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CORRELATION BETWEEN CHANGES IN HOST BEHAVIOUR AND OCTOPAMINE LEVELS IN THE TOBACCO HORNWORM *MANDUCA SEXTA* PARASITIZED BY THE GREGARIOUS BRACONID PARASITOID WASP *COTESIA CONGREGATA*

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Summary

The parasitoid wasp *Cotesia congregata* lays its eggs within the body of its host, the larval form of the tobacco hornworm *Manduca sexta*. Host behaviour appeared normal until approximately 8 h prior to the emergence of the parasitoids from their host at which time *M. sexta* feeding and locomotion declined irreversibly. This change in host behaviour may be to the advantage of the wasp since unparasitized *M. sexta* presented with wasp pupae ate them. Despite the decline in feeding and locomotion, hosts with emerged parasitoids had normal reflexes and showed no other signs of debilitation. Concomitant with the change in host behaviour, octopamine concentration measured using high-performance liquid chromatography with electrochemical detection (HPLC-ED) increased from 22.2±2.1 pg μl^{-1} to 143.7±7.8 pg μl^{-1} in the haemolymph of the host. In unparasitized *M. sexta*, however, increased octopamine levels were correlated with increased activity. We discuss possible explanations for the co-occurrence of high haemolymph octopamine levels and low behavioural arousal in parasitized *M. sexta*.

Key words: parasite, parasitism, host regulation, feeding inhibition, arousal, activity.

Introduction

The physiological control of behaviour is complex. The interconnections between different physiological systems, and the multiplicity of influences on the central nervous system (e.g. circadian rhythms, hormones, sensory stimuli; see Huber *et al.* 1989; Chapman and de Boer, 1995, for overviews) make it difficult to determine how behaviour is regulated on a mechanistic level. Despite this complexity, some parasites have evolved at least partial solutions to the problem of how behaviour is controlled. Some parasites can induce changes in specific patterns of host behaviour (see Hurd, 1990; Horton and Moore, 1993; Moore, 1993, 1995) and so they must have the ability to alter selectively the dynamics between the different physiological components that regulate behaviour. For this reason, it may be possible to use parasites as probes to aid in understanding the interplay between endocrinal, neural, immunological and other factors that control behaviour. Unfortunately, although there are a number of studies describing the behavioural changes that occur in parasitized animals (Horton and Moore, 1993; Moore, 1993, 1995), there is very little work examining the physiological mechanisms underlying these changes (see Adamo, 1997).

In some parasitic systems, the host belongs to the same phylogenetic class as the parasite (Beckage, 1993*a*,*b*, 1997) and so the parasite can probably synthesise most, if not all, of the neuroactive and/or hormonal substances used by the host. Since such parasites would not have had to evolve novel neuroactive substances, they may be the most likely to have evolved specific mechanisms for influencing host behaviour. Insect parasitoids are excellent examples of such systems. Insect parasitoids are usually flies or wasps whose larvae must develop within the body of another insect, although the adult wasp or fly is free-living (see Godfray, 1994).

The gregarious parasitoid wasp *Cotesia congregata* lays its eggs inside the body of its host, the larval form of *Manduca sexta* and other sphingids (Beckage and Riddiford, 1978). The eggs are co-injected with a polydnavirus that suppresses the immune system of the host (Lavine and Beckage, 1995, 1996). The egg hatches within the host's haemocoel releasing both first-instar *C. congregata* larvae and large secretory cells called teratocytes (Beckage and de Buron, 1994). The *C. congregata* larvae develop within the haemocoel, consuming haemolymph, but they do not physically damage the host's internal organs and they do not make any physical connections to the central nervous system. After about 12 days within the host, the *C. congregata* larvae moult to the third instar and emerge. The *C. congregata* larvae spin cocoons immediately after emergence.

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The pupae remain attached to the host's body, and the adult wasps emerge 4 days (males) or 5 days (females) later unless a larval diapause has been induced (Beckage and Riddiford, 1978, 1982, 1983; Beckage and Templeton, 1986).

During the time that the *C. congregata* larvae are within the host, parasitized *M. sexta* grow more slowly than controls but there is a 'dose-dependent' enhancement of host growth with more heavily parasitized hosts attaining higher final masses prior to wasp emergence (Beckage and Riddiford, 1978; Alleyne and Beckage, 1997; Alleyne *et al.* 1997). There are a number of concurrent physiological changes (see Beckage, 1993*a*,*b*), including changes in haemolymph proteins, ecdysteroid titres (Beckage and Templeton, 1986; Gelman and Beckage, 1995) and juvenile hormone titres (Beckage and Riddiford, 1982). Some changes in the host, such as the initial suppression in the host's cellular immune system and its failure to pupate, appear to be induced by the virus (Dushay and Beckage, 1993; Beckage *et al.* 1994; Lavine and Beckage, 1996).

After the parasitoids emerge, the host lives for another 2 weeks. During this time it does not appear to feed, its mass decreases and it seldom moves (Beckage and Riddiford, 1982, 1983). During parasitoid emergence, host respiration rate suffers a dramatic decline, probably indicating a decrease in the host's metabolic rate (Alleyne *et al.* 1997). Whether this is a result of their lack of motion and feeding, or is a major cause of it, is not known.

In the present study, we examine the behaviour of parasitized *M. sexta* and correlate changes in host behaviour with changes in the concentration of octopamine in the haemolymph. Octopamine is thought to play a role in producing an active behavioural state in insects (Davenport and Evans, 1984*a*,*b*; Orchard *et al.* 1993) partly because of the positive correlation between haemolymph octopamine concentration and activity level (Davenport and Evans, 1984*a*; Woodring *et al.* 1988). This suggests that one possible mechanism for decreasing host motor movements (including feeding) may be to depress host octopamine levels. We tested whether the host's permanent inactive behavioural state correlates with reduced octopamine titres.

Materials and methods

Insects

Rearing protocols for the *Manduca sexta* (L.) larvae and *Cotesia congregata* (Say) wasps were as described in Alleyne and Beckage (1996). Briefly, *M. sexta* larvae were reared on a modified artificial diet (Bell and Joachim, 1976) under a long-day photoperiod (17 h:7 h L:D) at 25 °C. Larvae were housed individually in plastic cups and fed *ad libitum*.

