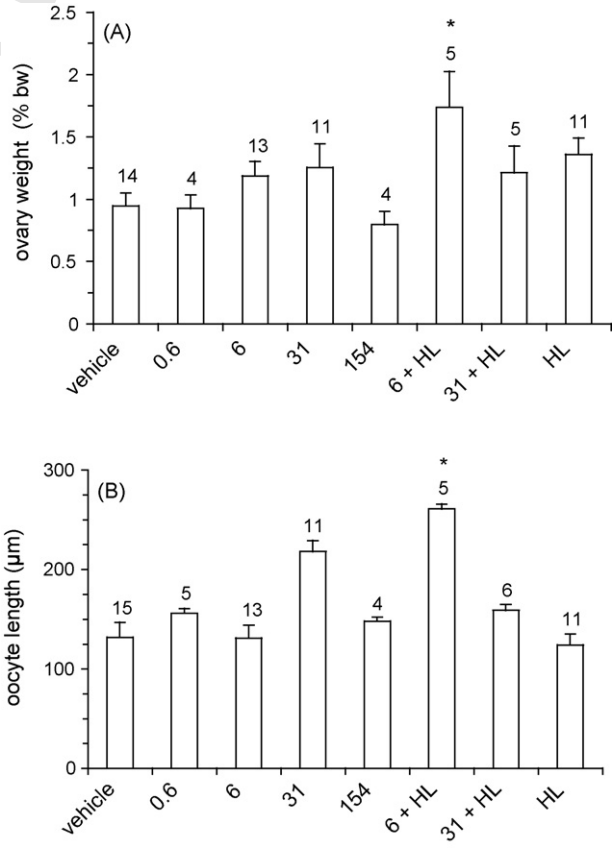

Fig. 6. Effect of 20E alone, 20E plus day 2 haemolymph, or day 2 haemolymph alone on ovarian development in on-host ticks. (A) ovary weight as %body weight, (B) oocyte length. Only ticks injected with 6 μg/g bw plus day 2 haemolymph had a statistically significantly greater ovary weight and oocyte length compared to the vehicle-injected group.

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Table 1
Ovarian development in virgin females injected while on the host with 20E or 20E plus haemolymph (HL) collected from day 2 engorged ticks

Treatment group (n)	Size of tick ($W \times T$; mm^2) ± S.E.M.[c]	Weight (mg) (see Fig. 5A)[c]	Ovarian growth phase[a] (number[b] of ticks and % at each stage)			
			1	2	3	4
Vehicle (15)	38.4 ± 5.8	303 ± 53	10 (67%)	5 (33%)	0	0
Haemolymph (12)	36.5 ± 6.2	271 ± 85	8 (67%)	2 (16.5%)	2 (16.5%)	0
20E (0.6 μg/g bw) (5)	39.3 ± 2.5	386 ± 46	2 (40%)	3(60%)	0	0
20E (6 μg/g bw) (13)	33.6 ± 4.1	277 ± 44	7 (54%)	6 (46%)	0	0
20E (6 μg/g bw + HL) (5)	72.8 ± 9.2	685 ± 115	1 (20%)	1 (20%)	1 (20%)	2 (40%)
20E (31 μg/g bw) (11)	52.1 ± 7.8	447 ± 87	2 (18%)	6 (54%)	3 (27%)	0
20E (31 μg/g bw + HL) (7)	28.4 ± 5.5	177 ± 50	4 (57%)	1 (14%)	2 (28%)	0
20E (154 μg/g bw) (4)	32.6 ± 5.7	220 ± 56	1 (25%)	3 (75%)	0	0

[a] See Section 2.6.2 for definition of phases.
[b] Number of ticks. In each tick, the length of eight of the apparently largest oocytes was measured (see Section 2.3).
[c] Tick weight and size ($W \times T$) were measured after 6 days after injections. See Section 2.7 for details.

treatment, none of the injected ticks had begun ovulation (OGP 5). Across all treatment groups, most tick ovaries remained in OGP 1 or 2, with no apparent uptake of yolk. Only ticks from the 20E plus haemolymph groups, as well as those injected with 31 μg 20E/g bw or haemolymph alone, contained ovaries in OGP 3 (Table 1). The greatest observed growth of the ovaries occurred in the 6 μg 20E/g bw plus haemolymph group; two of the five ticks in this group had ovaries in OGP 4, which were covered in large oocytes containing distinct yolk bodies and were brown in colour.

