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The caterpillar *Manduca sexta* brain shows changes in gene expression and protein abundance correlating with parasitic manipulation of behaviour

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The parasitic wasp, *Cotesia congregata*, manipulates the behaviour of its host, the caterpillar *Manduca sexta*. The female wasp injects her eggs and a symbiotic virus (i.e. bracovirus, CcBV) into the body of its host. The host's behaviour remains unchanged until the wasps exit the caterpillar, and then the caterpillar becomes a non-feeding "bodyguard" for the wasp cocoons. Using proteomic, transcriptomic and qPCR studies, we discovered an increase in antimicrobial peptide gene expression and protein abundance in the host central nervous system at the time of wasp emergence, correlating with the change in host behaviour. These results support the hypothesis that the wasps hyperactivate an immune-neural connection to help create the change in behaviour. At the time of wasp emergence, there was also an increase in bracoviral gene expression and proteins in the host brain, suggesting that the bracovirus may also be involved in altering host behaviour. Other changes in gene expression and protein abundance suggest that synaptic transmission may be altered after wasp emergence, and a reduction in descending neural activity from the host's brain provides indirect support for this hypothesis.

Keywords Neuroinflammation, Polydnavirus, Neural activity, Parasitic manipulation, Feeding, Neuroimmunology, Cytoskeleton, Extracellular matrix

Parasitic manipulators are parasites that enhance their fitness by altering their hosts' behaviour[1]. Our understanding of how parasitic manipulators alter host behaviour has progressed rapidly in the last few years[2–4]. Parasitic manipulators can exploit existing neuroregulatory networks in their host[5–7]. They can remodel these networks by secreting chemicals, e.g. venoms[8,9] or toxins[3,10] and/or by inducing changes in gene transcription within the host's neurons and/or glia[6,11]. Immune-neural connections may be especially prone to exploitation because parasites are pre-adapted to manipulate immune signaling to survive within the host[5]. Parasitic manipulators typically impact multiple neuromodulatory systems simultaneously[8,12] across a range of neural networks[1]. Using this multi-targeted approach, parasitic manipulators produce reliable changes in host behaviour[13], although the details remain unclear.

The interactions between the parasitic wasp *Cotesia congregata* and its caterpillar host, *Manduca sexta*, have been studied for decades[14,15]. However, the effect of the wasp on the host's central nervous system (CNS) is still poorly understood, despite the pronounced changes in host behaviour[16]. Given the extensive background information available for this host-parasite system, as well as on the neurobiology of the host[17], a more detailed examination of the effects of wasp parasitism on the host's CNS promises to provide important insights into how the wasp exerts control over its host's neural function.

Female *C. congregata* wasps co-inject venom, a polydnavirus, and wasp eggs into the body of *M. sexta*[14]. The venom is not necessary for wasp development[18] or altering host behaviour[14]. The polydnavirus of *C. congregata*

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(*C. congregata* Bracovirus, CcBV) is a domesticated virus that has become incorporated into the wasp's genome[19]. This non-replicating virus is made in the wasp's ovaries[20] and acts as a gene delivery agent, inserting its genes into the host's genome[19]. CcBV attacks host tissues such as the fat body[21], allowing the wasp to alter host physiology, optimizing the caterpillar host for wasp larval development[14]. CcBV gene expression can also be found within the host CNS soon after wasp oviposition[22], and at least some of these genes are translated into CcBV proteins[23]. The genes of other polydnaviruses (e.g. *Microplitis demolitor* bracovirus) also show expression in the brains of their host (e.g. *Pseudoplusia includens* (Lepidoptera)[24]). However, whether polydnaviruses promote parasitic manipulation of behaviour is unknown. *M. sexta* caterpillars show normal feeding and locomotion behaviour during wasp larval development, despite CcBV activity and the numerous physiological and endocrinological changes that occur within the host[14]. However, approximately 1 day prior to the wasps' exit from the host, host feeding and spontaneous locomotion decline dramatically, never to recover[16]. Once the 50–80 wasp larvae scrape their way through the host's body wall, the wasps spin cocoons that remain attached to the cuticle of the caterpillar, and eclose as adult wasps 4 to 5 days later[14]. The caterpillar loses all self-generated behaviours after the wasps emerge; however, it retains its defensive behaviours[16,25]. Host defensive behaviours appear to be crucial for wasp survival[26,27]. In the field, cocoons have a much lower survival without their host[26,27] and *M. sexta* defensive behaviours are thought to to repel predator attacks on the cocoons[27]. However, a living host is also a threat. *M. sexta* caterpillars will eat wasp cocoons[16,26]. The wasp larvae avoid this problem by inducing anorexia in caterpillars during their emergence[25,28], and the host never feeds again[16]. The host eventually dies of starvation several days after the wasps emerge from its body[16]. In essence, the caterpillar becomes a non-feeding (i.e. anorexic) "bodyguard" for the wasps, able to defend itself, and, by extension, the cocoons, but unable to feed on them. In other words, the "bodyguard" phenotype is a non-feeding caterpillar with intact defensive behaviours.