C. congregata adults were reared using first-instar *M. sexta* larvae for parasitization. Parasite cocoons were stripped from hosts approximately 24 h after the wasps emerged from fifth-instar host larvae (Beckage and Riddiford, 1983). Adult wasps eclosing from the cocoons were maintained at 25 °C in a 0.25 m^3 Plexiglas cage. Adults had continuous access to undiluted clover honey and distilled water.

For behavioural and hormonal studies, hosts were parasitized by exposing fourth-instar, day 0 *M. sexta* to mixed populations of *C. congregata* males and females in the Plexiglas rearing cage. Hosts were removed after being stung by *C. congregata* females and returned to 30 ml rearing cups. They moulted to the fifth instar and parasitoids emerged 10–12 days post-parasitization.

Parasitized hosts were usually compared with unparasitized animals of the same mass, even though these animals may not have been the same chronological age owing to different rates of growth and development. Parasitized *M. sexta* were also compared with age-matched *M. sexta* injected with teratocytes. This allowed us to control for changes in behaviour due to age alone. To control for the effects of decreased food intake in *M. sexta* after the parasitoids emerge, in another control group unparasitized *M. sexta* that were the same mass as parasitized animals were food-deprived for 1 day.

Assessment of behavioural changes induced by parasitism

At the onset of the fourth instar, control and parasitized *M. sexta* (both before and after parasitoid emergence) were weighed daily. Six hours after the onset of photophase, each animal was given a feeding trial. Food was removed from the *M. sexta* for 5 min prior to the trial. The animal was then placed on a fresh piece of diet for 10 min and the length of time that it actively chewed the food was recorded. A feeding bout was considered to have ended when two sequential bites were more than 10 s apart. The rate of chewing, or the bite rate, was also recorded during the 10 min trial. Data were recorded with the aid of an electronic event recorder (Eventlog, Conduit). This software also allowed us to determine the maximum bite rate during 10 s intervals.

Beginning 2 days prior to the expected emergence of the parasitoids, hosts were given feeding trials twice a day, once at 6 h after the start of photophase and another 12 h later. These measurements were continued until 2 days after parasitoid emergence. The onset and duration of parasitoid emergence was noted.

To determine whether host reflexes were altered during parasitism, we measured the time required to complete the 'righting response' in both control and parasitized animals. Control (mass-matched to parasitized *M. sexta*) and parasitized (both before and after parasitoid emergence) *M. sexta* were observed and the time required for each animal to right itself (i.e. placing both prolegs and abdominal legs onto the ground after being positioned ventral-side-up on a hard surface) was recorded. We also measured their ability to grasp a metal rod using their prolegs (i.e. encircling the rod with their abdominal ventral prolegs) and maintain this position for 2 min. We also determined the ability of each group of *M. sexta* to perform the proleg-withdrawal reflex and to move the head to the horn (located at the most caudal end of the abdomen) in response to a pinch (pinch reflex). The pinch was delivered by squeezing the base of the horns with watchmakers' forceps.

To determine the effect of parasitism on host locomotion, *M. sexta* had their most-caudal abdominal legs dipped in India

ink and were placed on a 22 cm×28 cm sheet of white paper for 10 min. Animals were allowed an 'acclimation' period of 5 min on the paper prior to the trial before measurements were taken. The length of the path trodden by the *M. sexta* was measured using a thread and ruler. Assessments were made of the behaviour of *M. sexta* 2 and 5 days prior to parasitoid emergence, on the day of parasitoid emergence and 1, 3 and 7 days after parasitoid emergence. Unparasitized *M. sexta* of the same mass as well as some heavier animals that were of the same instar (fifth) were also tested. To test the locomotion of animals starved for 1 day, and the fed controls for this trial, *M. sexta* were placed on linoleum instead of white paper because the starved animals tended to eat the paper. Control (fed) animals moved the same distance on both surfaces (*t*-test, $P>0.1$, $N=20$). The data for the locomotion, bite-rate, feeding-duration and righting-reflex tests were gathered using the same 20 animals per group.

Are C. congregata *larvae necessary to suppress host feeding behaviour?*

To determine whether the parasitoid, in the absence of the polydnavirus or teratocytes, could suppress host feeding, we transplanted *C. congregata* larvae into previously unparasitized hosts. Since the virus does not replicate in the wasp larvae and the larval stage contains only the integrated form of the virus within the wasp's genome (Stoltz, 1993; Lavine and Beckage, 1996), transplanted *C. congregata* will not introduce the virus into their new host. *M. sexta* that had been parasitized 8 days previously were chilled and then dissected under a sterile hood. The parasitoid larvae were floated out of the host by washing the body cavity with sterile Grace's medium. The *C. congregata* larvae were then washed gently by aspirating them into a syringe and releasing them into fresh Grace's medium two or three times. This was done to prevent teratocytes from being implanted with the parasitoids. An unparasitized *M. sexta*, in the first day of its fourth instar, was chilled and the area around the last abdominal leg was swabbed with 95 % alcohol prior to injection. Typically, 10 late first-instar *C. congregata* larvae were injected into the *M. sexta* using a modified Hamiliton syringe fitted with a blunt-ended 18 gauge needle. When the *C. congregata* larvae were sucked up into the syringe, care was taken not to aspirate any teratocytes. Another group of *M. sexta* were injected with approximately 50–100 teratocytes. Control *M. sexta* were injected with sterile Grace's medium. The maximum volume injected into any animal was 20 µl. The injected *M. sexta* were then returned to their individual containers with fresh food. One day after at least one of the transplanted *C. congregata* had emerged from its host, the host was given the battery of behavioural tests described above. Teratocyte-injected and saline-injected *M. sexta* were also given the same behavioural tests.

