Direct manipulation of insect reproduction by agents of parasite origin

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Reproductive output of female *Tenebrio molitor* beetles is reduced upon infection with metacestodes of the rat tapeworm, *Hymenolepis diminuta*. We are using this as a model to investigate the adaptive significance of parasite-induced curtailment of insect reproduction. Production of the yolk protein vitellogenin (Vg) in the insect fat body is significantly reduced both *in vitro* and *in vivo* by metacestodes. Synthesis can be measured by using [^{14}C]L-leucine incorporation, followed by immunoprecipitation. In this paper we demonstrate that a significant decrease in [^{14}C]Vg can be produced by an acetic acid extract of the parasite. Conclusive evidence is presented that the active component(s) originate from the metacestodes: an extract of parasites grown entirely axenically has similar deleterious effects. The developmental stage of the metacestode is important: immature (stage I–II) parasites had greater capacity to suppress Vg synthesis than mature ones (stage V–VI). Examination of the chemical nature of the effector molecule(s) revealed that acetic-acid-extractable, boiling-resistant, pronase-sensitive agents in the molecular mass range 10–50 kDa reduced Vg synthesis by 47.4%. These data suggest that metacestodes produce a modulator molecule that directly affects insect vitellogenesis and, therefore, that reduction of host fitness may confer a selective advantage upon the parasite.

Keywords: fat body; *Hymenolepis*; *Tenebrio*; vitellogenin synthesis

1. INTRODUCTION

A great many parasites and pathogens of insects undermine their hosts' reproductive output (Hurd & Webb 1997; Hurd 1990, 1998). Mechanical destruction of the gonads is rare; more usually morphological, physiological or behavioural changes in the host result in curtailment of egg production. The fact that this response to infection occurs in such widespread and diverse associations is suggestive of an adaptive strategy on the part of the parasite. A diversion of host resources away from egg production may provide a richer environment for parasite development and could even lead to increased longevity for the symbiotic association. However, downregulating reproduction may be a host response to alleviate the nutritional stress imposed by infection (Hurd 1990) or simply a by-product of other aspects of pathology generated by the infection (Dawkins 1990; Poulin 1994; Hurd & Webb 1997). In the majority of these associations, it is difficult to ascertain whether the parasite gains a selective advantage from a decrease in insect reproductive fitness, because situations in which the same parasite infects the same host species, but does not affect reproduction, are not available for comparison. We propose that an alternative approach is to gain an understanding of the mechanism underlying parasite-

induced fecundity reduction. If a parasite-derived modulator molecule can be identified, it is likely that the reduction in host fitness occurs directly, and is not a secondary effect resulting from other pathology or a host response (Hurd 1998). We are using a tapeworm–beetle model to investigate this issue.

Mealworm beetles, *Tenebrio molitor*, infected with intermediate stages of the rat tapeworm, *Hymenolepis diminuta*, display conspicuous changes in the quality and quantity of both developing oocytes (fecundity) and laid eggs (fertility) (Hurd & Arme 1986a). Egg production is retarded on two levels. In the fat body, synthesis of the yolk protein vitellogenin (Vg) is reduced (Hurd & Arme 1986b; Webb & Hurd 1996) and in the ovary, uptake of Vg is hindered (Hurd & Arme 1987a; Webb *et al.* 1997); eggs are therefore protein deficient and lack viability (Hurd & Arme 1986a,b).

Although ovarian performance appears to be down-regulated by host-derived factors (Major *et al.* 1997), alterations in the fat body seem to be directed by products of parasite origin. When fat-body tissue from non-infected beetles is cultured *in vitro* with isolated metacestodes, a marked decrease in Vg synthesis is noted (Webb & Hurd 1996). Previous experiments strongly suggest that nutrient robbery could not induce this sequence of events (Webb & Hurd 1996); rather, specific effector molecules probably elicit changes in the host.

Here, we extend the hypothesis that parasite products reduce fecundity and assess the response of fat body from non-infected insects to such modulatory molecules *in vitro*. An examination of the chemical nature of the active agent(s) is also initiated.

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2. MATERIAL AND METHODS

(a) *Host and parasite*

Adult *Tenebrio molitor* were maintained and infected with *Hymenolepis diminuta* eggs as described elsewhere (Hurd & Arme 1984; Major *et al.* 1996). Most parasite material was obtained by dissection of infected insects under a light microscope. Axenic culture of *H. diminuta* metacystodes was performed according to Hurd & Burns (1994), with a harvest of parasites at stage I–II, approximately 4–7 days post-hatching. Metacystodes were staged by using the descriptions of Voge & Heynemann (1957) and Hurd & Arme (1987b).

