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RESEARCH ARTICLE

The parasitic wasp *Cotesia congregata* uses multiple mechanisms to control host (*Manduca sexta*) behaviour

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ABSTRACT

Some parasites alter the behaviour of their hosts. The larvae of the parasitic wasp *Cotesia congregata* develop within the body of the caterpillar *Manduca sexta*. During the initial phase of wasp development, the host's behaviour remains unchanged. However, once the wasps begin to scrape their way out of the caterpillar, the caterpillar host stops feeding and moving spontaneously. We found that the caterpillar also temporarily lost sensation around the exit hole created by each emerging wasp. However, the caterpillars regained responsiveness to nociception in those areas within 1 day. The temporary reduction in skin sensitivity is probably important for wasp survival because it prevents the caterpillar from attacking the emerging wasp larvae with a defensive strike. We also found that expression of *plasmatocyte spreading peptide* (*PSP*) and *spätzle* genes increased in the fat body of the host during wasp emergence. This result supports the hypothesis that the exiting wasps induce a cytokine storm in their host. Injections of PSP suppressed feeding, suggesting that an augmented immune response may play a role in the suppression of host feeding. Injection of wasp larvae culture media into non-parasitized caterpillars reduced feeding, suggesting that substances secreted by the wasp larvae may help alter host behaviour.

KEY WORDS: Cytokine, Illness-induced anorexia, Sickness behaviour, Immune gene expression, Numbness

INTRODUCTION

Parasitic manipulators are parasites that alter host behaviour, leading to an enhancement of the parasite's reproductive success (e.g. Thomas et al., 2005). Some parasitic manipulators have co-evolved with their hosts for millions of years (Hughes et al., 2011). These ancient interactions are often multifaceted (Thomas et al., 2010), with the parasite able to exert control over the host's immune system and endocrine system as well as its behaviour (Beckage and Gelman, 2002). How parasites alter host behaviour remains poorly understood (Adamo, 2013; van Houte et al., 2013; Chetouhi et al., 2015). However, recent evidence suggests that changes in host behaviour require multiple parasitic effects on host physiology (e.g. de Bekker et al., 2014; Libersat and Gal, 2014). These results are superficially surprising, because the most parsimonious method of exerting control over the host would be to attack discrete neuroanatomical and/or neuropharmacological targets (Adamo, 2013). We examined this issue by studying the mechanisms by which the parasitic wasp *Cotesia congregata* alters the behaviour of its host, the caterpillar *Manduca sexta*. In this system, the wasps exit from a still-active host. Unless the wasps suppress the caterpillar's feeding and defensive reflexes, the caterpillar will destroy them (Adamo, 1998).

One common mechanism used by parasitic manipulators is the alteration of immune–neural connections (Adamo, 2013). Immune–neural connections may be especially susceptible to manipulation because parasites must interfere with host immune systems to survive. Altering the signals the immune system sends to the host's nervous system may thus be but a small evolutionary step for many parasites (Adamo, 2013). Immune–neural connections benefit the host by activating behavioural changes that promote recovery from illness (i.e. sickness behaviour: Aubert, 1999; Dantzer, 2004). For example, factors released by the immune system (e.g. cytokines) result in a reduction in appetite in animals (Adamo, 2012a). This phenomenon is called illness-induced anorexia (e.g. insects: Adamo, 2008, Yamaguchi et al., 2012). In both mammals (e.g. Murray and Murray, 1979) and insects (Adamo et al., 2010), illness-induced anorexia aids host recovery. Therefore, by manipulating immune–neural connections in the host, parasites could alter behaviours such as host feeding, even if they have no direct access to the brain (Adamo, 2012b, 2013), i.e. the parasites do not directly influence neural function. The parasitic wasp *C. congregata* pupates from cocoons attached to the outside of its caterpillar host. Unless the wasp suppresses host feeding, the host will consume the cocoons (Adamo et al., 1997). Activation of immune responses, even in the absence of a live pathogen, suppresses feeding in *M. sexta* (Dunn et al., 1994; Adamo, 2005). Therefore, manipulation of immune–neural connections could suppress feeding in this host.

A decline in feeding is just one of a series of precisely timed physiological and behavioural changes that the wasp requires of its host in order to successfully complete its life cycle (see Beckage and Gelman, 2002, for a review of this system). The female wasp injects approximately 150 eggs (Beckage and Gelman, 2002) into the body of the caterpillar, along with venom and polydnavirus. The venom and polydnavirus suppress the ability of the caterpillar to destroy the wasp's eggs. The wasp larvae develop within the blood space of the caterpillar (haemocoel). Here, they consume nutrients from the host's blood (haemolymph), but they do not damage tissue. During this period, the caterpillar continues to feed and behave normally (Adamo et al., 1997; Miles and Booker, 2000). At the end of wasp larval development, the wasp larvae make a final moult to their 3rd instar as they emerge through the host's cuticle (Fulton, 1940). Prior to this developmental stage, the wasps are ignored by the caterpillar's immune system (Lavine and Beckage, 1996), but an immune response becomes activated during wasp emergence (Adamo, 2005). Wasps unable to exit the host are encapsulated by the host's immune cells (Adamo et al., 1997). Once outside the host, the wasp larvae spin cocoons and remain tethered to the host by a

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strand of silk (Fulton, 1940). The wasp larvae then pupate, and emerge as adult wasps 4–5 days later (Beckage and Templeton, 1986).

About 24 h prior to wasp emergence, host feeding and movement begin to decline (Beckage and Riddiford, 1978; Miles and Booker, 2000; Adamo et al., 1997). By the time the wasps are actively emerging, host feeding has been permanently suppressed (Adamo et al., 1997). The lack of locomotion and feeding is not due to host debilitation (Adamo et al., 1997). For example, removal of the supraoesophageal ganglion induces hyperkinesis after the wasps emerge (Beckage and Templeton, 1986). Moreover, the caterpillar's defensive strike reflex remains intact after the wasps emerge (Adamo et al., 1997). Why caterpillars do not strike at the emerging wasps remains unknown; we examined this issue in this paper. After the wasps emerge, the caterpillar host becomes the cocoons' bodyguard (Kester and Jackson, 1996), as is seen in other parasitoid systems (e.g. Grosman et al., 2008; Harvey et al., 2011). (A parasitoid is an insect that develops by feeding off the body of another arthropod; Godfray, 1994.) The host can live for up to 2 weeks after wasp emergence; however, the caterpillar ultimately succumbs to starvation (Kester and Jackson, 1996).

