Haemolymph from female beetles infected with *Hymenolepis diminuta* metacestodes retards the development of ovarian follicles in recipient *Tenebrio molitor* (Coleoptera)

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SUMMARY

Infection with developing metacestodes of the rat tapeworm, *Hymenolepis diminuta*, is known to retard the accumulation of the yolk protein, vitellin, in the terminal ovarian follicles of the intermediate host, *Tenebrio molitor*. It is probable that this is the result of competitive inhibition of juvenile hormone binding at a microsomal binding site in the beetle follicular epithelium. Experiments were designed to test the hypothesis that inhibitor molecules were circulating in the haemolymph of infected beetles. Whole haemolymph, collected from male or female beetles at various stages post-infection, was injected into non-infected female recipients 2 days post-emergence. Ovaries were removed 3 days later and the vitellin content of the same sized follicles measured using an ELISA. The vitellin content of follicles from recipients of haemolymph from females infected with metacestodes at stage 1 and stage 3–4 was significantly reduced (24 and 27·9%) compared to sham-infected females. However, haemolymph from females infected with mature metacestodes did not affect the vitellin content. Results were thus comparable to those obtained by monitoring ovarian vitellin levels in female *T. molitor* with *bona fide* infections. Haemolymph from infected males did not affect ovarian vitellin content. These results indicate that molecules that can modulate vitellogenesis may be present in the haemolymph of females infected with developing metacestodes but that these factors disappear later in infection.

Key words: *Hymenolepis diminuta*, *Tenebrio molitor*, fecundity reduction, vitellogenesis, host–parasite interaction, juvenile hormone.

INTRODUCTION

Examination of a variety of parasitized insects has revealed down-regulation of female fecundity/fertility to be a common occurrence (Hurd, 1993; Hurd, Hogg & Renshaw, 1995). Lack of gonadal damage, coupled with rejection of a simple 'nutrient robbery' hypothesis (Hurd & Webb, 1997), have led to speculation of effects of a more complex nature. Putative modulatory factors may originate from the host, for instance, as the result of the mobilization of an immune response (De Jong-Brink, 1995). Alternatively, they may come directly from the parasite. We are using a tapeworm metacestode/beetle association, *Hymenolepis diminuta*/*Tenebrio molitor* to investigate mechanisms underlying fecundity regulation.

The acrotrophic ovarioles in *T. molitor* develop asynchronously. The oocyte matures in the terminal follicle, with yolk protein, vitellin, being transported through the haemolymph from the insect fat body, the site of synthesis of the precursor, vitellogenin. Follicles of similar size normally contain the same amounts of vitellin (Hurd & Arme, 1987). However, in infected females, the vitellin content of these follicles is significantly depleted. On day 3 post-infection, *T. molitor* follicles of 400–600 μm show a decrease of 50·2% in vitellin content, as compared to comparable follicles from non-infected females (Webb & Hurd, 1995*b*). Ovarian sequestration of vitellogenin is partly blocked due to a retardation in the development of patency of the follicular epithelial cells (Hurd & Arme, 1987) which, in turn, may be due to an inhibition of juvenile hormone III (JH-III) binding at microsomal binding sites of infected *T. molitor* follicle cells (Webb & Hurd, 1995*a*).

By injecting haemolymph from infected beetles into non-infected recipients and then monitoring vitellogenesis, we sought to demonstrate the presence of a circulating modulator factor. Vitellin content of the terminal follicles of ovaries of recipient females was used to assess the effect of haemolymph donated from infected insects. Decreased accumulation of vitellin, in parallel with beetles harbouring *H. diminuta* metacestodes, was demonstrated in experimental insects (Webb & Hurd, 1995*b*).

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MATERIALS AND METHODS

Maintenance of host and parasite

Hymenolepis diminuta (Arme strain) was routinely maintained in male Wistar rats, and tapeworm eggs recovered from faeces on a weekly basis by salt flotation in saturated NaCl (Hurd & Arme, 1984). *Tenebrio molitor* (Rice University Strain), was maintained at 26 °C (light/dark: 12 h/12 h, humidity 60–70 %). Pupae were sexed (Bhattacharya, Ameel & Waldbauer, 1970), and newly emerged adults placed in Petri dishes in ratios 10:3 females:males. This ratio ensured mating of all females. Food was withheld for 48 h post-eclosion, at which time beetles were fed parasite eggs in apple pulp (1 g w/w), or (controls) apple pulp alone for 24 h. Following infection, insects were fed on wheat bran only (*ad libitum*).