3.2.3. Effect of 20E and 20E plus haemolymph on haemolymph Vg-concentration

The effect of low doses of 20E on haemolymph Vg-concentration (1.33 ± 0.15 mg/ml; 18, for ticks injected with 0.6 or 6 μg 20E/g bw; data pooled) was just marginally not statistically significant (Kruskal–Wallis) compared to the vehicle-injected control (1.02 ± 0.33 mg/ml; 15, $p = 0.058$, Fig. 7), but there was a marked increase in haemolymph Vg-concentration in those ticks injected with 31 μg/g bw (6.0 ± 1.5 mg/ml; 10 vs. 1.02 ± 0.33 mg/ml; 15; $p = 0.00014$). Although 6 μg 20E/g bw had no significant effect on haemolymph Vg-concentration, the inclusion of haemolymph had a marked synergistic effect (8.5 ± 2.4 mg/ml; 5 for 20E plus haemolymph vs. 1.10 ± 0.12; 13, for 20E alone, $p = 0.00094$; Fig. 7).

3.2.4. Effect of 20E and 20E plus haemolymph on ovary Vg uptake

The Vt-content of the ovaries was measured using both the spectrophotometric assay (Fig. 8A) and the ELISA (Fig. 8B), 6 days after the last injection. As with the off-host injections, 20E did not stimulate Vg-uptake by the oocytes according to the spectrophotometric assay. However, with the ELISA method, Vt-accumulation by ovaries was significantly greater (Kruskal–Wallis) in ticks treated with day 2 haemolymph (11.7 ± 3.0 μg Vt/mg ovary, 11) or 20E plus day 2 haemolymph (55.9 ± 25.8 μg Vt/mg ovary, 5 for 6 μg 20E/g bw and 24.3 ± 7.0 μg Vt/mg ovary, 5 for 31 μg 20E/g bw) compared to vehicle-injected control groups (5.1 ± 1.5 μg Vt/mg ovary, 14; $p = 0.035$). Injection of 6 μg 20E/g bw plus haemolymph caused a significant increase over 6 μg 20E/g bw alone (4.5 ± 1.8 μg Vt/mg ovary, 12; $p = 0.0019$). The very high SEM in the ticks injected with 6 μg 20E plus haemolymph (Fig. 8B) arose because two of the five ticks in this group had ovaries with large

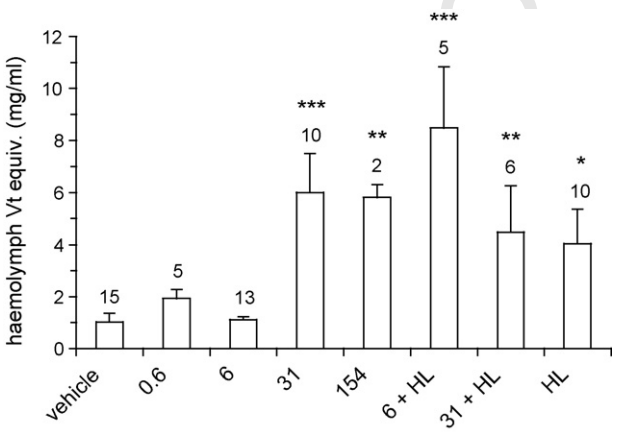

Fig. 7. Haemolymph Vg-concentration (measured as Vt-equivalents by ELISA) of on-host partially fed virgin females, 6 days after the indicated treatment. Haemolymph alone and 20E at 31 μg/g body weight with or without haemolymph caused a marked stimulation of Vg-synthesis.