(b) *Chemicals*

[U-¹⁴C]L-leucine (323 Ci mmol⁻¹) was obtained from ICN Pharmaceuticals Ltd, Basingstoke, Hampshire, UK. All other reagents were of analytical grade and purchased from Sigma Chemical Co.

(c) *In vitro synthesis of [¹⁴C]vitellogenin*

Day 9 post-emergence (day 6 post-infection) beetles were surface-sterilized with 70% (by volume) methanol and fat bodies were dissected in ice-cold *Tenebrio* saline (76 mM NaCl, 36 mM KCl). In some experiments, fat bodies were pooled, resuspended and divided between tubes, such that each contained one fat-body equivalent. This procedure enhanced the reproducibility of the data.

In vitro incubation (4 h, 26 °C) was carried out in a medium based on haemolymph amino-acid composition (Hurd & Arme 1984), filter-sterilized before use, and containing [¹⁴C]L-leucine (0.25 µCi, total volume 120 µl; Webb & Hurd 1996). After incubation, 2 × RIPA (radioimmunoprecipitation assay) buffer was added (0.3 M NaCl, 0.02 M Tris-HCl, 2% (by volume) Triton X-100, 0.2% (by mass) sodium dodecyl sulphate (SDS), 0.05 M sodium deoxycholate, pH 7.7; 120 µl) and the tissue disrupted by sonication (less than 25 s). The suspension was sedimented (16 500 g, 10 min). Two aliquots (12 µl) were removed from the supernatant and the [¹⁴C]Vg content was determined by immunoprecipitation.

(d) *Immunoprecipitation*

Details of antibody production and demonstration of Vg in the immunoprecipitate have been described previously (Webb & Hurd 1996). To each supernatant aliquot, 2 × RIPA buffer (12 µl) and anti-Vg polyclonal antibody (100 µl) were added and the mixture incubated on ice for 2 h. Protein A Sepharose (5 µg per 25 µl 1 × RIPA) was transferred to each tube and precipitation allowed to occur overnight at 0 °C. After sedimentation (3500 g, 15 min), the pellet was washed four times with 1 × RIPA (400 µl), then solubilized in 200 µl of 0.05 M NaOH. Ecocint scintillation cocktail (ICN) was added (4 ml) and [¹⁴C]Vg counted.

(e) *Extraction of H. diminuta metacystodes*

(i) *Methanolic extraction*

Approximately 1000 metacystodes were homogenized in methanol (0 °C, 500 µl) in an all-glass pestle and mortar. The homogenate was centrifuged (16 500 g, 5 min) and the resulting pellet re-extracted twice in methanol. The supernatants were pooled and the solvent evaporated under nitrogen.

(ii) *Acetic acid (HAc) extraction*

Metacystodes (ca. 1000) were heated in 1 M acetic acid (HAc) (500 µl, 60 °C, 10 min), cooled to 0 °C and homogenized on ice

with all-glass apparatus. After sonication (20 s), debris was sedimented (16 500 g, 1 min) and the pellet re-extracted twice in HAc. The supernatants were combined and the sample lyophilized.

'Blank' tubes were prepared as above for both extraction procedures, but contained no biological material. All extracts were stored at -20 °C until required and resuspended in fat-body incubation medium before use, at a concentration of 100 parasite equivalents per 100 µl.

(f) *Inclusion of latex beads and bovine serum albumin in the fat-body incubation*

To evaluate the role of either (i) a foreign body or (ii) a foreign protein on Vg synthesis *in vitro*, 10% (by mass) styrene divinylbenzene latex beads (mean diameter 90.7 µm ± 10%) or 29 µg BSA were included in the culture medium. (The quantity of BSA approximates the mean amount of protein in an acid extract of 100 parasites.)

(g) *Chemical nature of the metacystode effector*

(i) *Boiling*

During the extraction, boiling for 20 min was substituted for heating at 60 °C.

(ii) *The effect of pronase on the acetic acid extract*

200 U of the proteolytic enzyme pronase (EC 3.4.24.31) were incubated with an extract of 500 stage I–II parasite equivalents (1 h, 37 °C). The mixture was boiled, centrifuged (16 500 g, 10 min) and the clear supernatant removed for addition to five individual fat-body samples. A duplicate set of tubes contained parasite extract that had been preincubated with denatured pronase (boiled, 10 min).

(h) *Statistics*

Data were tested for normality, then for significance with a one-way ANOVA or, for determination of molecular mass range, Mann–Whitney *U*-tests (*p* ≤ 0.05).