Production of polyclonal antibodies

Monoclonal antibodies have previously been raised against *Tenebrio* vitellin for use in immunoassays (Webb & Hurd, 1995*b*). However, these have proved to be unstable in storage and polyclonal antibodies were deemed preferable for this work.

Collection and fractionation of antigen. *Tenebrio molitor* vitellin was obtained by the method of Webb & Hurd (1995*b*) which, in brief, consists of homogenizing 50 freshly excised *T. molitor* eggs over ice. Following centrifugation at 3500 ***g*** (10 min, 4 °C), the supernatant fraction was removed. The pellet was then resuspended and the process repeated once.

Approximately 1 ml of ovarian extraction buffer (50 mM Tris–HCl, 0·5 M NaCl, 0·02 % NaN_3; with protease inhibitors, 1 mM phenylmethylsulphonyl fluoride, 5 mM ϵ-aminocaprionic acid, 1 mM benzamidine, 10 μg/ml aprotinin and 2 μg/ml each of antipain, leupeptin, pepstatin and chymostatin, pH 8·4), was added to the pellet, which was then disrupted by ultra-sound sonication for 1 min. After settling, the suspension was further centrifuged at 16500 ***g*** (10 min, 4 °C), and the supernatant collected. The sample was dialysed against 100 volumes of buffer A (20 mM Tris–HCl, pH 7·5) for 18 h at 4 °C. The dialysate was applied to a DEAE–Sepharose CL-6B column, previously equilibrated with buffer A at 20 °C. Protein was eluted using a linear salt gradient generated from 50 ml of buffer A and 50 ml of buffer A with the addition of 0·5 M NaCl. Fractions of 4 ml were collected.

Protein was monitored at 280 nm to establish the protein peak, before quantifying using the Coomassie Blue binding method (BioRad) with BSA as standard. The major protein peak, eluting between 0·1 and 0·16 M NaCl, was subjected to SDS–PAGE analysis, which revealed only the four major subunits corresponding to those expected for *Tenebrio* vitellin (Webb & Hurd, 1995*b*). Aliquots of the immunogen were flash-frozen in liquid N_2 with a full complement of protease inhibitors, and stored at −80 °C.

Antibody production and purification. On day 1 mice (male, balb/c), were injected intraperitoneally with 100 μg antigen in 100 μl of phosphate-buffered saline (PBS) which was sonicated with Freund's Complete Adjuvant (1:1) for 30 sec. This was repeated on day 14 and on day 28 using Incomplete Adjuvant, and tail test-bleeds were taken. On day 38 blood was removed by cardiac puncture and the serum purified using an Econo-Pac A kit.

The specificity of the polyclonal antibodies was checked using tissues from non-infected 3-day-old female *Tenebrio molitor*. Purified vitellin and homogenates of whole ovary, fat body and muscle were boiled with equal volumes of denaturing buffer and loaded onto wells of a 7·5 % minigel prior to SDS–PAGE. The total protein content of samples was as follows: ovary, 12 μg; fat body, 8 μg; muscle, 25 μg; and purified vitellin, 25 μg per well. Broad range molecular weight markers 5 μg (BioRad) were run as a reference. Immediately following electrophoresis, Western blotting (Webb & Hurd, 1995*b*) was performed using polyclonal antibody at 1:100 dilution or non-immune serum at 1:100 dilution.

Collection and injection of haemolymph

Both female and male infected beetles were used to provide whole haemolymph for immediate injection into non-infected females. For injections, needles were made by pulling out 25 μl capillary tubes over a Bunsen flame. Calibration was by uptake of 4 μl of distilled water, previously expelled from a 10 μl Hamilton syringe onto Parafilm.