Transforming an actively feeding caterpillar into an anorexic "bodyguard" requires changes in multiple behaviours, while at the same time ensuring that the mechanisms needed for survival and defensive behaviour remain functional. As expected, during the this phase the host's sensory and motor systems remain operational[16,29–32]. However, the ability of the host to initiate feeding and spontaneous locomotion is greatly reduced[32]. One of the most parsimonious ways for the wasp to induce the "bodyguard" phenotype is to exploit an existing host network that naturally produces a similar behavioural phenotype. For example, reduced feeding, decreased locomotion and heightened defensive behaviours are observed in *M. sexta* during an immune response, and these changes are thought to benefit the caterpillar[16,28,33,34]. The details of how sickness behaviours are activated and maintained in insects is poorly understood, however immune-neural connections appear to be involved[35]. During an immune response, insects release immunomodulators (e.g. octopamine[36], cytokines[35]). These immunomodulators can activate receptors in the brain[37], and this activation is thought to induce sickness behaviours such as illness-induced anorexia[38], a phylogenetically conserved host behaviour[39] that promotes recovery in *M. sexta*[40,41]. The wasp larvae activate a massive systemic immune response as they scrape their way through the host's bodywall[28]. Therefore, wasps could exploit an immune-neural connection to create an anorexic bodyguard[16,25]. However, there is no direct evidence that systemic immune activation in the body of the *M. sexta* caterpillar has an impact on the caterpillar brain, although nematode infections can increase the expression of antimicrobial peptide (AMP) genes in the brain of another lepidopteran, *Galleria mellonella*[42]. We use qPCR to test whether an immune response in the body of *M. sexta* also produces an increase in immune gene expression in its brain. Such an activation would help explain how the wasp larvae could alter the neural function of the caterpillar without physically contacting its CNS. We also perform proteomics and transcriptomics analyses of the *M. sexta* CNS to help determine whether neuroinflammation (i.e. excessive immune activation within the brain[43]) occurs in the host concomitant with the change in host behaviour. Neuroinflammation is known to alter synaptic transmission[44]. Neuroinflammation is common in the hosts of parasitic manipulators and may be critical for host manipulation in some systems[45–48].

CcBV activity within the CNS could also activate neuroimmunological responses and potentially cause neuroinflammation. If viral genes and proteins increase in the brain at the time of wasp emergence from the host, CcBV could also play a role in altering host behaviour. We examine whether there is a burst of CcBV gene expression and/or protein abundance at the time of host behavioural change. We further examine whether the expression of specific CcBV genes, and/or the presence of certain CcBV proteins, within the caterpillar CNS correlates with the host's change in behaviour. Such a temporal correlation would suggest that specific CcBV genes and/or proteins play a role in mediating host behavioural change[49]. CcBV gene expression remains measurable within the CNS for at least six days after wasp emergence[50], and, therefore, appears to persist for the duration of "bodyguard" behaviour.

Finally, changes in neural activity are required to produce changes in behaviour. Transcriptomics and proteomics alone cannot demonstrate that neural activity has been altered. Nervous systems have powerful homeostatic mechanisms to maintain neural circuit function despite perturbations in ion channel performance or neurotransmitter abundance[51,52]. Therefore, we assessed neural activity descending from the brain during different stages of parasitism, allowing us to correlate transcriptomic, proteomic and electrophysiological changes within the CNS of the host with the expression of the "bodyguard" phenotype. We predict that: (1) systemic immune activity results in increased immune activity in the brain of *M. sexta*, (2) immune activity within the brain increases dramatically with the change in host behaviour; (3) CcBV gene expression and protein abundance increase when host behaviour changes, and (4) the change in host behaviour correlates with decreases in descending neural activity from the brain. Examination of the correlations across the different measures will provide insight into how the wasps alter the host's brain to create the "bodyguard" phenotype.