Determination of biogenic amine concentrations in the haemolymph

To determine whether the presence of the maturing parasitoids was correlated with changes in haemolymph biogenic amine levels, we assayed samples of haemolymph during the development of the parasitoids, at the time of parasitoid emergence, and 1, 3 and 7 days after parasitoid emergence. Unparasitized *M. sexta* had blood samples collected from them at the beginning and end of the fourth instar, during the moult to the fifth instar, on day 1 of the fifth instar (1.8–2.3 g), day 3 of the fifth instar (3.8–5.6 g) and during the wandering phase. To control for the effects of the decline in food intake in post-emergent hosts, unparasitized mass-matched *M. sexta* (fourth instar) were food-deprived for 1 day before haemolymph was collected. Haemolymph was also collected from teratocyte-injected animals 12 and 15 days after teratocyte injection. To test whether the presence of *C. congregata* larvae alone, without virus or teratocytes, influences octopamine levels, haemolymph was collected from *M. sexta* that had *C. congregata* larvae implanted in them. Haemolymph was removed 1 day and 3 days after the parasitoids emerged.

Haemolymph was collected from animals at the same time each day, 6 h after the start of photophase, by snipping the horn of *M. sexta* and collecting haemolymph in a 25 µl Hamilton syringe as it formed a bead at the tip of the horn. Samples of 5–10 µl were collected in approximately 10 s. A 5 µl sample was added to 40 µl of ice-cold 0.2 mol l^{-1} perchloric acid immediately after collection. The samples were then spun at 8 500 ***g*** for 5 min at 4 °C and the supernatant was added to 60 µl of HPLC-grade water (Sigma) and transferred to an autosampler tube. The supernatant was frozen at –80 °C prior to use.

Chromatographic separations of octopamine and other compounds were achieved using a Vydac C-18 HS-54–15 HPLC column (15 cm×4.6 mm, 3 µm particles) protected by a Vydac C-18 guard column. Chemicals and HPLC-grade water were obtained from Sigma Chemical Co. (St. Louis, MO, USA) except for perchloric acid (J. T. Baker Inc., Jackson, TN, USA). The mobile phase (after Downer and Martin, 1987) contained 70 mmol l^{-1} monobasic sodium phosphate, 0.5 µmol l^{-1} EDTA, 0.1 mmol l^{-1} 1-octanesulphonate (Na^+ salt), with 15 % methanol and 5 % acetonitrile. The pH of the buffer was adjusted to 5.5 using sodium hydroxide. The mobile phase was thoroughly stirred and vacuum-filtered through a 0.22 µm filter and degassed. The mobile phase was run isocratically at 0.85 ml min^{-1}. The HPLC pump, autosampler, guard cell and dual-channel coulometric detector were from ESA Inc. (Chelmsford, MA, USA). The first electrode was set at a potential of 0.35 V and the second electrode at 0.73 V. The guard cell was set at 0.8 V. Identification of compounds (e.g. octopamine and dopamine) was based on comparison of retention times with standards, which were run at the beginning and end of each daily series. Peaks were also identified on the basis of changes in retention times or peak height as a function of systematic changes in chromatographic conditions, including pH, percentage organics and applied channel voltage (see Linn *et al.* 1994, for details).

Peak heights and/or area were measured and compared with a calibration curve made by injecting known amounts of each

compound where the peak identity was known. The value of the peak was then converted to $pg\,\mu l^{-1}$ haemolymph. Chromatograms for octopamine were recorded on a Spectra-Physics dual-channel Data-Jet integrator, which was interfaced with a Spectra-Physics SX-386 computer system for data analysis.

The effects of octopamine on M. sexta *feeding and locomotion*

To determine whether the observed increase in octopamine is involved in the decline in feeding in parasitized hosts, normal *M. sexta*, day 1, fifth instar, were given injections of octopamine (20 µl, 10^{-6} mol l^{-1} to 10^{-2} mol l^{-1}), as well as its antagonist phentolamine (20 µl, 10^{-2} mol l^{-1}). A third group was injected with both 10^{-2} mol l^{-1} octopamine and 10^{-2} mol l^{-1} phentolamine and a fourth group was injected with the vehicle for phentolamine, 10 % methanol. The 20 µl injections were given just behind the last abdominal leg using a 25 µl Hamilton syringe. Any animal that exhibited profuse bleeding was excluded from further study. To determine whether octopamine antagonists could induce feeding in parasitized animals, the cocoons were removed and *M. sexta* were given 20 µl of 10^{-2} mol l^{-1} phentolamine 1 day after the parasitoids had emerged from the host. Control animals were injected with the vehicle (10 % methanol).

The following procedures were used to monitor feeding behaviour: prior to injection, the animal was weighed and then 2 min after the injection the animal was placed on a piece of preweighed food; 3 h later, animals were observed in their containers and their bite rate was monitored for 5 min using an electronic event recorder (Eventlog, Conduit); 6 h after injection, the animal and its food was reweighed. Locomotion was also measured in animals injected with octopamine using the method described above.

Could changes in host behaviour be adaptive?

To determine whether suppression in host feeding behaviour benefits the wasp, *C. congregata* cocoons were placed on the substratum in front of unparasitized *M. sexta* (fifth instar, day 1). In a second test, five wasp cocoons were glued using cyanoacrylate onto the dorsum of control animals (N=5). The cocoons were affixed onto the cuticle in the dorsal abdominal area in order to mimic their normal placement on parasitized hosts. The number of cocoons consumed by the *M. sexta* during the 1 h trial was recorded. During these trials, their artificial diet was unavailable.

Statistics

Where possible, parametric statistics were used (Sokal and Rohlf, 1981) and values in the text and figures represent means ± 1 S.D. unless otherwise stated. For many measurements, the data were not normally distributed; for example, bite rate and locomotion sometimes fell to 0 in *M. sexta* after parasitoid emergence. We used non-parametric statistics in these cases (Meddis, 1984), and these data are presented as medians and quartiles.

Data points with no error bars in the figures denote data points that had errors too small to be shown in the units indicated on the graph.