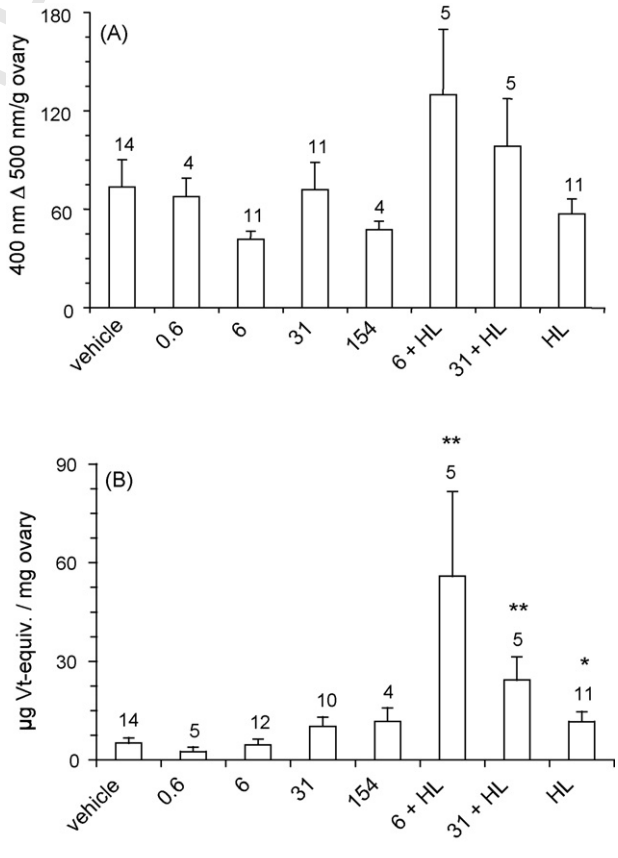

Fig. 8. Vt-content of the ovaries of on-host partially fed virgin females, 6 days after treated as indicated. Vt-content was measured by (A) the spectrophotometric assay (the difference in absorbance at 400 and 500 nm, normalized to ovary weight is expressed here as "400 nm Δ 500 nm/g ovary"), and (B) the ELISA. Although 20E alone did not significantly stimulate yolk uptake, haemolymph with or without 20E did so when measured by the ELISA.

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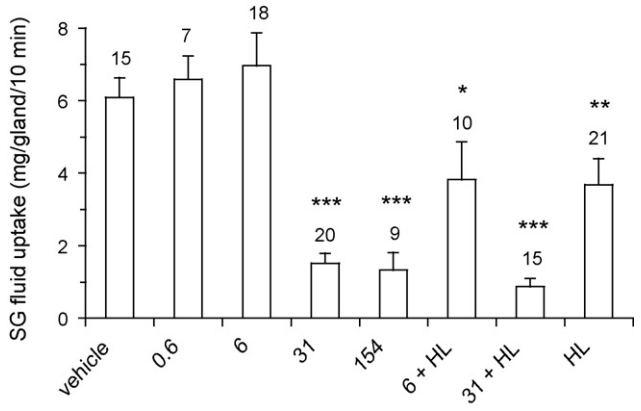

Fig. 9. Fluid secretory competence of isolated salivary glands (SGs) from on-host partially fed virgin females, 6 days after the indicated treatment. Although low concentrations of 20E (0.6 and 6 μg 20E/g bw) did not significantly reduce fluid uptake, the higher doses did with or without haemolymph.

brown oocytes with yolk spheres (Table 1). These ticks had ovary Vt-contents of 112 and 123 μg Vt/mg ovary-considerably greater than those seen in any of the other groups.

3.2.5. Effect of 20E and 20E plus haemolymph on salivary fluid secretory competence

Salivary fluid uptake was measured 6 days following injection (Fig. 9). Salivary glands from ticks treated with 31 and 154 μg/g bw (1.52 ± 0.28 mg/gland/10 min; 20, and 1.32 ± 0.49 mg/gland/10 min; 9, respectively) took up markedly less fluid (Kruskal–Wallis) than vehicle-injected controls (6.09 ± 0.53; 15, $p = 0.000053$). Although salivary fluid uptake in ticks injected with 0.6 and 6 μg 20E/g bw (6.59 ± 0.66 mg/gland/10 min; 7, and 6.96 ± 0.90 mg/gland/10 min; 18, respectively) was similar to that of the controls, co-injection with day 2 haemolymph significantly reduced salivary fluid uptake to 3.83 ± 1.04 mg/gland/10 min; 10, $p = 0.047$). There was no statistically significant synergistic effect of haemolymph on ticks injected with 31 μg 20E/g bw compared to ticks injected with 31 μg 20E alone (0.87 ± 0.22 mg/gland/10 min; 15, vs. 1.52 ± 0.28 mg/gland/10 min; 20, $p = 0.153$). Injections of haemolymph alone also caused a statistically significant reduction in salivary fluid uptake (3.68 ± 0.72 mg/gland/10 min; 21) relative to vehicle-injected controls (6.09 ± 0.53; 15, $p = 0.035$).

4. Discussion

In this study we attempted to reconcile the difference between the results of our earlier work and those of Thompson et al. (2005). Previously we determined, in off-host *A. hebraeum*, that exogenous 20E stimulated Vg-synthesis, but not oocyte development (Lunke and Kaufman, 1993; Friesen and Kaufman, 2002, 2004). Since then, Thompson et al. (2005) demonstrated in on-host virgin *D. variabilis*, that exogenous 20E stimulates both Vg-synthesis and yolk uptake. Although here we used the method of Thompson et al. in *A. hebraeum*, we were unable to induce a marked degree of yolk-uptake, even with 20E-doses five times greater than that tested by them (Figs. 6 and 7). However, we demonstrated the efficacy of our technique inasmuch as 20E-injections did stimulate salivary gland degeneration (Fig. 9) and Vg-synthesis relative to vehicle-injected ticks (Fig. 7) as demonstrated previously in off-host ticks.