Results and discussion
Overview
Changes in protein abundance (Table S1, Table S4) and gene expression (Fig. 1) within the CNS of parasitized *M. sexta* correlated with the stage of wasp development. Using the brains of parasitized caterpillars prior to the change in host behaviour (i.e. pre-emergent brains) as a baseline to remove the effects of parasitism without behavioural change, we found over 200 genes changed in expression as hosts adopted the "bodyguard" phenotype (Fig. 1), although the number of changes declined by three days after the wasps had emerged from the caterpillar (i.e. 3-Days Post emergence, Fig. 1). Interestingly, most of the changes in gene expression were an upregulation (Fig. 1), even though the host suffers a dramatic decline in its resting metabolic rate during the "bodyguard" phase (approximately 40%[53]). The change in host behaviour also correlated with the change in abundance of over 100 proteins within the CNS (Table S4). However, it should be noted that given the total number of genes and proteins (Fig. 1, Table S1), these products represent less than 5% of *M. sexta* genes and proteins. Unfortunately some of the genes showing the largest changes in expression could not be identified.

The burst in gene expression and change in protein abundance that occurs during wasp emergence probably requires a trigger from the exiting larvae. This trigger could be the massive immune response that occurs during wasp emergence (i.e. an immune-neural signal[28,31]), and/or the ecdysteroid pulse that occurs 1 day prior to host emergence[14], and/or secretions from the wasp larvae[31] themselves. There are receptors for immune signaling molecules[34] and ecdysteroids[54,55] in the *M. sexta* brain.
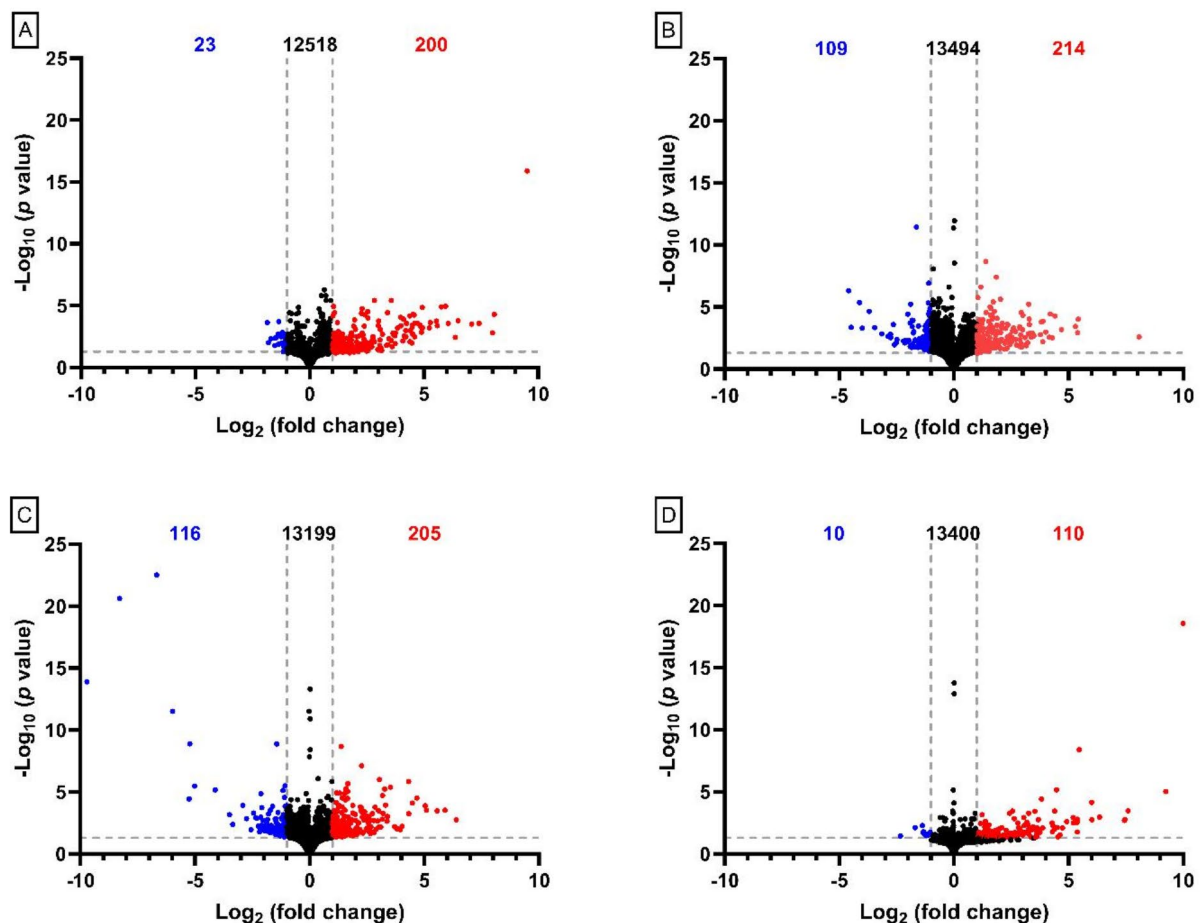