Results

Behavioural changes during parasitism

Although parasitized *M. sexta* weighed less than unparasitized controls 5 days after parasitism (Fig. 1), their feeding duration (Fig. 2), bite rate (Fig. 3), locomotion (Fig. 4) and righting reflex (Fig. 5) before the *C. congregata* larvae emerged were not significantly different from those of controls. After *C. congregata* emerged, the host moved less (Fig. 4) and, even more dramatically, spent much less time feeding (Fig. 2) and bite rate declined (Fig. 3). The decline in bite rate was less pronounced if the bite rate was measured only during feeding bouts and not averaged over interbout intervals, although the decline was still statistically significant (controls, 14.8±3.6 bites per 10 s; pre-emergence, 14.7±2.5 bites per 10 s;

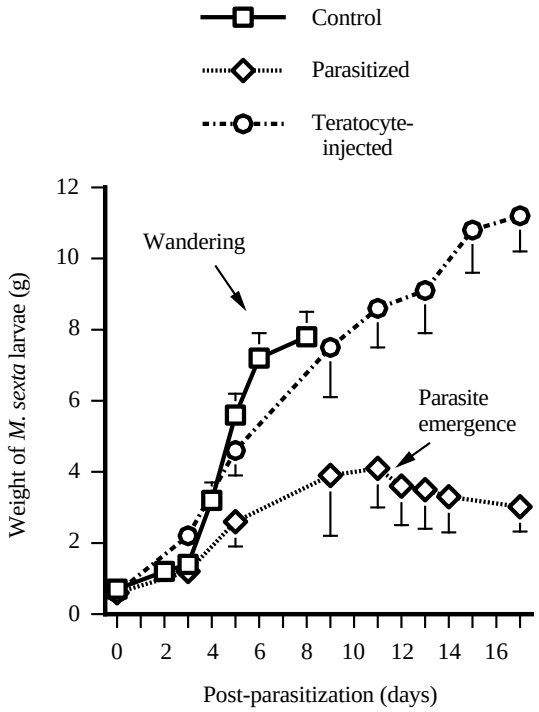

Fig. 1. Differences in mass gain over time of unparasitized (control, N=15), parasitized (N=15) and teratocyte-injected (N=15) *M. sexta*. Five days after parasitization of fourth-instar, day 0 *M. sexta*, parasitized animals weighed significantly less than controls [ANOVA, $F(2, 42)=140.7$, $P<0.001$; Bonferroni *post-hoc* analysis, $P<0.01$]. Both control and teratocyte-injected *M. sexta* exhibited an increase in mass with time (test for trends, control $Z=4.2$, teratocyte $Z=2.7$; $P<0.01$ for both). However, after the *C. congregata* larvae emerged from their hosts, the host's mass gradually declined ($Z=1.8$, $P<0.05$). Arrows denotes the day that the unparasitized *M. sexta* started wandering behaviour or the day of parasitoid emergence from parasitized *M. sexta*. Error bars denote one standard deviation from the mean.

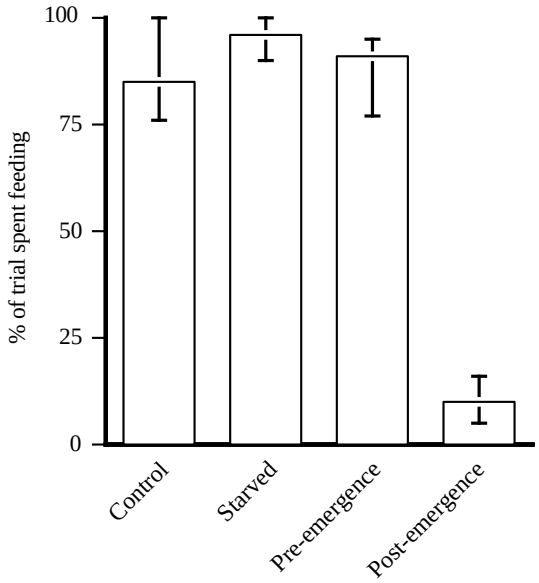

Fig. 2. The effect of parasitism on the time *M. sexta* spent feeding. There were no changes in the time parasitized *M. sexta* spent feeding prior to parasitoid emergence (pre-emergence, measured 2–4 days prior to emergence; Non-parametric test for contrasts, Z=0.23, P>0.1). One day after parasitoid emergence, however, parasitized *M. sexta* (N=20) spent significantly less time feeding than control (N=20), starved (N=20) or pre-emergence (N=20) *M. sexta* (Z=14.9, P<0.001). The columns represent the median value and error bars denote first and third quartiles.

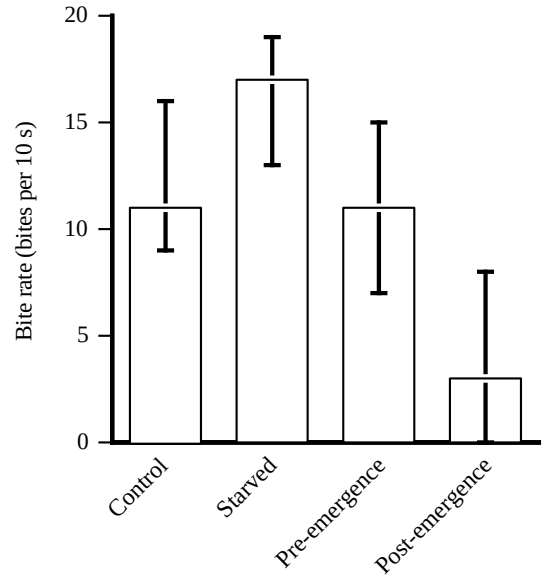

Fig. 3. Overall bite rate declined after parasitoid emergence. Prior to parasitoid emergence (pre-emergence), bite rate does not differ significantly from that of controls (test for contrasts, Z=0.63, P>0.1); however, 1 day after emergence bite rate declined (Z=2.14, P<0.01). 24 h of food deprivation significantly increased bite rate over that of controls (Z=2.02, P<0.05). Each group contained 20 animals. Columns represent median values and error bars denote first and third quartiles.