Four μl of haemolymph from non-infected or infected beetles were collected from the wound produced by removal of a leg at the level of the coxa. This was immediately injected into starved 2-day-old females via the dorsal abdominal surface, posterior to the prothorax. Infected donors were dissected to check for the presence and stage of infection. All beetles used as infected donors had more than 10 cysticercoids of the correct stage. Later dissection of recipients confirmed that no parasites had been transferred with haemolymph from infected insects. An additional set of control beetles were sham injected by piercing the dorsal abdominal surface with an empty microcapillary needle to control for the possible effects of wounding.

Beetles serving as haemolymph donors were infected by starving for 48 h prior to exposure to *H. diminuta* eggs mixed with apple for 24 h. Control donors were also starved and then exposed to apple alone. Haemolymph was taken at one of 3 time-points (1 sample/beetle), to ensure the presence of distinct developmental stages of cysticercoids in the

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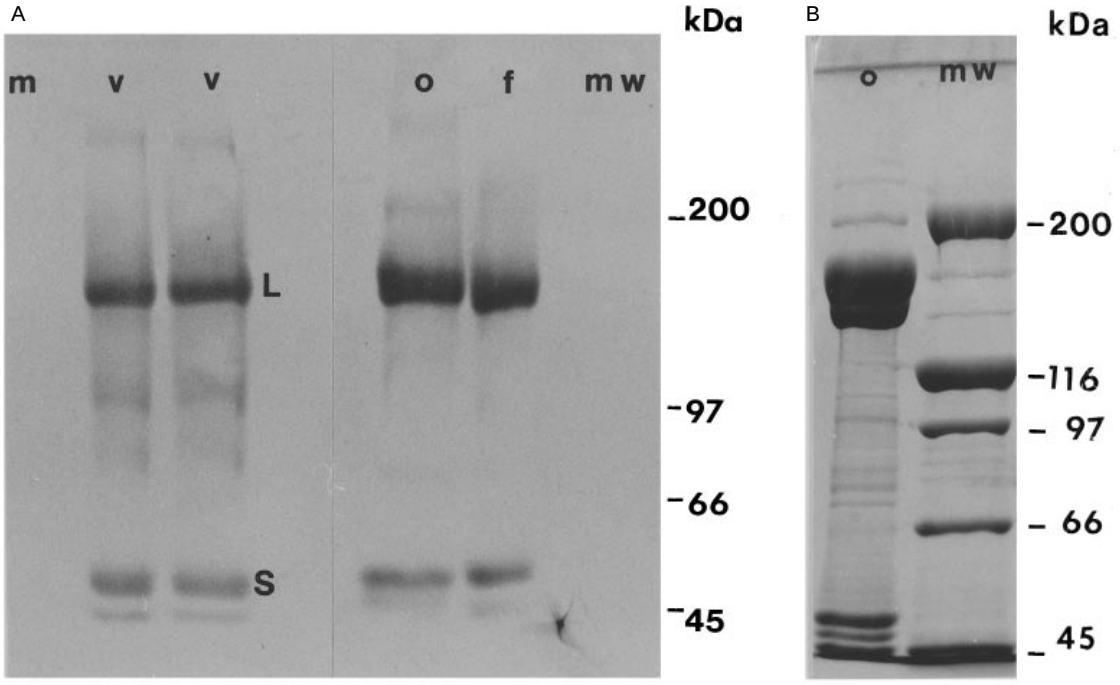

Fig. 1. (A) Western blots of *Tenebrio molitor* tissues reacted with polyclonal antibodies raised against *T. molitor* vitellin. *Tenebrio* ovary, 12 μg/lane (o); *Tenebrio* fat body, 8 μg/lane (f); *Tenebrio* muscle, 25 μg (m); *Tenebrio* vitellin 10 μg (v); molecular weight markers, broad range, 5 μl (mw). (B) Coomassie blue-stained 7·5 % SDS–polyacrylamide gel containing *Tenebrio* ovary 12 μg (o) and molecular weight markers, broad range 5 μl (mw). Vitellin large subunits (154 and 145 kDa), not fully resolved (L), vitellin small subunits (56 kDa and 45 kDa) (S) (Webb & Hurd, 1995*b*). Position of molecular weight markers is indicated.

haemocoel. These time-points were as follows. (1) Female and male donor beetles on day 2 post-infection, cysticercoids at stage 1 (Hurd & Burns, 1994). Both infected and control insects were 5 days post-eclosion. (2) Female donor beetles on day 6 post-infection, cysticercoids at stage 3 to early stage 4. Infected and control insects were 9 days post-eclosion. (3) Female and male donor beetles on day 12 post-infection. Mature cysticercoids (infective to rats). Infected and control insects were 15 days post-eclosion.