The wasp larvae are both necessary and sufficient to trigger the decline in feeding (Adamo et al., 1997; Beckage and Gelman, 2002). Implanting wasp larvae into 4th instar non-parasitized caterpillars leads to the same changes in host behaviour as occur with naturally parasitized caterpillars, even without the injection of venom and polydnavirus (Lavine and Beckage, 1996; Adamo et al., 1997). However, if venom, polydnavirus and teratocytes (cells derived from the hatching wasp larvae) are injected into a caterpillar without wasp larvae, the caterpillar continues to feed, becoming abnormally large because it is developmentally arrested at the larval stage (Beckage and Gelman, 2002). In many hosts, some wasp larvae remain behind after the other wasp larvae have left (Beckage and Riddiford, 1978). They may be important for maintaining the cessation of feeding during the days after the larvae have severed their physiological contact with its host (i.e. after wasp emergence) (Cooper and Beckage, 2010). We examined this issue in this paper.

The haemolymph concentration of the neurohormone octopamine increases during an immune response in *M. sexta* (Adamo, 2010), and it also increases dramatically at the time of wasp emergence (Adamo et al., 1997). Octopamine is an important part of the insect stress response (Roeder, 1999) and is also increased during an immune response (Dunphy and Downer, 1994). The increase in octopamine appears to play a role in the suppression of host feeding (Adamo et al., 1997). It has been shown to suppress peristaltic activity in the foregut (Miles and Booker, 2000), thereby interfering with the caterpillar's ability to swallow. However, elevated haemolymph octopamine concentrations alone do not result in a non-moving, non-feeding caterpillar. In fact, elevated octopamine levels tend to correlate with increased activity in most insects (e.g. Linn et al., 1994). Therefore, the wasp larvae need to do more than increase host haemolymph octopamine concentrations in order to produce the desired behavioural phenotype in their host.

An immune challenge also activates insect cytokines such as plasmatocyte-spreading peptide (Matsumoto et al., 2012; in *M. sexta*, Skinner et al., 1991; Eleftherianos et al., 2009) and spätzle (An et al., 2010). Insect cytokines are important regulators of insect immunity (e.g. Tsuzuki et al., 2012), and they are also involved with CNS function (Noguchi et al., 2003). Gene expression of insect cytokines is elevated during infection by some parasitic wasps (e.g. *Microplitis manilae*), and can also depress feeding when injected (Wang et al., 1999; Yamaguchi et al., 2012). Therefore, the wasps could depress host feeding behaviour by over-activating cytokines in the host (i.e. by producing a cytokine storm). We tested whether gene expression for cytokines is up regulated in the caterpillar during wasp emergence. We also measured the expression of three immune genes, *attacin-1*, *lysozyme* and *serpin-3*, to assess how the host's immune system responds to the presence of the wasps as well as to any observed changes in cytokine gene expression. For example, normal *M. sexta* increase expression of *attacin-1* (an antimicrobial peptide) and *lysozyme* (an antimicrobial protein) in response to increased Spätzle-1 (An et al., 2010). In this study, we demonstrate the multiple indirect mechanisms that contribute to the manipulation of a single host behaviour.

MATERIALS AND METHODS

Reagents

All chemicals are from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted.

Animals

Tests were carried out on fifth instar larvae of *M. sexta* (Linneaus 1763) obtained from colonies at either SUNY Binghamton or Dalhousie University. *Manduca sexta* larvae were parasitized by allowing *Cotesia congregata* (Say 1836) females to oviposit into 3rd instar larvae. After parasitization, larvae were returned to their individual chambers (7 cm×10.5 cm height), fed *ad libitum* on a wheatgerm-based diet (Binghamton colony, del Campo and Miles, 2003; Dalhousie colony, pre-prepared diet from Recorp Inc., Georgetown, ON, Canada) and allowed to develop. The wasps emerge during the caterpillar's 5th and final larval instar (de Buron and Beckage, 1997). For some studies, parasitized caterpillars were shipped to Dalhousie University. Colonies of both *M. sexta* (del Campo and Miles, 2003; Adamo et al., 2016) and *C. congregata* (Miles and Booker, 2000) were maintained as previously described.

The study was approved by the University Committee on Laboratory Animals (Dalhousie University; I-11-025) and was in accordance with the Canadian Council on Animal Care.

Are stranded wasp larvae required for host behavioural change?

The number of wasp larvae emerging from each parasitized caterpillar was recorded (N=35). One day later, caterpillars were dissected and the number of wasp larvae remaining (i.e. stranded) in the caterpillar was noted; 90% of wasp larvae emerge within the first 24 h of wasp emergence (Adamo et al., 1997). Cocoons from each caterpillar were collected and the eclosion rate was determined. All caterpillars showed the characteristic lack of spontaneous movement and feeding after wasp emergence. Caterpillars that harboured more than an ecologically relevant number of wasp larvae (i.e. more than 200: Fulton, 1940; Alleyne and Beckage, 1997) were excluded from the study.

Test of skin sensitivity during wasp emergence

Manduca sexta use their mandibles to strike at objects that activate skin sensory cells (called a defensive strike: Walters et al., 2001; McMackin et al., 2016). However, this strike would probably result in the death of the emerging wasp. To test whether this defensive strike is suppressed during emergence, we used von Frey filaments (Stoelting, Wood Dale, IL, USA) to deliver a known force to the skin, using a method modified from McMackin et al. (2016). At the first sign of wasp emergence (i.e. the protrusion of wasp mouthparts through the host body wall; see Table 1), caterpillars were removed from their container and placed on a small tray. Using the results

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Table 1. Stages in the *Cotesia*/*Manduca* system

	Stage 1 (12–24 h pre-emergence)	Stage 2 (wasp emergence)	Stage 3 (days post-emergence)
Wasp	• Secrete ecdysteroids • Migrate to body wall • Damage host fat body	• Scrape hole in host exoskeleton • Secrete proteins, including chitinase • Exit host • Spin cocoon	• No physical connection to host • Metamorphosis
Caterpillar	• Feeding slows	• Numbness around wasp exit hole • Wound melanization • Feeding stops • [OA] increases • Increase in *PSP+spätzle* pro-protein expression • Subtle CNS changes	• Sensation around wasp exit hole returns • Intact reflexes • No feeding • Gradual decline in [OA] • Decrease in *PSP+spätzle* pro-protein expression • Large CNS changes

Each stage requires a different behavioural phenotype from the host. For references, see text.
OA, octopamine.