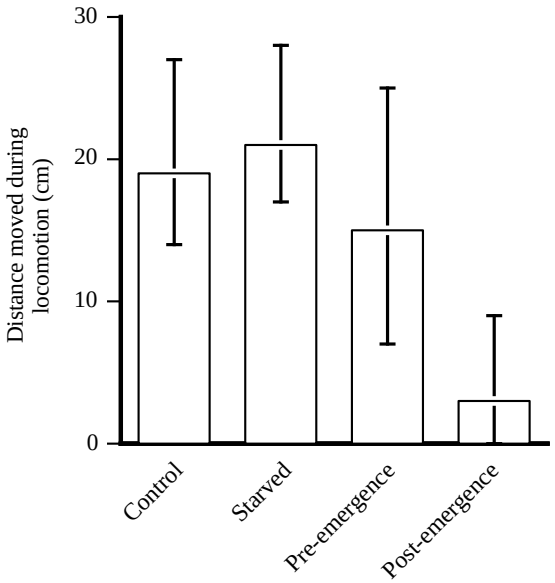

Fig. 4. Locomotion declined after parasitoid emergence. Prior to parasitoid emergence (pre-emergence) there was no significant decrease in the distance travelled by parasitized *M. sexta* (test for contrasts, Z=1.2, P>0.05); however, locomotion declined significantly from control and pre-emergence values after the parasitoids emerged (Z=7.2, P<0.001). Each group contained 20 animals. Columns represent median values and error bars denote first and third quartiles.

post emergence, 9.8±4.2 bites per 10 s; N=20, test for contrasts, Z=2.72, P<0.05).

There was no evidence of general debilitation. The speed of the righting reflex of *M. sexta* with emerged parasites did not differ significantly from that of controls (Fig. 5). *M. sexta* with emerged parasites exhibited both normal proleg retraction reflexes (in 15 out of 15 animals tested) and pinch reflexes (15/15). All parasitized *M. sexta* with emerged wasps were able to support themselves with their prolegs for 2 min (15/15), as could control animals (15/15) and *M. sexta* tested prior to parasitoid emergence (15/15).

The change in host feeding behaviour and locomotion was measurable 8 h prior to parasitoid emergence (Fig. 6), which was slightly after the host begins to decline in mass (Alleyne and Beckage, 1996). When *M. sexta* were tested 7 and 10 days after parasitoid emergence, the animals showed no sign of recovery in either feeding behaviour or locomotion (non-parametric test for trends, Z=0.41, P<0.1). The changes were permanent until the death of the host 14.2±5.6 days later (N=11).

Most of the *C. congregata* larvae within a single host emerged within 12 h of each other. However, in four out of 36 hosts (11 %), some *C. congregata* larvae continued to emerge for up to 48 h after the first parasitoid emerged.

Are C. congregata *larvae necessary to suppress host feeding behaviour?*

Teratocyte-injected animals continued to increase in mass (Fig. 1) and did not exhibit the depression in feeding seen in

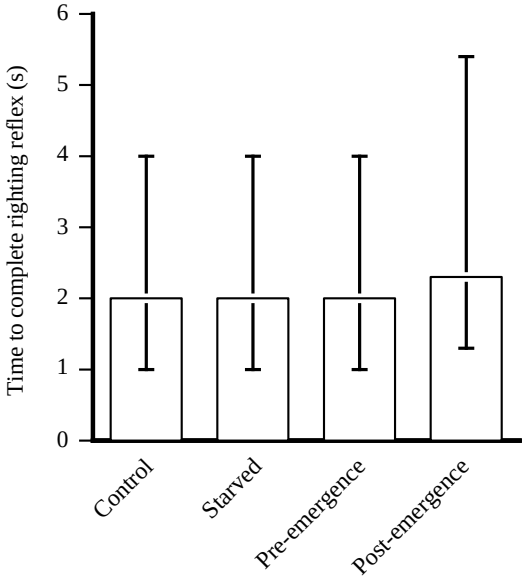

Fig. 5. The time to complete the righting reflex did not change significantly after parasitoid emergence. There were no significant differences between control, starved, pre- and post-emergence parasitized *M. sexta* in the time required for them to complete the righting reflex (test for contrasts, Z=0.42, P>0.1). Each group contained 20 animals. Columns represent median values and error bars denote first and third quartiles.

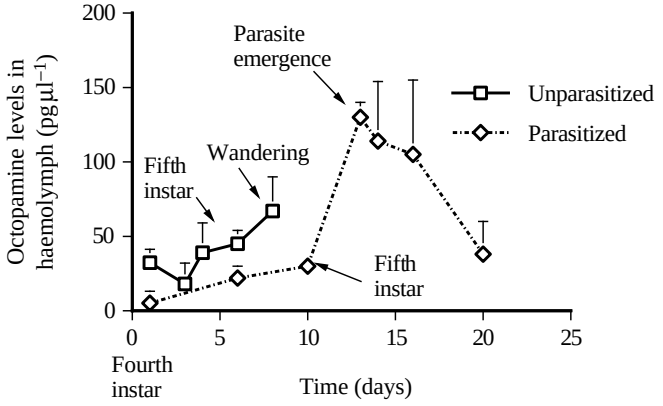

Fig. 7. Octopamine concentrations in the haemolymph of *M. sexta* during the last two larval instars and during parasitism by *C. congregata*. The first blood samples were taken during the first day of the fourth instar, 1 h after being parasitized. Octopamine level in the haemolymph increases during the fifth instar in control (unparasitized) animals, but increases to even higher levels in parasitized animals at the time of parasitoid emergence [ANOVA, F(11, 115)=5.6, P<0.01; Bonferonni *post-hoc* tests, P<0.05]. Levels remain elevated above pre-emergence levels in parasitized hosts for at least 3 days (Bonferroni *post-hoc* tests, P<0.05). The data points represent means and the error bars standard deviations. Sample sizes (listed in order of days past the fourth instar) were: unparasitized, 10, 10, 26, 7, 13, parasitized, 8, 7, 7, 16, 9, 8, 6.

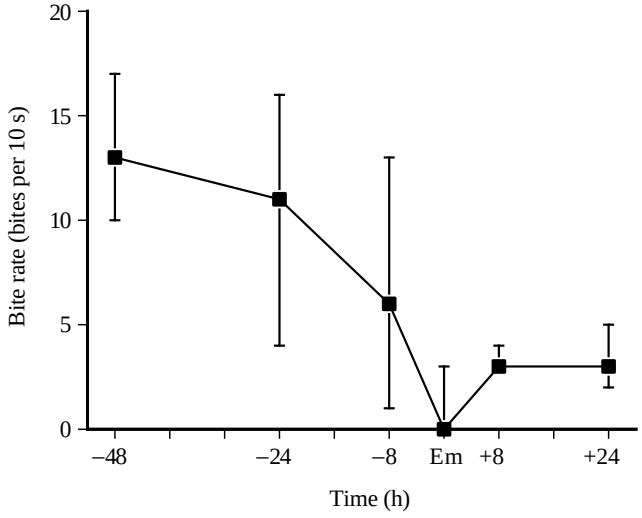

Fig. 6. Bite rate fell sharply just prior to parasitoid emergence (Em) (test for trends, repeated measures, Z=2.95, P<0.01). Data points represent median values and error bars denote first and third quartiles. N=11.