3. RESULTS

(a) *Effect of methanolic and HAc extracts on T. molitor Vg production*

Approximately 100 stage I–II parasite equivalents were added to each fat-body sample and [¹⁴C]Vg synthesis estimated. Methanolic extracts were found to have no significant effect on the amount of Vg produced, whereas acid extracts caused a 25% reduction (*p* = 0.019; figure 1). Addition of methanolic or acid blank extracts did not bring about any significant changes in Vg synthesis. Boiling the acetic acid extract did not lessen its influence on Vg production.

(b) *Acetic acid extracts of stage I–II and stage V–VI metacystodes*

As shown in table 1, only extracts from the immature stage I–II metacystodes were effective in reducing Vg output; those from mature parasites did not result in any appreciable change. In the absence of parasite extracts, inclusion of foreign particles or foreign protein (latex beads or BSA) had no significant influence on Vg synthesis.

Pronase action destroyed the active component of the parasite extract: the amount of Vg detected in tubes with pronase was the same as in tubes containing incubation

Figure 1. Production of [^{14}C]vitellogenin by fat-body tissue from individual non-infected insects, incubated (4 h, 26 °C) with medium alone (control), a methanolic or acetic acid extract of 100 stage I–II parasite equivalents (MeOH and HAc, respectively) and with preparations containing no biological material (MeOH and HAc blanks). Bars represent the mean and standard error ($n=8$ –20 fat bodies; the [^{14}C]Vg content was determined in duplicate for each). Data were only significantly different from control values with the addition of an acetic-acid parasite extract ($p=0.019$).

Table 1. Age-specific differences in the activity of the metacystode factor(s)

(Metacystodes were collected when immature (stage I–II) and when fully encysted (stage V–VI). Acetic acid extracts were made and added to the fat-body *in vitro* assay system at a concentration of 100 metacystodes per 100 μl . Data are the mean of 18 determinations $\pm$ s.e.m.)

incubation	cpm mg^{-1} fat-body dry mass	s.e.m.
control	1577	185
stage I–II metacystodes	788	52
stage V–VI metacystodes	1342	149

medium alone. Heat-inactivated pronase was unable to degrade the parasitic effector molecule(s) (figure 2).

(c) **The effect of an acetic acid extract from axenically cultured *H. diminuta***

Acetic acid extracts of parasites collected from infected beetles and those cultured axenically yielded significant decreases in [^{14}C]Vg (29% and 35% reductions, respectively).

(d) **Approximate molecular mass range of the active factor(s)**

By means of Millipore microfuge filter tubes (molecular mass cut-off 10 kDa and 50 kDa), metacystode extract was fractionated into size ranges of less than 10 kDa, 10–50 kDa and over 50 kDa, lyophilized and resuspended in fat-body incubation medium at a concentration of 100 parasite equivalents per 100 ml. The most active component in the extract, determined by its ability to reduce Vg production, was found to be of molecular mass 10–50 kDa (figure 3). Fractionation of the parasitic product appears to have increased its potency: whole acid extract causes a 25–29% reduction in Vg synthesis,

Figure 2. The effect of pronase on the activity of the acetic acid parasite extract. The extract was incubated (1 h, 37 °C) with 200 U of the proteolytic enzyme pronase. The mixture was boiled to denature the enzyme, then the sample was centrifuged and the clear supernatant added to individual fat bodies (100 parasite equivalents per fat body). A parallel treatment was carried out with previously boiled pronase. Data are the mean and standard error of at least 20 fat bodies, each experiment being carried out in duplicate.

Figure 3. Fractionation of parasite acetic acid-extractable material by means of molecular mass cut-off filters. After fractionation, the samples were lyophilized and reconstituted in fat-body incubation medium before use. In each case, the concentration of parasite extract is 100 stage I–II metacystode equivalents per 100 μl . For each data set, $n=18$.

whereas the 10–50 kDa fraction results in a decrease of approximately 47%.

4. DISCUSSION

We have obtained evidence to support the hypothesis that *Tenebrio* vitellogenin synthesis is directly affected by a factor(s) produced by tapeworm metacystodes. Our data indicate that this modulator molecule(s) may be peptide-like in nature. These data further suggest that the host's reproductive output may, at least in part, be modified directly by the parasites and that host manipulation may thus confer a selective advantage on the parasite.

The processes controlling the transcription of insect vitellogenin genes and the translation of Vg mRNA are not fully understood; it is therefore difficult to propose a mode of action for this metacystode modulating factor. In

most, but not all insects, juvenile hormone (JH) governs Vg synthesis (for reviews see Engelmann 1983; Wyatt & Davey 1996). In the locust, *Locusta migratoria*, it appears that the brain produces peptides that act antagonistically to enhance or depress the stimulatory effects of JH (Carlisle & Loughton 1979, 1984; Carlisle *et al.* 1987; Glinka & Pshennikova 1990; Girardie *et al.* 1987, 1991, 1996; Richard & Girardie 1992). In addition, as the ovary swells with mature oocytes, adipokinetic hormone is secreted from the corpora cardiaca and suppresses Vg production (Moshitzsky & Applebaum 1990). Such stimulatory and inhibitory neuropeptides are thought to act at the translational level (Glinka & Triseleva 1989; Glinka *et al.* 1994).