Fig. 1. Overall change in gene expression in the supraesophageal ganglion (i.e. brain) of *M. sexta* caterpillars. (A) Gene expression levels of Pre-emergence caterpillars compared to Unparasitized caterpillars. (B) Gene expression pattern of Emergent caterpillars compared to Pre-emergent caterpillars. (C) Gene expression pattern in 1-Day Post-emergent caterpillars compared to Pre-emergent caterpillars. (D) Gene expression pattern of 3-Days Post-emergent caterpillars compared to Pre-emergent. In all graphs the number of downregulated genes is indicated in blue on the top left of the graph, the number of upregulated genes is indicated by the red number in top right of each graph, and the number of unchanged genes is indicated in black in the top center. A grey horizontal dashed line indicates the significance cut-off for the false discovery rate of 0.05. Two grey vertical dashed lines indicate a 2-fold change, which was the chosen cut-off for significance.

Parasitism resulted in changes in the expression of genes and in the abundance of proteins related to gene transcription, cytoskeleton architecture, intracellular transport, the extracellular matrix and immune defense (Table S3, Table S4, S5, Figs. S1 – S23). These changes began prior to the change in host behaviour. However, there is a deepening in many of these changes as the caterpillar transitions into the "bodyguard" phenotype (e.g. Tables 1, 2, 3, 4, 5, 6, 7, 8). We focus on the changes in three physiological networks that we believe are most likely to be involved in creating the this phenotype.

Immune activity in the brain

Our results support our hypothesis that the "bodyguard" phenotype is produced, at least in part, by activating immune-neural connections. We confirmed that a systemic immune challenge in unparasitized *M. sexta* induced an increase in gene expression for 2 different antimicrobial peptides (*gloverin and attacin-1*) and a pattern recognition molecule (*hemolin*) in the brain (Fig. 2), correlating with the expression of illness-induced anorexia[28]. This result demonstrates that immune activity in the brain correlates with the expression of sickness behaviour. AMPs themselves may act as neural signals, although the details remain unclear[56,57].

The caterpillar exhibits a massive systemic immune response at wasp emergence[28,31] and, as predicted, parasitized caterpillar brains showed proteomic (Table 1), transcriptomic (Table 2) and qPCR (Fig. 2) evidence of increased immune activity at this time, compared with parasitized caterpillars prior to wasp emergence. Moreover, expression of CcBV genes (Table 3) and the abundance of CcBV proteins (Table 4) increased after the wasps emerge, and this increase could also initiate immune activity in the brain as a host response. Although there was also an increase in immune activity in the brain before the wasps emerge, compared with unparasitized controls (Fig. 2; Tables 1 and 2), caterpillar behaviour remained normal[31] during this smaller increase in immune activity within the CNS.

The large and long-lasting increase in immune activity that occurs in the brain of *M. sexta* after the wasps emerge (Tables 1 and 2) could be producing neuroinflammation. In mammals, increased immune-neural activity causes neuroinflammation[58], resulting in altered neural function[59]. Such hyperactivation of immune-neural connections could help produce this behavioural phenotype by heightening illness-induced anorexia and other sickness behaviours (e.g. lack of locomotion and enhanced defensive behaviours)[34]. In *D. melanogaster*, increased AMP production in the brain[60] and hyperactivation of immune pathways[61] also leads to neural damage. Neuroinflammatory damage is key to creating a bodyguard host in the *Dinocampus coccinellae-Coleomegilla maculata* wasp-ladybug system[46]. The exiting wasp larva induces an increase in the production of the wasp's symbiotic RNA virus within the host's brain[46] Electron microscopy of the *C. maculata* brain suggest that the increase in viral production damages the host brain, leading to a partially paralyzed, trembling host that sits on top of the wasp cocoon[46]. This behaviour is sufficient to repel arthropod predators, increasing wasp reproductive success[62]. However, such uncoordinated behaviour would not produce the bodyguard phenotype observed in *M. sexta*. *M. sexta* defends the cocoons using behaviours such as the defensive strike, that requires a coordinated motor response[63]. Moreover, the polydnavirus in *C. congregata* (CcBV) is non-replicating, and, therefore, cannot damage the brain in the same way as *D. coccinellae*'s RNA virus. Further studies are needed to determine the extent (if any) of neuroinflammatory damage and its location within the *M. sexta* CNS.