from McMackin et al. (2016) and Walters et al. (2001), we started with a filament with a bending force of 9.8 mN. This filament elicits a defensive strike in 50% of control *M. sexta* (McMackin et al., 2016). The filament was pressed against the area within 1 mm of the emerging wasp at a 90 deg angle (i.e. perpendicular to the skin) until it bent. Whether or not this elicited a defensive strike was recorded. If there was no response, thicker filaments were used to a maximum bending force of 58.8 mN. Filaments larger than this depressed too wide an area, decreasing the precision of the stimulus. The process was repeated at a distance of 5 and 10 mm from the emerging parasitoid. A bright light and dissecting microscope were used to ensure that the direction was such that the filament was not contacting an area from which another parasitoid was emerging. If the caterpillar did not respond to the thickest filament, a score of +60 mN was given. Five spots (i.e. emerging wasps) were tested on 10 different caterpillars and the median score (i.e. threshold value) for the five spots was recorded as the score for that animal. One day after emergence, the sensitivity of the area within 1 mm of the emerged wasps of the same 10 caterpillars was re-tested. Unfortunately, we could not predict the precise locations on the caterpillar where the wasps would emerge. Therefore, those areas on the host could not be tested pre-emergence. For comparison, skin sensitivity of 10 control caterpillars (5th instar-day 2) was also tested.

Evidence that wasp larvae secrete material into their host

Wasp larvae were surgically removed from *M. sexta* caterpillars approximately 1 or 6 days prior to emergence and placed in sterile Petri dishes (35 mm×10 mm) containing 500 µl of Grace's media. Each dish held approximately 10 wasp larvae. Dishes were incubated for one day on a shaker under a sterile laminar flow hood. After the older wasps had moulted (at least 50% in each dish), 100 µl of media was injected into control 5th instar-day 1 caterpillars using a 100 µl Hamilton syringe. As a control, 100 µl of media from a dish with younger mid-instar wasp larvae was collected and also injected into 5th instar-day 1 caterpillars. As a final control, 100 µl of media without incubating wasp larvae was injected into 5th instar-day 1 caterpillars. Caterpillars were weighed before the injection as well as 24 h later.

To determine whether the wasp larvae secrete substances during emergence, wasp larvae that had begun to emerge (i.e. were aligned with their mouthparts scraping against the body wall) each had a small strip (0.5 mm×1 mm) of no. 2 Whitman filter paper placed into their mouthparts. After 15 min, the filter strips were collected and soaked for 15 min in 100 µl of sample buffer (0.5 mol l^{-1} Tris-HCl pH 6.8, 4% SDS, 25% glycerol, 0.01% Bromophenol Blue). Filter paper was removed and samples were stored at −80°C.

The supernatant was added in an equal volume of sample buffer, heated at 95°C for 5 min and loaded onto a 12% SDS-PAGE gel and electrophoresed at 150 V for 90 min (Laemmli, 1970). Broad-range unstained molecular markers were run on each gel (Bio-Rad, Hercules, CA, USA). Proteins were visualized using silver staining (Swain and Ross, 1995) and spots of interest were excised from the gel for identification by mass spectrometry, as follows. Excised spots were placed separately into 1.5 ml Eppendorf tubes, washed 3 times each for 10 min with 100 µl of 50% acetonitrile (ACN) in 25 mmol l^{-1} NH_4HCO_3 and then dehydrated in 200 µl of 100% ACN for 10 min. Proteins were reduced with 200 µl of 10 mmol l^{-1} dithiothreitol (DTT) in 25 mmol l^{-1} NH_4HCO_3 at 56°C for 1 h, followed by alkylation in 200 µl of 25 mmol l^{-1} NH_4HCO_3 containing 55 mmol l^{-1} iodoacetamide in the dark at room temperature for 45 min. The reduced and alkylated gel pieces were then washed 2 times with 100 µl of 25 mmol l^{-1} NH_4HCO_3 and 50% ACN with 25 mmol l^{-1} NH_4HCO_3 for 10 min. Gel pieces were dehydrated with 200 µl of 100% ACN for 20 min and placed in a speed vac for 10 min to remove residual ACN. Gel pieces were rehydrated by adding 20 µl of 12.5 ng ml^{-1} trypsin in 25 mmol l^{-1} NH_4HCO_3 and incubated at 37°C overnight. Finally, the tryptic peptides were eluted from the gel with two extractions using 20 µl of 5% formic acid and concentrated using a speed vac. All samples were analysed by LC/MS/MS using an LC Packings HPLC system equipped with a 5 cm×300 µm PepMap C_{18} column. Separation was carried out using a linear gradient from 10% to 50% B over 20 min (A: 5% ACN, 0.5% formic acid, B: 90% ACN, 0.5% formic acid) at 5 ml min^{-1}. The HPLC was interfaced to an Applied Biosystems (Waltham, MA, USA) MDS SCIEX Q TRAP mass spectrometer via a nanoflow source. Data were acquired in the information-dependent acquisition mode. The specific mass to charge ratio (m/z) values of the tryptic peptides was measured using a high resolution scan (250 amu s^{-1}). This scan was used to generate a peak list of peptides for tandem MS analysis. The tandem MS spectra were submitted to the database search program MASCOT (Matrix Science, London, UK) in order to identify the proteins. Additionally, sequences were searched against NCBI and SWISSPROT.

Changes in immune gene expression

RNA extraction and cDNA generation

Abdominal fat body makes the majority of immune proteins in *M. sexta* (Zhang et al., 2014), including the cytokine plasmatocyte-spreading peptide (PSP; synthesized in an inactive pro-form; Wang

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et al., 1999) and the pro-form of another insect cytokine, spätzle (An et al., 2010). Control fat body was excised from 5th instar-day 2 larvae. Although there is no evidence that the fat body in *M. sexta* contains different compartments (Willott et al., 1988), sampling was always taken from fat body adhering to the left body wall within abdominal segments 6 and 7.

To assess the effect of the wasps on host immune gene expression, fat body was also collected from parasitized *M. sexta*: (1) approximately 3 days prior to the predicted day of wasp emergence, (2) at the time of wasp emergence, (3) 1 day after wasp emergence and (4) 3 days after wasp emergence (Table 1).

To determine the typical effect of a microbial immune challenge on normal, non-parasitized fat body, non-parasitized 5th instar-day 1 larvae were injected with a 60 µl mixture of heat-killed *Serratia marcescens* (Gram negative bacterium, 1/10 LD50 dose, Microkwik culture, Carolina Biological, Burlington, SC, USA). The LD50 doses had been determined during an earlier study (Adamo et al., 2016). Fat body was collected 1 day (5th instar-day 2) and 3 days (5th instar-day 4) after the injection.