M. sexta with emerged wasps. *M. sexta* with transplanted *C. congregata* larvae (N=4) showed no significant change in locomotion (18.1±4.7 cm) or bite rate during feeding bouts (14.2±4.3 bites per 10 s) compared with controls injected with Grace's medium until the day prior to parasite emergence (*t*-tests, P<0.05). After the parasites emerged, the host showed a decline in locomotion (median 5.2 cm; 0, 8, first and third quartiles) and bite rate (median 3 bites per 10 s; 0, 5, first and third quartiles) that was not significantly different from that observed in naturally parasitized hosts (non-parametric planned comparisons, Z=0.6, P>0.1). As with naturally parasitized hosts, the time required to complete the righting reflex was not significantly different from that of controls (Z=0.31, P>0.1).

M. sexta injected with Grace's medium alone (N=10) did not differ significantly in their behaviour from controls (locomotion 18.7±6.2 cm; bite rate 13.8±4.1 bites per 10 s; *t*-tests P>0.1) and successfully pupated as adults. Six out of 14 teratocyte-injected animals entered the wandering stage, but none successfully pupated. All died as larval–pupal intermediates. Of the *M. sexta* implanted with *C. congregata* larvae, four out of 23 had *C. congregata* larvae emerge from them (average number of emerged larvae 2.3±0.5). Of the other 19 *M. sexta*, six pupated into adults while the others died as larval–pupal intermediates.

Haemolymph octopamine levels

Fig. 7 shows the normal changes in concentration in octopamine in the haemolymph during the last two larval instars of *M. sexta*. Octopamine concentration increased during the fifth instar (Fig. 7). Octopamine levels in controls were significantly higher than those found in parasitized *M. sexta* until the time of parasite emergence (Fig. 7) at which time the octopamine concentration in the haemolymph increased dramatically and remained elevated for at least 3 days before returning to baseline values.

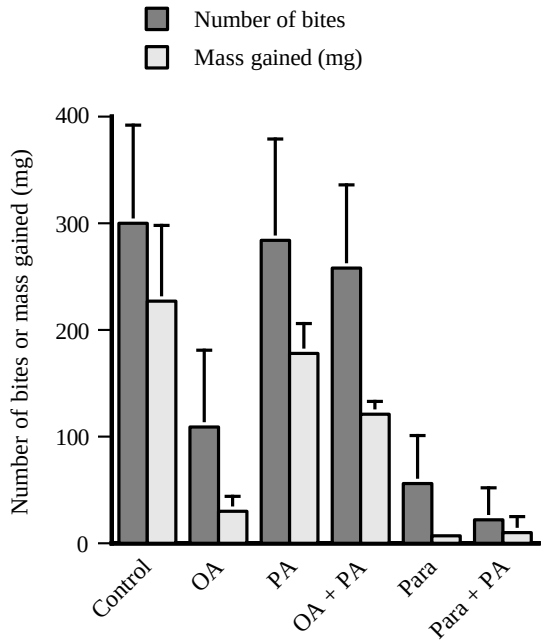

Fig. 8. Phentolamine (PA) (10^{-2} mol l^{-1}) blocked octopamine-induced (10^{-2} mol l^{-1}) inhibition of feeding in normal animals, but did not lead to increased feeding in *M. sexta* with emerged parasitoids. Using either mass gained during the trial or the number of bites during a 10 min test as a measure of feeding, feeding of octopamine-injected (OA) controls declined [ANOVA, bite rate, $F(5,33)=28$, $P<0.0001$; mass gain, $F(5,33)=8.6$, $P<0.001$; Bonferroni pairwise adjustments, $P<0.001$]. Phentolamine-injected animals did not differ from controls (Bonferroni pairwise adjustments, $P>0.1$, both measures). Animals co-injected with octopamine and phentolamine did not differ from controls (Bonferroni pairwise adjustments, $P>0.1$, both measures). Phentolamine had no significant effect on the feeding of parasitized (Para) *M. sexta* with emerged wasps (Bonferroni pairwise adjustments, $P>0.1$, both measures). Columns represent means and standard deviations. Control, $N=7$; octopamine-injected (OA), $N=7$; phentolamine-injected (PA), $N=6$; octopamine/phentolamine co-injected (OA+PA), $N=6$; post-emergence *M. sexta* (Para), $N=6$; post-emergence *M. sexta* phentolamine-injected (Para+PA), $N=7$.

Octopamine levels in *M. sexta* that had *C. congregata* implanted within them were not significantly different from those found in naturally parasitized *M. sexta* measured 1 day (133.7±13.1 pg μl^{-1}) and 3 days (128±11.3 pg μl^{-1}) after the parasitoids had emerged ($Z=0.71$, 0.83, $P>0.1$, $N=4$). Food deprivation for 24 h in late-fourth-instar larvae induced an increase in octopamine titre (7.8±2.2 pg μl^{-1}, $N=8$) compared with fed controls (4.8±2.8 pg μl^{-1}, $N=8$, *t*-test, $P<0.05$). *M. sexta* implanted with teratocytes exhibited no significant increase in octopamine levels compared with fifth-instar controls 12 days (22.1±8.6 pg μl^{-1}, $N=4$) and 15 days (29.3±16.2 pg μl^{-1}, $N=3$; *t*-test, $P<0.1$) after implantation.