LOC#	Protein	Fold Change (log$_2$)			
		Pre-emergence	Emergent	1-Day Post	3-Days Post
LOC115447387	Attacin-1	**[3/0]**	**2.22**	**2.64**	**3.12**
LOC115453768	Attacin 2	**1.54**	-0.36	**3.29**	**2.38**
LOC115450302	Gloverin	**3.00**	-0.74	**2.09**	**1.86**
LOC115449303	Hemolin	**2.15**	-1.5	**2.47**	**1.95**
LOC115453674	Immulectin-2	**[0/3]**	[1/3]	[0/0]	[0/0]
LOC115456357	Immulectin-3	**[0/3]**	[0/0]	[0/0]	[0/0]
LOC119192142	Plasminogen activator inhibitor 2, macrophage-like	**[0/3]**	**[3/0]**	[0/0]	**[3/0]**
LOC115445410	Serpin-13	**[0/3]**	[0/0]	[0/0]	[0/0]
LOC115448700	Lysozyme	-1.11	0.34	**2.83**	**1.47**
LOC115443095	Immunolectin-A precursor	-1.24	[2/3]	**[0/3]**	[2/3]
LOC115453218	CLIP domain-containing serine protease 2-like	0.029	0.05	0.23	**2.21**

Table 1. Immune proteins identified as having significantly altered abundance in the brain of *M. sexta* during at least one time point during parasitism. Highlighted in red indicates an increased abundance at that timepoint, highlighted in blue indicates a reduced abundance at that timepoint. To be considered significant the Fold change must have been a minimum of 2 and a p-value of less than 0.001 must have been achieved (false discovery rate correction). p-values can be found in table S4. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline. [#/#] Indicate incidents in which a protein was below detection limit in either the experimental group (numerator), or the baseline group (denominator), e.g. 0/3 in the pre-emergence column means that 0 of the 3 biological replicates from pre-emergent caterpillars had detectable amounts of a particular protein, but 3/3 of the control samples did.

LOC#	Gene	Fold Change (log$_2$)			
		Pre-emergence	Emergent	1-Day Post	3-Days Post
LOC115450302	*gloverin*	**4.60**	**5.31**	**4.04**	**5.35**
LOC115443503	*WAP four-disulfide core domain protein 18-like*	**3.67**	**1.47**	0.12	**3.19**
LOC115453509	*cecropin*	**4.48**	**4.01**	0.09	**3.87**
LOC115449094	*peptidoglycan recognition protein, chromosome 20*	**1.93**	**3.41**	0.10	**3.25**
LOC115454320	*cecropin-A-like*	**4.52**	**4.16**	0.06	**4.39**
LOC115449408	*putative defense protein Hdd11-like*	**4.57**	**2.31**	0.05	**6.01**
LOC115447396	*attacin-E*	**2.05**	0.08	0.08	**4.29**
LOC115447387	*attacin 2*	**2.68**	0.11	0.04	**3.66**
LOC115449088	*peptidoglycan recognition protein 3, chromosome 20*	**2.51**	0.05	0.03	**5.38**
LOC115441359	*peptidoglycan recognition protein 3, chromosome 9*	**4.23**	0.08	0.00	**4.30**
LOC115449911	*phenoloxidase-activating enzyme*	0.05	**1.77**	**1.74**	**1.97**
LOC115449930	*serine protease Hayan-like*	0.03	**2.13**	0.77	**2.43**
LOC115443863	*serine protease inhibitor dipetalogastin*	0.01	**3.16**	0.06	**5.21**
LOC115452581	*serine protease snake*	0.06	**1.39**	**1.37**	0.92
LOC115443044	*lysozyme*	0.09	**3.30**	0.10	**2.92**
LOC115449303	*hemolin*	0.02	**4.69**	0.07	**5.20**
LOC115452515	*phenoloxidase-activating enzyme*	0.07	**1.34**	0.28	0.96
LOC115450759	*serine protease persephone*	-0.03	**1.47**	0.11	1.16
LOC115439859	*G-protein coupled receptor Mth2*	0.01	**1.36**	0.23	0.04
LOC115454286	*phenoloxidase-activating enzyme*	0.07	**3.78**	0.05	1.96
LOC115450929	*phenoloxidase subunit 2*	0.02	**-1.78**	**-1.48**	**-1.17**
LOC115443156	*serine protease inhibitor*	0.04	**-1.85**	**-5.26**	**-2.33**
LOC115451915	*protein toll*	0.04	0.40	**1.39**	0.07
LOC115446883	*nuclear factor NF-kappa-B p100 subunit*	0.03	0.96	**1.13**	0.04
LOC119188533	*defense protein 3-like*	0.01	-0.01	0.00	**4.62**
LOC115447383	*defense protein 3*	0.01	0.02	0.01	**4.53**
LOC115453334	*melanization protease 1*	0.00	0.04	0.03	**3.38**
LOC115454305	*lebocin-3*	0.01	0.06	0.03	**6.35**
LOC115441347	*peptidoglycan-recognition protein LB*	0.05	0.06	0.06	**4.91**
LOC115453672	*bactericidin B-5P-like*	-0.01	0.08	0.01	**3.68**

Table 2. Immune gene mRNA transcripts from the brain of *M. sexta* identified as having significantly altered abundance during at least one time point during parasitism. Highlighted in red indicates an upregulation at that timepoint, highlighted in blue indicates a downregulation at that timepoint. To be considered significant, a p-value < 0.05 must have been achieved, and a Fold change of at least 2. p-values can be found in table S3. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline.