To mimic the effect of an immune challenge by a multicellular organism (e.g. a wasp larva), 5th instar-day 1 caterpillars were injected with 20 µl of 25 mg 500 μl^{-1} A25 Sephadex beads in PBS using the methods described by Lavine and Beckage (1996). These beads lead to an encapsulation response similar to that induced by stranded wasp larvae (Lavine and Beckage, 1996). Sephadex beads were disinfected in 95% ethanol prior to injection. Fat body was collected from caterpillars 1 day later (5th instar-day 2) or 3 days later (5th instar-day 4).

To control for handling and injury stress, fifth instar-day 1 caterpillars received a sham injection by piercing them with a dissecting pin disinfected in 95% ethanol. Fat body was collected 1 day later (5th instar-day 2) and 3 days later (5th instar-day 4). Because immune function changes with age in *M. sexta* (Beetz et al., 2008; Booth et al., 2015), we also collected fat body from control 5th instar-day 4 caterpillars to test whether any observed changes in gene expression in 5th instar-day 4 caterpillars could be attributed to ageing.

Fat body tissue was immediately stabilized in RNA*later* (Qiagen, Hilden, Germany) and stored at −80°C. Tissues were lysed with Qiazol (Qiagen) and disrupted using a TissueRuptor (Qiagen). RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen). Samples were processed for RNA extraction and cDNA generation following guidelines to preserve sample quality (Taylor et al., 2010). Steps adhered to the manufacturer's instructions and included a DNase 1 treatment (RNase-Free DNase, Qiagen) step to remove genomic DNA contamination. The integrity of total RNA samples was assessed using denaturing 'bleach gel' electrophoresis (Aranda et al., 2012). The purity and concentration of extracted total RNA was determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only samples with an A_{260}/A_{280} ratio greater than 1.8 were used. The concentration of extracted total RNA samples ranged from 10 to 380 ng μl^{-1}. cDNA was synthesized using iScript (Bio-Rad). cDNA concentration was assessed using Qubit 1.0 fluorometer and Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). Sample cDNA concentration was normalized to 100 ng μl^{-1} and samples were stored at −80°C.

Quantitative real-time PCR

To determine the relative expression of immune-related genes in the fat body relative to controls, cDNA levels were measured by quantitative real-time PCR (qPCR) following methods developed for this species in our lab (Adamo et al., 2016). We used previously reported primer sets for *M. sexta* genes (Table 2) (Adamo et al., 2016). For reference gene selection, we tested *ubiquitin*, *glycerol-3-phosphate dehydrogenase* (*G3PDH*) and *βFTZ-F1*, all of which have previously been found to be relatively stable during immune challenges in *M. sexta* (Adamo et al., 2016). We used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes (Anderson et al., 2004), using the quantitative cycle (C_q) values of five biological samples for each candidate reference gene for each treatment. We found that only one gene showed significant stability under all conditions (*ubiquitin*). Combining ubiquitin with any other gene significantly degraded the stability score. Therefore, ubiquitin was used as the reference gene.

Primers were purchased from IDT (www.idtdna.com) and stored at −20°C at a working stock of 10 µmol l^{-1}. Mean efficiencies of target and reference gene primers were calculated by construction of a standard curve using serial dilutions of fat body cDNA. The efficiencies for the target and reference genes ranged from 0.9 to 1.0.

We confirmed primer specificity by end-point PCR followed by analysis on a 1.5% TBE agarose gel. PCR products were treated with ExoSap (Affymetrix, Santa Clara, CA, USA) and sequenced. Mega 4 (www.megasoftware.net/) and Bioedit (www.mbio.ncsu.edu) were used to edit sequence data and produce contiguous alignments. Homologous sequences were identified using BLAST searches (www.ncbi.nlm.nih.gov) with overlapping regions removed.

qPCR was performed following MIQE guidelines as described by Taylor et al. (2010) using the Bio-Rad iCycler CFX-96. For each biological sample and gene, we prepared a 25 µl reaction mixture containing 1 µl sample cDNA, 12.5 µl SYBR Green Supermix (Bio-Rad), 1 µl forward primer (10 µmol l^{-1}), 1 µl reverse primer (10 µmol l^{-1}) and 9.5 µl RNase-free dH_2O. Reactions were performed in 96-well plates with a CFX96 real-time system (Bio-Rad). The reaction proceeded as follows: initial denaturation (95°C: 3 min), followed by 45 cycles of denaturation (95°C: 30 s), annealing (52°C: 45 s) and extension (72°C: 30 s). In some trials, the annealing temperature was 54 or 50°C. PCR reactions were followed by melt curve analysis to ensure a single product and no-

Table 2. Forward and reverse primer sequences for target genes and reference genes

Primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Efficiency	Reference
ProPSP	ATGAAGTTATTTTTTATAGTT	TCAAAATGTAAGTTTGCATCT	0.95	Eleftherianos et al., 2009
ProSpätzle	AGTGACCAGTAAGCCAACAAC	CGAAGAGCCAAACGAGTAAATG	0.96	An et al., 2010
Attacin-1	GCAGGCGACGACAAGAAC	ATGCGTGTTGGTAAGAGTAGC	0.93	An et al., 2010
Lysozyme	GTGTGCCTCGTGGAGAATG	ATGCCTTGGTGATGTCGTC	1.0	An et al., 2010
Serpin-3	GATTCCTCGCGATTCGATGC	CATTTACGTCATTAAGTTTCATG	0.97	Zhu et al., 2003
*Ubiquitin**	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	0.98	Kumar et al., 2012
*βFTZ-F1**	CGTGCCTCCTACAATAGTGCTT	AATCCCTAGCGGTTACTGACC	1.0	MacWilliam et al., 2015
*G3PDH**	CGATTAAGGAACTTGAGGACG	ATAAGGAAGCGGATGCAAGG	1.0	Mészáros and Morton, 1996

Asterisks denote reference genes.

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template controls to eliminate primer-dimer errors. C_q values for each sample and gene target were calculated in CFX Manager (v. 3.1, Bio-Rad). Each biological sample was run with two technical replicates, and no-template controls were run for each gene target. Data were calculated as fold-change in expression of target genes in test animals against control (non-parasitized) animals using the Relative Expression Software Tool (REST, v. 1, 2009) program. The normalized expression ($\Delta\Delta C_q$) was calculated as the relative quantity of the target gene normalized to the quantities of the reference genes. We did not find any significant differences in the expression of the genes tested between control 5th instar-day 2 and 5th instar-day 4 *M. sexta*. Therefore, all gene expression was tested relative to 5th instar-day 2 controls so that all groups would be compared against a common control.