The effect of octopamine on M. sexta *feeding and locomotion*

Injections of 20 µl of 10^{-6} mol l^{-1} octopamine (approximately 20 picomoles per larva) had no significant influence on feeding when compared with controls ($Z=0.92$, $P>0.1$, $N=7$). Injections of 10^{-2} mol l^{-1} (approximately 200 nanomoles per larva) octopamine inhibited feeding in unparasitized *M. sexta*, and this inhibition could be blocked by the octopamine antagonist phentolamine (Fig. 8). Parasitized *M. sexta* with emerged parasites showed no significant change in their feeding behaviour when injected with phentolamine (Fig. 8). This was true even when phentolamine injections were given every 12 h for 3 days (test for contrasts, repeated measurements, $Z=0.23$, $N=6$, $P>0.1$). Octopamine-injected (10^{-2} mol l^{-1}) and unparasitized *M. sexta* increased their locomotion (28.7±7.1 cm, $N=10$) compared with saline-injected controls (21.3±6.8 cm, $N=10$; test for contrasts, $Z=3.18$, $P<0.01$). Food deprivation for 24 h also increased locomotion in unparasitized *M. sexta* (25.3±4.2 cm) compared with fed controls (19.7±5.7 cm; test for contrasts, $Z=2.9$, $N=10$, $P<0.01$).

Possible adaptive significance in the change in host behaviour

All 15 *M. sexta* presented with cocoons fed on them and four out of five *M. sexta* that had cocoons glued onto their cuticle ate at least one cocoon.

Discussion

The feeding behaviour of parasitized *M. sexta* began to decline significantly approximately 8 h before the wasps emerged (Fig. 6). After parasitoid emergence, hosts showed only a small decline in their maximum bite rate, but a large decrease in the duration of their feeding bouts and an increase in the interval between these bouts. Hence, the tendency to feed, rather than the ability to eat, appeared to be most strongly affected by parasitism.

Although hosts with emerged parasitoids rarely fed, they responded appropriately to most sensory stimuli (i.e. they performed the righting, grasping and tail-pinch responses normally). The host's chemosensory hairs show no change in threshold sensitivity after parasitoid emergence (J. Frazier, personal communication), suggesting that the change in feeding is not due to depressed sensory responsiveness. The hosts scored above zero on the locomotion test (Fig. 4) and righting behaviour was normal (Fig. 5), demonstrating that they did not lack muscular strength or coordination. These results, coupled with the observation that the hosts live for another 2 weeks after the parasitoids emerge, suggest that the decline in feeding and locomotion did not occur because the animals were about to expire. The largest behavioural deficit appeared to be a decreased tendency to initiate motor movements, as if the host was in a permanently low level of arousal or in an inactive behavioural state.

There is evidence to link the change in the host's behaviour with changes in the host's central nervous system. Beckage and Templeton (1986) found that hosts with emerged parasitoids locomoted almost continuously if the supraoesophageal ganglion was removed. Larvae treated in this way cannot feed and, therefore, we do not know whether their tendency to feed

was also increased by this operation. Nevertheless, this result, in accordance with our results showing that reflexes remain intact in parasitized *M. sexta*, suggests that the changes in host behaviour were due to changes within the central nervous system rather than to damage to peripheral structures such as muscles or sensory organs.

Immunohistochemistry has revealed differences in the staining of some of the neuropeptides present in the central nervous systems of unparasitized and parasitized *M. sexta* before and after parasitoid emergence (Gelman and Beckage, 1995; Zitnan *et al.* 1995*a*,*b*). However, since almost all of these changes occur after behavioural changes in the host, it is not clear whether there is a causal relationship. Changes in proctolin staining, however, did occur at about the time of emergence (Zitnan *et al.* 1995*a*) but these data were collected on naturally parasitized hosts so it is not known whether the changes were due to the effects induced by the polydnavirus (and therefore probably not critically involved in the suppression of feeding) or by the parasitoid.

Although there is little evidence as to how the presence of the parasitoid influences behaviour, a direct effect of the parasitoid on the host's central nervous system is possible. Hormones such as ecdysteroids can be found in the medium used to culture *C. congregata* larvae (Gelman *et al.* 1997) indicating that the wasp larvae may secrete substances into the host *in vivo*.

The presence of the parasitoid larvae is necessary and probably sufficient to induce a decline in host feeding. Injections of polydnavirus alone do not suppress feeding; in fact, virally infected *M. sexta* continue to feed, often becoming very large (12 g or more) before dying as larval–pupal intermediates (Dushay and Beckage, 1993; Reed and Beckage, 1997). Teratocytes injected without larvae or virus have a similar effect on the host which also continues to feed (Fig. 1). Only when parasitoid larvae were present did host feeding cease. However, we cannot exclude the possibility that we inadvertently transferred some teratocytes with the *C. congregata* larvae during our implantations. Not only is the presence of the polydnavirus not required for the suppression of feeding in the host, but it is also not necessary for the increase in octopamine levels that occurs concomitantly with the change in host behaviour since implanted parasitoids were able to induce an increase in host octopamine levels comparable to that seen in naturally parasitized hosts.

After parasitoid emergence, elevated octopamine titres in the host were correlated with decreased locomotion and feeding behaviour, and the effect on feeding could not be reversed by the octopamine antagonist phentolamine. However, in unparasitized *M. sexta*, as in other insects (e.g. see Davenport and Evans, 1984*a*,*b*), increased octopamine titres were correlated with increased locomotion, which is the inverse of the relationship found in *M. sexta* after the parasitoids emerged. Unlike the suppression of feeding in these post-emergent hosts, the depressive effect of octopamine on feeding in unparasitized *M. sexta* was blocked by phentolamine. This latter observation demonstrates that phentolamine is capable of blocking octopamine-induced changes in feeding behaviour. These data suggest that the increase in octopamine in parasitized *M. sexta* is unlikely to be the direct cause of changes in behaviour and raise the intriguing question of how hormonal octopamine becomes decoupled from activity in post-emergent *M. sexta*.

When acting as a hormone in the haemolymph, octopamine probably does not directly induce changes in central nervous system function (Adamo *et al.* 1995), unlike its effect when released within the neuropile. It appears that, while octopamine released within the central nervous system or as a local neuromodulator may induce an increase in behavioural state, hormonal octopamine may be only indirectly related to the increase in behavioural responsiveness, for example by facilitating the release of energy compounds from the fat body (see Orchard *et al.* 1993). Injections of octopamine into the haemocoel may increase activity in *M. sexta* because the unphysiologically large amounts injected affect receptors within the central nervous system. Behaviourally effective doses of octopamine injected into the haemocoel tend to be well above normal circulating levels (by a factor of 100–1000 times above circulating levels; e.g. Ismail and Matsumura, 1992). Raising neurohormonal levels of octopamine, therefore, is unlikely to lead directly to large changes in behaviour but could induce changes in behaviour indirectly, as we discuss below.