LOC#	Protein	Pre-Emergent	Emergent	1-Day Post	3-Days Post
CcBV_30.1	Hypothetical protein	+	+	+	+
CcBV_13.4	EP2-1	+	+	-	+
CcBV_36.2	BV7-6	+	+	+	+
CcPL9.004	CcPL9.004	+	+	+	+
CcBV_1.5	EP1-3	+	+	-	+
CcBV_5–13	CcV1	+	+	+	+
CcBV_31.9	Hypothetical protein	-	+	-	-
CcBV_19.2	Cystatin 1	+	+	+	+
CcBV_22.1	BV7-1	-	+	+	+
CcPL4.001	CcPL4.001	-	+	+	+

Table 3. CcBV proteins found in the supraesophageal ganglion (i.e. brain) during at least one of the sampled timepoints. '+' indicates that the protein was found to be present at that time point. '-' indicates that it was absent, or below detection threshold at that time point.

Cytoskeleton and extracellular matrix (ECM)

Although not part of our original hypothesis, both the proteomic (Table 5) and transcriptomic analyses (Table 6) showed strong changes in the protein abundance and gene expression of molecules related to the cytoskeleton (e.g. actin, tropomyosin, troponin-1) and extracellular matrix (e.g. hemicentin, nidogen, integrin beta 6, teneurin), including those thought to be directly involved in synaptic transmission (e.g. filamin A[64], tropomyosin[65] and teneurin[66]). Changes began before the wasps emerged, when the caterpillar still has normal behaviour. There was a pronounced upregulation of gene expression, and an increase in protein abundance, of molecules that are key for intracellular transport (e.g. actin, paramyosin, troponin 1, Tables 5 and 6). However, at the time of wasp

LOC#	Gene	Fold Change (Log2)					
		Emergent	p	1-Day Post	p	3-Days Post	p
CcBV_26.5	hypothetical Protein	2.20	< 0.05	2.75	< 0.01	2.58	< 0.01
CcBV_14.6	protein-Tyrosine Phosphatase W	3.41	< 0.001	2.85	< 0.05	-	-
CcBV_17.2	protein-Tyrosine Phophatase Y	4.47	< 0.001	-	-	3.41	< 0.01
CcBV_31.5	hypothetical Protein	4.04	< 0.001	-	-	-	-
CcBV_3.4	hypothetical Protein	3.02	< 0.001	-	-	-	-
CCBV_32.1	hypothetical Protein	2.27	< 0.05	-	-	-	-
CcBV_32.9	hypothetical Protein	2.06	< 0.05	-	-	-	-
CcBV_3.3	hypothetical Protein	1.95	< 0.05	-	-	-	-
CcBV_7.5	EP1-5	1.89	< 0.05	-	-	-	-
CcBV_8.2	EP1	1.81	< 0.05	-	-	-	-
CcBV_26.4	hypothetical Protein	1.71	< 0.05	-	-	-	-
CcBV_19.5	cystatin 2	1.47	< 0.05	-	-	-	-
CcBV_31.2	hypothetical Protein	-	-	4.82	< 0.001	-	-
CcBV_31.3	hypothetical Protein	-	-	3.25	< 0.05	-	-
CcBV_26.3	ankyrin-6	-	-	1.91	< 0.01	-	-
CcBV_10.5	hypothetical Protein	-	-	1.83	< 0.05	2.85	< 0.01
CcBV_10.1	protein-Tyrosine Phosphatase E	-	-	-	-	4.63	< 0.0001
CcBV_1.9	protein-Tyrosine Phosphatase P	-	-	-	-	4.54	< 0.0001
CcBV_1.3	EP1-1	-	-	-	-	4.21	< 0.001
CcBV_10.2	protein-Tyrosine Phosphatase S	-	-	-	-	4.17	< 0.0001
CcBV_26.1	protein-Tyrosine Phosphatase Δ	-	-	-	-	4.06	< 0.001
CcBV_26.7	protein-Tyrosine Phosphatase ε	-	-	-	-	3.76	< 0.001
CcBV_1.5	EP1-3	-	-	-	-	3.72	< 0.001
CcBV_1.8	protein-Tyrosine Phosphatase M	-	-	-	-	3.33	< 0.001
CcBV_18.7	hypothetical Protein	-	-	-	-	3.27	< 0.001
CcBV_1.4	EP1-2	-	-	-	-	3.12	< 0.01
CcBV_18.5	CRP2	-	-	-	-	2.92	< 0.01
CcBV_1.7	protein-Tyrosine Phosphatase L	-	-	-	-	2.88	< 0.01
CcBV_1.2	protein-Tyrosine Phosphatase I	-	-	-	-	2.63	< 0.01
CcBV_26.6	protein-Tyrosine Phosphatase A	-	-	-	-	2.6	< 0.01
CcBV_1.10	protein-Tyrosine Phosphatase Q	-	-	-	-	2.21	< 0.01
CcBV_10.3	protein-Tyrosine Phosphatase T	-	-	-	-	2.02	< 0.05
CcBV_1.11	protein-Tyrosine Phosphatase D	-	-	-	-	2.00	< 0.05
CcBV_7.3	histone 4	-	-	-	-	1.87	< 0.05
CcBV_7.4	protein-Tyrosine Phosphatase R	-	-	-	-	1.61	< 0.05