Evidence that the insect cytokine PSP can suppress feeding

PSP was synthesized by GenScript (Piscataway, NJ, USA) based on the sequence ENFAGGCATGYLRTADGRCKPTF (Skinner et al., 1991; Wang et al., 1999). In 5th instar *M. sexta*, the concentration of PSP is 1.2 µg ml^{-1} haemolymph (Skinner et al., 1991), although the level of naturally activated PSP is unknown. High doses (e.g. 1 µg per caterpillar) of synthetic PSP (active form) induce physiological abnormalities such as paralysis and rupture of the hindgut. We therefore used a range of doses (0.1 to 0.3 µg per larva) that did not induce these abnormalities.

Caterpillars were used for injections within 1 day of their moult to the fifth instar (5th instar-day 1). They were anaesthetized by placing them on ice for about 10 min. Each member of a weight-matched pair was injected with either 30 µl of sterile saline or 30 µl of sterile saline with PSP. Phenylthiocarbamate was applied to the tip of the needle of both groups to prevent activation of the peptide by oxidation. After injection, the larvae were returned to the ice for an additional 10–20 min. Each larva was then placed into a separate container with a pre-weighed piece of diet, and left undisturbed in an environmental chamber at 27°C. Caterpillars and their food were weighed daily for the next 2 days.

Statistical analysis

Data were analysed using SPSS (v. 22.0) and GraphPad Prism (v. 5.0); qPCR data were analysed using CFX Manager v. 3.1 (Bio-Rad) and the REST program (2009; http://rest.gene-quantification.info). The REST program uses a randomization (Bootstrapping) technique in which 10,000 reallocations of the sample values are used to determine how often results as extreme as those observed would occur by chance. Other data were tested for normality using Shapiro–Wilk tests. *Post hoc* treatment comparisons used Dunnett's test. Data that were not normally distributed were analysed using non-parametric tests. Sample sizes were determined based on pilot studies for the skin sensitivity and qPCR studies. For the other experiments, sample size calculations were based on literature values.

RESULTS

Are stranded wasp larvae required for host behavioural change?

Wasps successfully eclosed as adults even if no wasp larvae remained stranded inside the host (2/35 caterpillars). Caterpillars without stranded larvae also stopped feeding (N=2). The greater the number of wasp larvae inside a host, the greater the number of stranded wasps (Pearson's correlation, r=0.54, P=0.0012, N=35 caterpillars). The average total number of wasp larvae per caterpillar was 108.5±43.8 and the average number of stranded larvae was 17.7±27.5 (N=35 caterpillars).

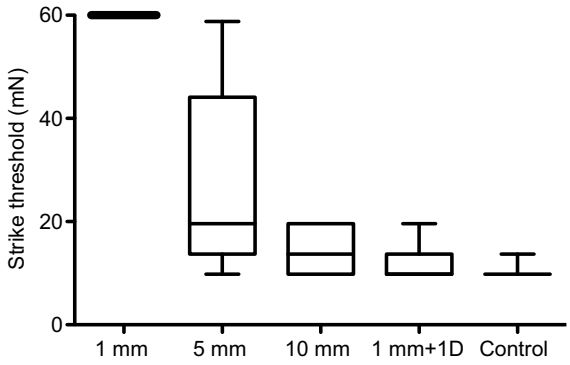

Fig. 1. Threshold for a defensive strike. Box and whisker plot. Bars represent first and third quartiles, with the internal line representing the median. Error bars denote maximum and minimum values; 1 mm, 5 mm and 10 mm represent the distance between the stimulus and emerging wasp; 1 mm+1D represents caterpillars tested 1 mm from an emerged wasp, 1 day after emergence. No caterpillars made a defensive strike when tested 1 mm from an emerging wasp. Sample sizes: N=10 parasitized caterpillars, N=10 controls.

Test of skin sensitivity during parasite emergence

On the day of emergence, caterpillars made no response when stimulated within 1 mm of an emerging wasp (0/10 caterpillars). However, caterpillars responded with a defensive strike if stimulated 5–10 mm away from the emerging wasp (Fig. 1; Friedman test=16.63, P<0.0001, N=10 caterpillars). One day later, caterpillars had regained their sensitivity in the areas from which the wasps had emerged (Fig. 1; sign test, P=0.002, N=10). Control caterpillars responded reliably to the von Frey filaments with a defensive strike (10/10 caterpillars; Fig. 1).

Evidence that wasp larvae secrete material into the host

The 5th instar-day 1 caterpillars injected with media from culture dishes containing wasp larvae that had moulted to the final instar gained less mass (N=14) than those injected with media containing mid-instar wasp larvae (N=10), or media that held no wasp larvae (N=22) (1-way ANOVA, $F_{2,43}$=18.9, P<0.0001, using initial body mass as a covariate in the analysis; Fig. 2).

Filter paper strips taken from the mouth of emerging wasps (N=4) showed at least eight distinct bands that were found across replicates (see Fig. S1, Table S1). Of the proteins identified, at least one appeared to be from the wasp because it was identified as a hymenopteran protein. It was a chitinase-like protein (*Bombyx mori*, accession no. Q9GV28; see Table S1). The other identified proteins were from the host (Table S1). No proteins identifiable as known hymenopteran venom proteins were found.

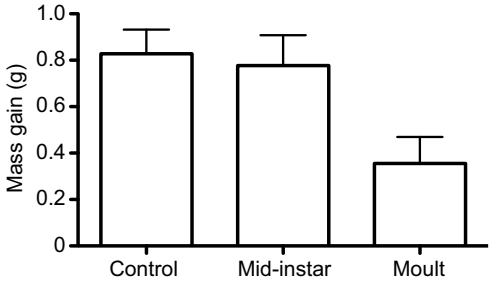

Fig. 2. Mass gain 24 h after injection with culture media, controlling for initial mass. Bars denote means and error bars represent s.e.m. Sample sizes: control, N=22; media containing mid-instar wasps, N=10; media containing moulting wasps, N=14.

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Changes in immune gene expression

A sham challenge (sterile poke) had no effect on immune gene expression 1 day (Fig. 3A) or 3 days (Fig. 3B) later.