Integration of a parasitic factor into a similar scheme to the above is not easy. Is the parasite factor mimicking an endogenous insect hormone or neuropeptide, which normally regulates the manufacture of Vg? Is this occurring via manipulation or exploitation of a secondary messenger signal transduction cascade? The factor represses vitellogenesis during an incubation period of only 4 h; this observation may suggest that there is insufficient time for gene activation, transcription and translation. Furthermore, *H. diminuta* metacestodes do not disturb the local concentration of juvenile hormone (directing Vg production): they possess neither juvenile-hormone-degrading enzymes nor the hormone itself (T. J. Webb, M. Major, D. W. Borst & H. Hurd, unpublished observations). It would follow, therefore, that the parasite factors might modulate the efficiency of Vg translation. Unfortunately, there are many gaps in our knowledge of *T. molitor* vitellogenesis that prevent us from making direct extrapolations from other systems. For instance, we have no evidence of the afore-mentioned insect peptides playing the same role in *Tenebrio*.

It is interesting to note that the effector may only be present in early-stage (I–II) parasites and not in mature stages (V–VI), as reaffirmed in the present study and with the use of whole, isolated metacestodes (Webb & Hurd 1996). It is thus produced at a time when the parasite biomass is very small, but growing rapidly, and metabolic demands on the host may be accelerating. The effects of the parasite factor *in vivo* are, however, long lived, persistent and non-reversible by endogenous regulatory mechanisms as tissue taken from beetles at all stages of infection *in vivo* synthesized less Vg (Hurd & Arme 1986b; Webb & Hurd 1996).

Demonstration of an effector molecule in the acetic acid, but not the methanolic, extract has certain implications for future isolation of the factor: hydrophobic or lipophilic molecules are unlikely candidates. Properties of the factor include heat stability (activity persists after 10 min boiling), pronase sensitivity, and solubility in aqueous medium and, hence, suggest peptide-like characteristics. The molecular mass is between 10 and 50 kDa. Its heat stability suggests a rigid structure and, together with the finding that there was some overspill of the active factor into the fraction with molecular mass less than 10 kDa, it could be either that the true molecular mass is at the lower end of the 10–50 kDa range or that more than one factor is involved. Further fractionation, isolation and N-terminal sequence information may reveal whether the factor(s) is (are) novel or

homologous with known (neuro)peptides of insects or helminths.

H. diminuta metacestodes can successfully infect a range of insects (Miyazaki 1991) and are known to affect the fertility of other tenebrionid beetles (Keymer 1980; Maema 1986). In addition, there is some evidence that *H. diminuta* induces changes in the egg production of an unnatural host, the locust (*Locusta migratoria*) artificially infected with metacestodes (Hurd & Arme 1987c). Although the regulation of insect vitellogenesis is complex, with many orders and even species within the same order exhibiting widely different mechanisms (Wyatt & Davey 1996), the metacestode factor may operate at a common junction and, thus, prove potent in many insects.

The question as to why the metacestode should specifically reduce the production of Vg, but not other proteins, in the fat body (Webb & Hurd 1996) also deserves some explanation. Metacestodes are known to possess uptake sites for several monosaccharides and amino acids (Arme 1988) but cannot use Vg itself (Webb & Hurd 1996). As they compete directly for amino acids circulating in the haemolymph, all host tissues could be depleted of nutrients. In female beetles, egg production puts a heavy demand on amino-acid pools, yet is not essential to the survival of the host. A normal rate of egg production combined with the burden of an infection may not be sustainable, and host death could ensue. Read (1990) discussed the positive effect that parasite manipulation of the host's reproductive effort may have on the trade-off between fecundity and longevity. He suggested that a decreased cost of reproduction could pay off in terms of host survival. Data from our laboratory (Major 1997) support this concept. In the absence of predators, *T. molitor* infected with *H. diminuta*, supplied with apples and bran *ad libitum*, survived longer than non-infected counterparts maintained under identical conditions.

The quality of the parasite environment may thus be enhanced if host nutrient partitioning is specifically manipulated to the detriment of egg production (Hurd 1998). In the course of evolution, the parasite may have developed a means of dictating the rate of vitellogenin production, using a control point already established in the host.

We thank Dr Mary Major for maintaining both the beetle and tapeworm life cycles and the BBSRC for financial support. We are also grateful to two anonymous referees for useful comments.

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