Table 4. CcBV mRNA transcripts from the brain of *M. sexta* identified as having significantly altered abundance during at least one time point during parasitism. To be considered significant a p-value of less than 0.05 must have been achieved, and a Fold change of at least 2. Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline.

emergence, these proteins declined in abundance, compared with their pre-emergence amounts. Once the wasps had emerged from the host, these proteins then surged in abundance to levels higher than that observed prior to wasp emergence (Table 5). Furthermore, after wasp emergence, additional proteins involved in axonal transport (e.g. Atlastin, Dynactin subunit 6, Vesicle transport protein Sect. 20) changed in abundance, and additional genes (*atlastin*, *dynein heavy chain 1*) changed in expression (Table 6). The increasingly large changes in genes and proteins related to intracellular transport suggest that axonal transport is probably disrupted after the wasps emerge. Disruption in axonal transport leads to a decline in synaptic transmission in *D. melanogaster*[67] Also correlating with the change in host behaviour, were changes in gene expression (*hemicentin-1* and *2*, *nidogen* and *integrin beta 6*) and protein abundance (Hemicentin-2-like and Teneurin-A) of molecules that are important in the ECM (Tables 5 and 6). These changes are also likely to depress synaptic transmission[68].

Viruses commonly alter cellular architectural proteins, and, therefore, some change in cytoskeleton proteins are expected in virally infected insect cells[69]. Moreover, the CcBV virus appears to target the cytoskeleton, at least in *M. sexta* hemocytes[70]. CcBV-induced changes in hemocyte cytoarchitecture contribute to the survival of the wasp larvae by reducing host hemocyte activity[71]. If the virus produces similar changes in the microglia of the *M. sexta* brain, it could alter the functioning of these cells[67]. Such changes could also impact immune-neural signaling.

LOC#	Protein	Fold Change (log$_2$) Pre-Emergence	Emergent	1-Day Post	3-Days Post
LOC115447545	Actin, muscle-type A2	[3/0]	[0/3]	2.01	2.08
LOC115455744	Paramyosin, long form	3.68	-3.56	2.65	2.04
LOC115451171	Tropomyosin-1	2.85	-2.88	2.22	2.02
LOC115446640	Troponin I	2.46	[0/3]	2.69	2.04
LOC115442031	Troponin T, skeletal muscle	4.48	-4.94	2.54	1.79
LOC115456032	Twitchin	3.83	-2.86	3.52	1.96
LOC115449243	Unconventional myosin-Va	2	-1.9	3.2	2.5
LOC115444681	Myosin light chain 1	[3/0]	-3.27	2.35	1.89
LOC115447513	LIM domain-binding protein 3	2.81	-2.65	2.83	0.5
LOC115444875	PDZ and LIM domain protein Zasp	2.67	-2.35	2.94	1.01
LOC115443889	Muscle-specific protein 20-like	[3/0]	[0/3]	3.33	0.67
LOC115448529	Flightin	[3/0]	[0/3]	0.52	0.56
LOC115453254	Filamin-A	[3/0]	[0/3]	-2.73	3.16
LOC115439755	Myosin regulatory light chain 2	4.66	[2/3]	2.02	2
LOC115440915	Jupiter microtubule associated homolog 1-like	-0.08	[0/3]	[0/3]	[0/3]
LOC115454499	Hemicentin-2-like	0.3	[0/3]	[0/3]	[0/3]
LOC115453237	Wiskott-Aldrich syndrome protein family member 3 SCAR	0.15	[0/3]	[2/3]	[2/3]
LOC119189930	Myophilin-like	[0/0]	[3/0]	[3/0]	[3/0]
LOC115452617	Teneurin-a	[0/0]	[3/0]	[3/0]	[3/0]
LOC115441485	Atlastin	0.008	0.411	-3.06	-1.53
LOC115449723	Dynactin subunit 6	-0.22	0.42	[0/3]	-0.77
LOC115453699	Muscle LIM protein Mlp84B	[3/2]	[2/3]	3.01	1.17
LOC115445275	Vesicle transport protein SEC20	0.78	-0.62	[2/3]	[0/3]
LOC115448845	Hemicentin-1-like	[0/3]	-0.37	-0.03	0.19