The presence of the wasps altered cytokine gene expression. Expression of *PSP* was significantly increased relative to 5th instar-day 2 controls (N=6) at the time of wasp emergence (Fig. 3A; P=0.003, N=6). It was significantly depressed prior to wasp emergence (P=0.001, N=5) and 1 day (P=0.001, N=5) after wasp emergence. *Spätzle* gene expression was marginally elevated at emergence (Fig. 3A; P=0.06, N=6). Expression of *PSP* and *spätzle* was not significantly different from controls 3 days after wasp emergence (N=6; Fig. 3B).

The presence of the wasps also altered the expression of other immune genes. In parasitized *M. sexta*, *attacin-1* expression was elevated at the time of wasp emergence (Fig. 3A; P=0.03, N=6). *Lysozyme* gene expression was reduced in caterpillars pre-wasp emergence (P=0.006, N=5). *Serpin-3* gene expression was reduced prior to wasp emergence (Fig. 3A; P=0.001, N=5) and 1 day (Fig. 3A; P=0.01, N=5) and 3 days (Fig. 3B; P=0.002, N=7) post-emergence.

Immune challenge altered immune gene expression. *Attacin-1* gene expression was elevated 1 day after challenge with either heat-killed bacteria (P=0.004, N=6) or Sephadex beads (Fig. 3A; P=0.007, N=6). There was no increased expression relative to controls 3 days after challenge (Fig. 3B; N=6). *Lysozyme* gene expression was elevated in immune-challenged caterpillars (heat-killed bacteria, P=0.006, N=6; Sephadex beads, P=0.003, N=6), 1 day, but not 3 days (N=6), after the challenge. However, there were no significant changes in *PSP* expression or *spätzle* expression 1 day (N=6) or 3 days (N=6) after caterpillars were challenged with either heat-killed bacteria or Sephadex beads (Fig. 3).

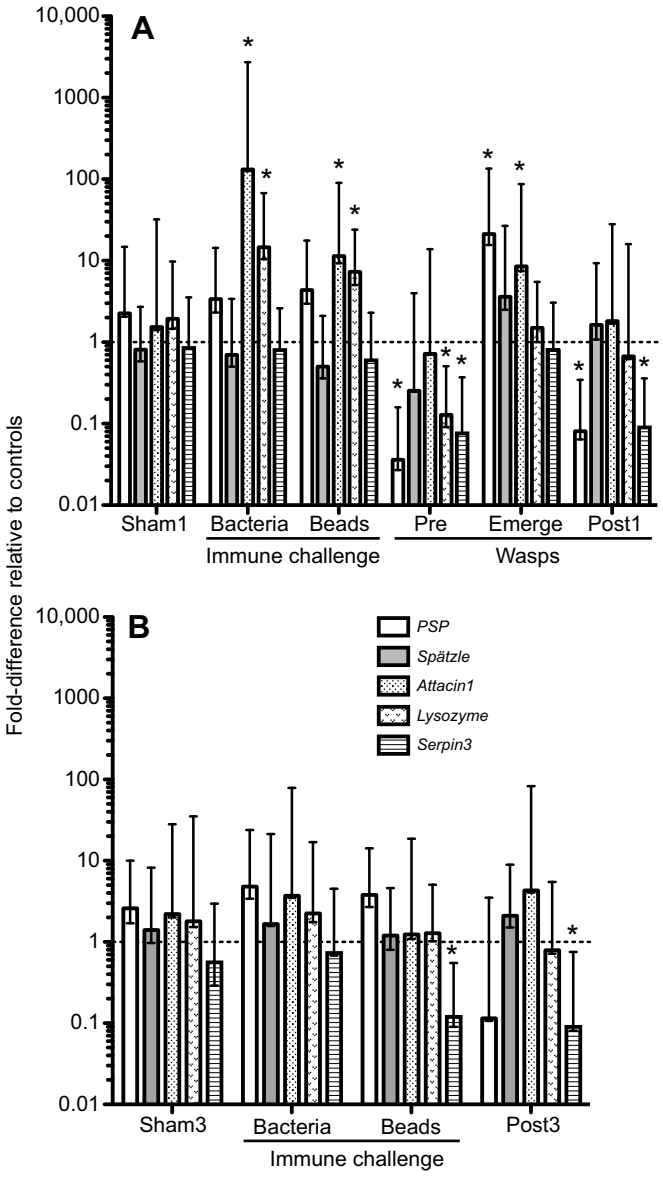

Fig. 3. Gene expression during parasitism and immune challenge. Bars are means and error bars denote 95% confidence intervals. The dashed line denotes the normalized control value. Asterisks denote values significantly different (P<0.05) from control (5th instar-day 2 caterpillars, N=6). (A) Effects of the wasps on caterpillar gene expression approximately 3 days prior to wasp emergence (Pre), during wasp emergence (Emerge) and 1 day post-emergence (Post1). The effects of a sham challenge (sterile poke, assessed 1 day later; Sham1) and two different immune challenges (heat-killed bacteria and Sephadex beads, assessed1 day post-challenge) are included for comparison. (B) Effects of the wasps on caterpillar gene expression 3 days after wasp emergence (Post3). The effects of a sham challenge (sterile poke, assessed 3 days later; Sham3) and two different immune challenges (heat-killed bacteria and Sephadex beads, assessed 3 days after the challenge) are included for comparison.

Evidence that insect cytokines can suppress feeding

Caterpillars injected with PSP (0.1 µg, N=10; 0.2 µg, N=16; 0.3 µg, N=26) reduced weight gain compared with controls (N=20) (Fig. 4; 1-way ANOVA, $F_{3,68}$=2.85, P=0.04, controlling for initial mass). Caterpillars injected with 0.3 µg PSP (N=26) gained significantly less mass than controls (Dunnett's q=2.442, P<0.05). Similarly, caterpillars given the 0.3 µg dose (N=26) ate less than controls (N=20) ($F_{3,68}$=2.78, P=0.04, controlling for initial weight, *post hoc* comparison, Dunnett's q=2.563, P<0.05). There was no significant effect on weight gain 2 days after injection ($F_{3,68}$=0.6, P=0.62).

DISCUSSION

Manduca sexta's main occupations as a caterpillar are eating and protecting itself from attack. The parasitic wasp manipulates both of these drives as it exits the body of its host. It desensitizes the section of the body wall that is damaged during wasp emergence, preventing the expression of the host's defensive strike. This desensitization probably prevents the caterpillar from killing the wasps as they exit. The caterpillar is numb, but not paralysed, and will respond to nociceptive stimuli elsewhere on the body (Fig. 1). The localized desensitization is temporary, however. Within a day, the caterpillar's defensive strike recovers and it becomes a bodyguard for the cocoons (Kester and Jackson, 1996).