Recipient females were all starved for 48 h post-adult eclosion and injected on day 2. In all cases, the effect of haemolymph injections into non-infected female recipients was monitored on day 3 post-injection by quantitative assessment of the amount of incorporated ovarian vitellin per oocyte. Insects were maintained at 26 °C and fed a diet of wheat bran *ad libitum* until dissection and removal of follicles over ice. Six control and 6 infected beetles were examined for each treatment. A total of 36 follicles was assayed from each group of 6 beetles.

Development of an ELISA

Polyclonal antibodies (as described above) were used to monitor ovarian vitellin content in *T. molitor*. Freshly excised follicles measuring between 400 and 600 μm from beetles on day 3 post-injection were placed individually in wells of tissue culture plates over ice. Then 100 μl of *Tenebrio* saline (76 mM NaCl, 36 mM KCl), containing a cocktail of protease inhibitors (as before), was added to each well and the whole mixture was sonicated for 10 sec (Vibra Cell, Sonics and Materials, Connecticut, USA).

The vitellin content of 0·4 μl aliquots of each follicle solution was determined by ELISA (Harlow & Lane, 1988), using polyclonal antibodies diluted 1:100 with PBS (400 μg protein/100 μl). Vitellin, from the same source as that used as the immunogen, was employed in the range 5–100 ng/well for the production of standard curves for protein quantification.

Statistical analysis

Vitellin titres are expressed as arithmetic means $\pm$S.E.M. Values were compared by ANOVA using Minitab statistical packages.

RESULTS

Polyclonal antibodies

Resolution of the 2 large vitellin subunits (154 and 145 kDa) was not always achieved with the minigel system; however, comparison of Western blots with the original gel (not shown) indicated that the polyclonal antibodies raised against *T. molitor* vitellin cross-reacted with both of the major large

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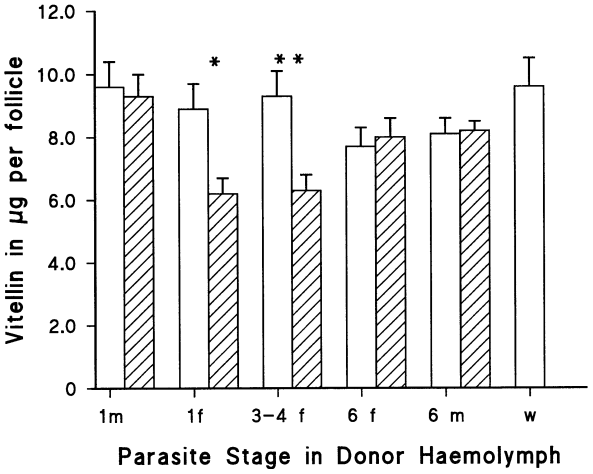

Fig. 2. Vitellin content of follicles (400–600 μm), taken from beetles on day 5 post-emergence, assessed by ELISA. Recipients of 4 μl haemolymph from non-infected beetles (□) and from infected beetles (▨). Recipients of haemolymph from male beetles infected with stage 1 metacestodes (1m) and mature metacestodes (6m), female beetles with stage 1 metacestodes (1f), stage 3–4 metacestodes (3–4f) and mature metacestodes (6f), and females wounded but not injected (w). Mean values ± S.E.M., $n = 36$ follicles in each case. * Shows significant difference from control at $P = 0{\cdot}005$ and ** at $P = 0{\cdot}0038$.

subunits and the 2 small subunits of purified vitellin (56 and 45 kDa) and also with these subunits of ovarian vitellin and vitellogenin in the fat body. No cross-reactivity with other components of these tissues or with muscle tissue was observed (see Fig. 1). No response was observed with pre-immune serum. The antibodies were thus suitable for using to quantify vitellin in follicles.