Food deprivation induces an increase in octopamine levels in the haemolymph of locusts (Davenport and Evans, 1984*a*) as well as in unparasitized *M. sexta* (Ismail and Matsumura, 1992; this study). Ismail and Matsumura (1992) showed that injections of octopamine elevated sugar (glucose and trehalose) levels in *M. sexta* and that, as also shown in the present study, very high octopamine levels suppressed feeding in *M. sexta*. They suggested that octopamine induces anorexia in unparasitized *M. sexta* by elevating haemolymph sugar levels and thereby activating a negative feedback loop inhibiting feeding. This postulated negative feedback loop could contribute to the lack of feeding in the post-emergent host with high levels of hormonal octopamine.

Another possible interpretation of our results is that octopamine levels may increase in post-emergent parasitized *M. sexta* because of the decline in feeding and locomotion as opposed to being causally involved in these changes. After the parasitoids emerge, the host lives for another 2 weeks without feeding and presumably relies on energy stores for survival. In support of this hypothesis, Dahlman (1970) found that trehalose levels remain constant in parasitized *M. sexta* after the parasitoids emerge. Even with their low rate of metabolism (Alleyne *et al.* 1997), trehalose levels would not be expected to remain stable in a non-feeding animal without any liberation of energy stores. At emergence, some fat body remains in the host, and this gradually atrophies (Beckage and Templeton, 1986), suggesting that the host does mobilise its energy stores after the parasitoids emerge. The observed elevation in octopamine levels may be the physiological response of the host to its non-feeding state and the levels may decline with

time as the dying host's energy demands decline. The lack of feeding and locomotion would then be due to some other parasitoid-dependent effect.

It is also possible that the octopamine found in the post-emergent animal comes from a non-neural source. Octopamine plays a role in mediating insect immune responses (Baines *et al.* 1992; Dunphy and Downer, 1994; Diehl-Jones *et al.* 1996) and octopamine concentration increases in the haemolymph during bacterial infection (Dunphy and Downer, 1994). Could *C. congregata* larvae trigger an immune response during emergence and thereby induce an increase in host octopamine levels? *C. congregata* larvae do not induce the immune response of encapsulation during their development; however, they do cause significant damage to the integument upon emergence including activation of the cuticular phenoloxidases and melanization at the wound site where the parasitoids penetrate the epidermis and cuticle (Alleyne and Beckage, 1997). Moreover, after most of the wasp larvae have emerged, those that do not vacate the host die and are encapsulated. Typically, hosts are left with some wasp larvae 'stranded' in the haemocoel that fail to initiate emergence (Beckage and Riddiford, 1978). The observed increase in octopamine levels may be related to these immunological events and the octopamine concentration may then decline with time as the immune response triggered during parasitoid emergence dissipates. However, although octopamine levels in the haemolymph (haemocytes plus plasma) have been shown to increase in response to a bacterial infection, it has yet to be shown that octopamine levels increase in the plasma during encapsulation, which is a different immunological response (see Ratcliffe, 1993, for a review).

Determining the causes of the changes in host behaviour will require a better understanding of the physiology of the host during parasitism. We know that intermediary metabolism is altered (Thompson, 1993) and that the rate of metabolism is reduced after the wasps emerge (Alleyne *et al.* 1997). However, we do not know the effect of parasitism on receptors for octopamine and other substances, the effect of parasitism on octopamine located within the central nervous system, lipid levels in parasitized *M. sexta*, or the source of the host's neurohormonal octopamine, to list a few areas in which more information is needed. Moreover, octopamine is not the only regulator of energy metabolism. The peptide adipokinetic hormone (AKH) is released during food deprivation in larval *M. sexta* and is thought to be important in regulating energy reserves in the fat body (Siegert and Ziegler, 1983; Ziegler, 1990). The relationship between octopamine and AKH in regulating energy metabolism is poorly understood in *M. sexta* and the potential effects of high levels of octopamine could be exacerbated or ameliorated by the effect of parasitism on the levels of peptides such as AKH. For a more complete understanding of such interactions, peptide levels will need to be measured as well.

Octopamine levels have been measured in the haemolymph of another parasitized insect. The common armyworm *Mythimna separata* is attacked by the parasitoid wasp *Apanteles kariyai*, and this host also shows an increase in octopamine levels during parasitism (Shimizu and Takeda, 1994). In this system, octopamine levels increase prior to parasitoid emergence. Shimizu and Takeda (1994) interpreted the increase in octopamine levels as a response to the 'stress' of parasitism. However, increases in octopamine levels due to 'stress' should correlate with an increased behavioural state, and this does not appear to be the case in the armyworm. It should be noted that these results are at odds with those of Noguchi *et al.* (1995) who found that octopamine levels were not changed during parasitism in the same host parasitized by the same species of wasp.

The suppressed feeding of the host may be advantageous for the *C. congregata* larvae since four out of five unparasitized *M. sexta* that had *C. congregata* pupae attached to their cuticle ate cocoons containing the pupae. Although this is not conclusive evidence, it does suggest that there may have been strong evolutionary pressure on the parasitoid selecting for *C. congregata* larvae able to suppress host feeding after parasitoid emergence. Killing the host immediately after emergence would be one obvious way of preventing host predation, but as the pupae remain attached to the host for 4–5 days, a decomposing host may increase pupal mortality by encouraging bacterial and/or fungal growth or the presence of scavengers. Interestingly, the host retains its defensive reflexes, such as the bite response to a pinch, after parasitoid emergence. These reflexes may help protect the pupae from predators and other parasitoids. Because the change in the host's behaviour has a plausible adaptive function, it seems reasonable to suggest that a specific physiological mechanism has evolved to induce the observed decline in feeding and locomotion. Further investigation of this system is likely to provide an increased understanding of the neural and hormonal mechanisms that are involved in regulating behavioural responsiveness in arthropods.

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