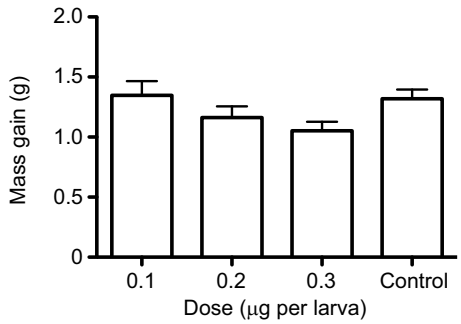

Fig. 4. PSP reduces mass gain. Bars denote means and error bars denote s.e.m. Sample sizes: 0.1 µg, N=10; 0.2 µg, N=16; 0.3 µg, N=26; control, N=20.

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Manduca sexta can also kill the wasps by eating the cocoons (Adamo et al., 1997); therefore, suppression of host feeding is critical for successful wasp development. The wasps appear to suppress host feeding, in part, by activating a massive immune response as they exit the host (Adamo et al., 1997; Adamo, 2005). Supporting this hypothesis, we found evidence that the exiting wasps activate a cytokine storm. We found an increase in gene expression of *PSP* and a marginal increase for *spätzle* (Fig. 3). If the increased gene expression during wasp emergence leads to greater cytokine protein production and activation, then this would result in a surge in cytokine levels (i.e. a cytokine storm). Given our results (Fig. 4), such large amounts of activated PSP would suppress feeding.

Because we did not measure the concentration of activated PSP and spätzle, we do not have direct evidence that cytokines were activated at the time of wasp emergence. Increases in gene expression do not always lead to increased protein production (Zhang et al., 2014). Moreover, even if the proteins were produced, the pro-proteins need to be activated (Zhong et al., 2012). However, the dramatic drop in haemocyte number that occurs during wasp emergence supports the hypothesis that cytokine levels are high at this time (Adamo, 2005). Cytokines like PSP reduce circulating haemocyte numbers and alter haemocyte behaviour (Wang et al., 1999). Further evidence includes the increase in *attacin-1* gene expression that occurs during wasp emergence (Fig. 3). *Attacin-1* gene expression is initiated by the toll receptor pathway, and activated spätzle is a ligand for this receptor (An et al., 2010).

The increased expression of cytokine pro-proteins in the fat body of parasitized *M. sexta* may be an unusual response to immune challenge. Immune challenge had no effect on the expression of the spätzle pro-protein in non-parasitized *M. sexta* (Fig. 3); a similar result has also been found in another study (An et al., 2010). A previous study has found a modest increase in *PSP* gene expression after an immune challenge (approximately 2.6 times; Gunaratna and Jiang, 2013), although we did not find a significant increase (Fig. 3B). In other words, the exiting wasps seemed to induce an unusually large response compared with other immune challenges. The injection of heat-killed bacteria is admittedly a limited, artificial challenge, but the injection of Sephadex beads will elicit the same type of response as that by the wasp larvae at the time of emergence (Lavine and Beckage, 1996). Therefore, it is possible that the wasps do more than simply activate a normal immune response as they exit the host. They may manipulate (i.e. augment) the response for their own advantage. The increase in pro-cytokine gene expression may be part of that manipulation.

Gene expression of another insect cytokine, stress-responsive peptide (*SRP*), increases in the cutworm *Spodoptera litura* soon after parasitism by the wasp *Microplitis manilae* (Yamaguchi et al., 2012). *SRP* gene expression increases in the cutworm after various forms of damage (e.g. a cut leg) and is thought to be a host response to parasitism (Yamaguchi et al., 2012). SRP reduces growth and feeding and these changes do not appear to benefit the parasite (Yamaguchi et al., 2012). Therefore, in this system, SRP appears to be released by the host as part of its defence against the parasite. However, other parasitic wasps appear to exploit host cytokines for their own benefit (Hayakawa et al., 1998). We unexpectedly found a decline in *PSP* expression prior to wasp emergence and again after wasp emergence. The function of this decline remains unknown. By controlling the concentration of cytokines, parasites can exert a powerful control over host behaviour.

Octopamine also activates cell-mediated immunity, enhancing haemocyte mobility and phagocytosis (Huang et al., 2012). Combined with the PSP response (Wang et al., 1999; Eleftherianos et al., 2009), these two compounds would augment cell-mediated immunity. Such haemocyte activation probably decreases the risk of the host bleeding to death from the wasps' exit wounds or acquiring an infection through them. An increase in antimicrobial peptide production (e.g. attacin-1) would also reduce the risk of infection. A dead host results in dead wasps (Adamo, 1998); the wasps need a living bodyguard to successfully eclose. Octopamine and the cytokines probably help keep the host alive for the wasps, as well as depressing feeding.

Metabolic rate slows after wasp emergence, possibly as a response to the caterpillar's non-feeding state (Alleyne et al., 1997). This reduction may explain, in part, the decline in *serpin-3* gene expression 3 days after wasp emergence. However, other genes, such as *attacin-1* and *lysozyme*, were not significantly affected (Fig. 3B). It is interesting that it is an inhibitor (i.e. *serpin-3*) of the phenoloxidase pathway, a critical component of insect immunity (Gonzalez-Santoyo and Cordoba-Aguilar, 2012), that is reduced. Inhibiting the inhibitor may be an energetically efficient way of squeezing greater immune function out of a resource-challenged host (Adamo et al., 2016).

We predict that any parasitic mechanism of manipulation that requires an energetic investment by the wasp must benefit that individual wasp larva, and not create benefits solely for the group. Even though parasitic wasps are haplodiploid (Chapman, 1998), the relatedness among offspring will vary (e.g. between brothers and sisters). This system sets up the potential for conflict among siblings regarding investment in host manipulation. Instead of expending energy manipulating the host, 'cheating' wasp larvae could use that energy for growth, enhancing individual fitness. The cheater could rely on the efforts of its siblings instead. Perhaps for this reason, the mother wasp seems to bear the brunt of the cost for host manipulation in this system and supplies not only the eggs but also the venom and polydnavirus (Beckage and Gelman, 2002). We predict that the manipulation costs that the wasp larvae bear individually will be those necessary for the survival of the individual wasp larva (e.g. secretions to numb the body wall of the host). For example, the wasp larvae appear to produce a chitinase during emergence. Chitinases are a component of some wasp venoms (Moreau and Asgari, 2015). A chitinase could help the wasps dissolve the chitin of the host's exoskeleton, something each wasp must do in order to emerge from the host. If the larvae do secrete something that augments cytokine activation in the host, we suspect that such a substance will also promote individual survival, as well as suppress feeding (Fig. 4). For example, a substance that promoted cytokine activation probably aids clot formation in the hole left by an individual exiting wasp. If the hole does not clot, the leaking haemolymph will prevent the silk strand from holding the cocoon to the caterpillar and the wasp will be lost (S.A.A., personal observation).