Haemolymph injections

The vitellin content of follicles of 400–600 μm from non-injected beetles 5 days post-emergence that had been wounded to simulate injection ($9{\cdot}5 \pm 0{\cdot}7$ μg/follicle) did not differ from follicles taken from females of the same age that had been injected with haemolymph from non-infected beetles (see Fig. 2). Therefore, donor haemolymph did not normally affect follicle development.

Females receiving haemolymph from female beetles infected with stage 1 or stage 3–4 metacestodes contained significantly reduced amounts of vitellin (24 %, stage 1; 27·9 %, stage 3–4) (see Fig. 2). However, haemolymph from females with mature metacestodes and from all male beetles had no significant effect on vitellin content.

DISCUSSION

Down-regulation of the sequestration of the yolk protein, vitellogenin, by ovarian follicles of *T. molitor*, has previously been reported to be induced by *H. diminuta* metacestodes (Hurd & Arme, 1986*a*, *b*; Webb & Hurd, 1995*b*). Our work shows, for the first time, that transference of haemolymph from infected female beetles into non-infected recipients can simulate the effect of infection on egg production.

The fact that the decrease in follicle vitellin is not so great as that found *in vivo* (24 and 27·9 % in our work as against 50·2 % in *bona fide* infections), as reported by Webb & Hurd (1995*b*), suggests that 4 μl is not the optimal dose for donor haemolymph. However, more than 4 μl of haemolymph is difficult to obtain from 1 beetle and it was considered desirable not to use pooled samples.

Recent work (Webb & Hurd, 1995*a*) has indicated that a modulatory factor may exist in infected beetles, which acts as a JH-III microsomal binding protein inhibitor in the follicle cells. Diminished JH-III binding to follicular epithelium cells may cause the delay in patency reported by Hurd & Arme (1987), thus delaying the passage of vitellogenin into the developing oocyte. In turn, this may be part of the mechanism underlying the delay in egg production, resorption of developing follicles and reduction in egg viability which results in fertility reduction in *H. diminuta* infected *T. molitor* (see reviews by Hurd, 1993 and Hurd & Webb, 1997).

If, as our work suggests, a modulatory factor(s) has indeed been transferred from 1 insect to another by haemolymph injection, 2 questions now arise. Firstly, what is the source of this modulatory factor(s)? Does it come directly from the parasite or is it induced in the host as part of a response mechanism to the presence of the parasite? Secondly, is its mode of action direct, i.e. does it act as a JH-III binding protein site antagonist, or does it induce the production of unknown host antagonists?

Trematodes of the genus *Schistosoma* are known to reduce or prevent reproduction in their snail hosts. *Trichobilharzia ocellata* infections of *Lymnaea stagnalis* have been used as a model to investigate the mechanisms underlying fecundity reduction. In this association, it has been demonstrated that a host-derived peptide, schistosomin, present in the haemolymph of infected snails, competitively binds to hormone-receptor site and thus interferes with the action of gonadotrophic hormones. It is now thought that schistosomin is released by cells of the internal defence system. It is likely that its production is induced in stress situations (reviewed by De Jong-Brink, 1995).

It is too early in our investigations to be able to draw parallels with this trematode/snail system and these findings appear to provide evidence in favour of a host-derived factor(s) which modulates vitellogenin uptake. In these experiments, haemolymph from male infected beetles did not induce a reduction in follicle vitellin content even though parasite densities in donor males were within the same range

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as females. This suggests that the putative modulatory factor is only produced by the female hosts and does not come directly from the parasite.

In contrast, using an *in vitro* fat body incubation system, Webb & Hurd (1996) have demonstrated a significant, dose-dependent inhibition of the synthesis of the yolk protein precursor, vitellogenin, when 2 or more developing metacestodes of *H. diminuta* were added to the medium. A parasite-product is likely to be involved in this inhibition although it is not yet apparent whether it acts directly on the synthetic pathway or via mediators produced by fat body cells. It is conceivable that parasite-induced down-regulation of vitellogenin synthesis and ovarian sequestration may be controlled by different mechanisms. The question of host or parasite as instigator of coleopteran fecundity reduction thus remains open.

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