The evidence presented here for a haemolymph-borne VUF remains tentative. Ovary weight, oocyte size and Vt-content of ovary in off-host ticks were all significantly augmented by injection of 20E plus engorged haemolymph (Fig. 2). Likewise, oocyte growth was significantly stimulated by 20E plus >CW haemolymph (Fig. 2C), although data for the other two parameters (ovary weight and Vg-uptake) did not quite reach statistical significance. Notwithstanding these somewhat encouraging results, it must be noted that the magnitudes of the increases shown here were small relative to the results of Thompson et al. (2005), and small relative to the increases normally observed in engorged ticks at OGP 5. For example, the maximum mean haemolymph Vg-titre we observed here in off-host ticks was about 9 mg/ml in ticks treated with 20E plus engorged haemolymph (Fig. 3) and 8.5 mg/ml in on-host ticks (Fig. 7). In comparison, Vg-titres in normal engorged *A. hebraeum* peak at about 40 mg/ml at the time of oviposition (Friesen and Kaufman, 2002). Similarly, the maximum mean ovary weight observed here for treated ticks was 1.3% bw for off-host ticks (Fig. 2A) and 1.7% bw for on-host ticks (Fig. 6), compared to about 7% bw in normal engorged ticks around the time of oviposition (Friesen and Kaufman, 2002). Finally, the degree of oocyte growth stimulated by 20E plus >CW haemolymph was also much less (97 μm in off-host ticks and 132 μm in on-host ticks) than that occurring normally in ovaries at OGP 5 (mean of 425 μm; Friesen and Kaufman, 2003).

Engorged haemolymph markedly increased the effect of 20E alone in stimulating Vg-synthesis in off-host ticks (Fig. 3). This effect cannot be explained by the endogenous ecdysteroid content of day 2 engorged haemolymph. Friesen and Kaufman (2002) reported the haemolymph ecdysteroid concentration to be about 50–60 ng/ml on day 2 post-engorgement. Consequently, injecting 10 μl of such haemolymph into a tick would have added only about 5 ng 20E on top of the injected load of 450 ng/g bw.

At least one possibility to explain the rather small effect of injected haemolymph in this study is that bolus injection of haemolymph could result in any putative VUF being diluted to only a near-threshold concentration. Haemolymph volume in partially fed *A. hebraeum* constitutes about 25% bw (Kaufman et al., 1980). The partially fed females used here for off-host injections (100–220 mg) are thus estimated to have haemolymph volumes in the range of 25–55 μl. Injection of 4 or 9 μl of haemolymph into such ticks would result in a dilution of between 3.8- and 14.8-fold. We tried to mitigate this, and the possibility that VUF is labile, by applying three bolus injections in the off-host experiments, but this was not considered for the on-host experiments because of significant damage to the cuticle that would result.

Salivary glands from the vehicle-injected off-host ticks accumulated only 1.34 ± 0.11 mg/gland/10 min (Fig. 4), whereas those from vehicle-injected on-host ticks accumulated 6.09 ± 0.53 mg/gland/10 min (Fig. 9). The reason for this discrepancy is that fluid secretory competence was measured in the off-host ticks 10 days following removal from the host (see Section 2.2.1). Salivary glands from small partially fed ticks lose about 75% of their fluid secretory competence when removed and kept off the host for 4 days or more (Kaufman, 1983; Harris and Kaufman, 1984). In contrast, fluid secretory competence of salivary glands from the on-host ticks was measured on the day of removal from the host (see Section 2.2.2).

In conclusion, there seems to be a real difference between *A. hebraeum* and *D. variabilis* with respect to how they control yolk uptake. In the latter, injection of 20E alone appears to be sufficient to stimulate full egg development, whereas in the former, some factor in addition to 20E is required for this process. Our data here suggest that this putative VUF should be detectable in the haemolymph of engorged ticks, although we have not yet determined optimal experimental conditions for establishing this.

Uncited references

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Chinzei et al. (1992), Chinzei and Yano (1985), Connat et al. (1985) and Kaufman (1989).

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Acknowledgements

Research in this laboratory is generously funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada. The visit of A.S. to the University of Alberta was generously supported by a grant from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (*CNPq*) of Brazil. We are grateful to Prof. Carlos Termignoni, Centro de Biotecnologia and Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Brazil, for general advice and for reviewing the manuscript. We also thank Dr. M. Belosevic, University of Alberta, for allowing us the use of his microtitre plate reader.

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