Some parasitoids consume the entire host, precluding the need for host behavioural manipulation at the end of the parasitoid's development (Godfray, 1994). However, these parasitoids lose the opportunity to use the host as a bodyguard (Harvey and Malcicka, 2016) during the parasitoid's vulnerable pupal stage (e.g. Kester and Jackson, 1996). Even among wasp parasitoids from the same genus, the host's post-emergent behaviour can vary (Harvey et al., 2008). This observation suggests that examining differences in behavioural modification mechanisms across wasp species could be useful in determining the key evolutionary steps that led to specific changes in host behaviour (Ponton et al., 2006).

This study suggests that host feeding is suppressed via multiple mechanisms (e.g. mediated by both increased octopamine and PSP;

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Table 1). It is likely that even more mechanisms contribute to the cessation of host feeding (e.g. Adamo and Shoemaker, 2000). In non-parasitized animals, immune challenge induces only a temporary reduction in feeding, not a permanent cessation (Adamo et al., 2007), suggesting that additional wasp-induced mechanisms are needed. Moreover, 3 days after the wasps emerge, the intensity of immunohistochemical staining for a wide range of neuropeptides within the host's supraoesophageal ganglion increases sharply (Zitnan et al., 1995). These changes do not occur in age-matched controls or in starved caterpillars (Zitnan et al., 1995). Some of the neuropeptides affected by the wasps (e.g. allatotropin; Zitnan et al., 1995) are important for feeding regulation in insects (Audsley and Weaver, 2009). These results suggest that the wasps exert effects on the central nervous system that are long lasting (Zitnan et al., 1995). Other parasitic wasps have been shown to affect the central nervous systems of their hosts (e.g. Dheilly et al., 2015; Shi et al., 2015).

Therefore, *C. congregata* wasps appear to use complex, multifactorial mechanisms to alter host behaviour (Table 1). Some of this complexity may be the result of the gregarious nature of the parasitoid. This gregariousness may reduce selection for the secretion of compounds that have direct neural action (e.g. reduced feeding). Such a system would select for cheaters. However, indirect, multipurpose methods that also benefit individuals might enjoy a greater selective advantage, even though this will lead to complex methods of behavioural control. This paper provides evidence that the *C. congregata*/*M. sexta* system exhibits: (1) exploitation of immune–neural connections, (2) genomic/proteomic changes in the host, and (3) multiple, seemingly redundant, mechanisms to influence a single behaviour. Increased understanding of these mechanistic details will provide us with a novel perspective on the neural mechanisms regulating behaviour.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
S.A.A. and C.I.M. developed the question. S.A.A., C.I.M., R.H.E., I.K. and K.F.T. developed the necessary methods and performed the studies. S.A.A. analysed the data and wrote the paper. C.I.M., R.H.E., I.K. and K.F.T. critically read and revised the manuscript.

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Supplementary information
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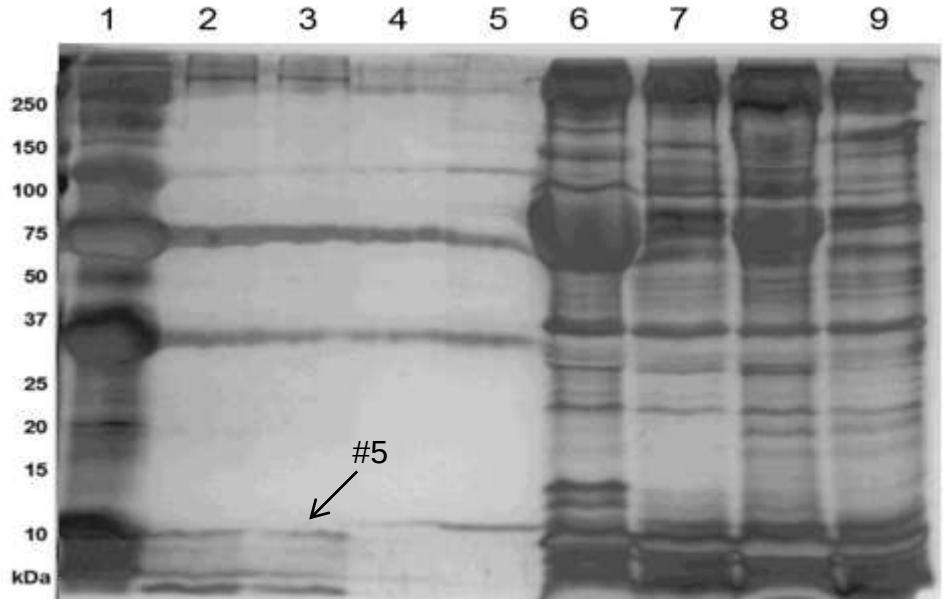

Figure S1. SDS-PAGE of proteins found in the mouth of emerging *Cotesia congregata* wasps. Lane 1 is unstained broad range molecular mass marker, lane 2-3 is filter paper containing material from the mouthparts of emerging *C. congregata* larvae, lanes 4, 5 are filter paper alone. The remaining lanes (6-9) are proteins from blood of *Manduca sexta* (for future analyses). The arrow denotes band #5. This band held the only hymenopteran protein identified (chitinase-like protein). The molecular weights (in kilodaltons) for the protein bands sent out for identification were approximately: 250, 75, 50, 25, 8, 6, 5, 2 (see supplementary Table 1).

Table S1. Proteins found in the mouth of emerging *Cotesia congregata* wasps. The proteins were identified by mass spectrometry and blastp. (also see supplementary Fig. 1).

Band number	Approximate Molecular Weight of Band (kilodaltons)	Protein Identity from Blastp	Species
1	250	Apolipophorin Transferrin Alaserpin	*Manduca sexta* “ “ “ ”
2	75	Insecticyanin	*Manduca sexta*
3	50	Apoliphorin	*Manduca sexta*
4	25	Apoliphorin	*Manduca sexta*
5	8	Apoliphorin Chitinase-like protein	*Manduca sexta* *Bombyx mori*
6	6	Insecticyanin	*Manduca sexta*
7	5	Apolipophorin Glyceraldehyde-3-phosphate dehydrogenase	*Manduca sexta* *Bemisia tabaci*
8	2	Elongation factor 1 alpha	*Myzus persicae*