Table 5. Cytoskeleton and Extracellular Matrix (ECM) proteins identified as having significantly altered abundance in the brain of *M. sexta* during at least one time point during parasitism. Highlighted in red indicates an increased abundance at that timepoint, highlighted in blue indicates a reduced abundance at that timepoint. To be considered significant a p-value of less than 0.001 must have been achieved. P-values can be found in table S4. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline. [#/#] Indicate incidents in which a protein was below detection limit in either the experimental group (numerator), or the baseline group (denominator), e.g. 0/3 in the pre-emergence column means that 0 of the 3 biological replicates from pre-emergent caterpillars had detectable amounts of a particular protein, but 3/3 of the control samples did.

Neural function

Even before the wasps emerge from the host, both the transcriptomics and proteomics results showed changes in the gene expression and protein abundance of molecules important for neuronal function (e.g. potassium channels, Table 7). Although these changes were insufficient to produce a change in host behaviour, they could facilitate the production of the "bodyguard" phenotype, when combined with the additional changes that occur after wasp emergence (Tables 7 and 8). However, the dynamic nature of neural networks[52] makes interpreting these changes challenging. Moderate changes in neurotransmitter synthesis, or the number of receptors, can be compensated for by the brain's homeostatic mechanisms[51]. Similarly, different ion channel subtypes can often compensate for each other[52,72]. Additionally, synaptic mechanisms tend to keep firing rates within a set-point range, despite perturbations[73]. Therefore, changes in proteomics and transcriptomics of the host brain could be a direct effect of parasitism, but could also be a sign of the activation of host homeostatic mechanisms (also see discussion in[52,74]).

CcBV

CcBV is known to rapidly enter host cells and is transcribed within an hour of wasp oviposition in fat body and hemocytes[21]. We found that bracovirus gene expression occurs in every region of the CNS within 24 h (Fig S24). How CcBV enters the brain this quickly remains unknown, and whether it enters all brain areas simultaneously is also unknown. Once in the cell, CcBV genes appear to make a number of proteins that could interfere with intracellular signaling pathways[75], and have been shown to be immunosuppressive when expressed in immune tissues[76]. Unfortunately, nothing is known about their effects in the CNS.

Some CcBV proteins could be found in all regions of the CNS at all time points (e.g. CcV1, Table 4, S24), however two proteins occurred in the brain only during and after wasp emergence (BV7-1 and CcPL4.001) or in the ventral nerve cord at emergence (Table 4, S24). The timing of the appearance of these two proteins makes them good candidates for further studies on the possible involvement of CcBV proteins in changing host behaviour.

Transcripts for CcBV genes could be detected at all late-stage parasitism timepoints measured (Pre-emergent, Emergent, 1-Day Post, and 3-Days Post) (Table 4).

Likely overall effect of neuroparasitic changes

The cytoskeleton and ECM results suggest that synaptic transmission may be reduced in parasitized caterpillars after the wasps emerge. Such an effect would be consistent with previous research that showed that immunohistochemical staining for multiple neuropeptides increases after wasp emergence, likely caused by a

LOC#	Gene	Pre-Emergence	Fold Change (log$_2$) Emergent	1-Day Post	3-Days Post
LOC115446098	*actin, muscle-tyoe A2*	**5.24**	-0.06	-0.040	-0.001
LOC115453254	*filamin-A*	**2.33**	-0.13	-0.031	-0.009
LOC115453699	*muscle LIM protein 1*	**4.41**	-0.08	-0.047	-0.002
LOC115443891	*myophilin*	**4.08**	-0.06	-0.021	-0.002
LOC115447480	*myosin heavy chain, muscle*	**4.83**	-0.06	-0.041	-0.001
LOC115444681	*myosin light chain 1*	**4.14**	-0.07	-0.037	-0.001
LOC115439755	*myosin regulatory light chain 2*	**3.65**	-0.10	-0.058	-0.003
LOC115447152	*myosin-11*	**1.60**	-0.12	0.033	0.002
LOC115455744	*paramyosin, short form*	**5.28**	-0.06	-0.035	-0.002
LOC115442184	*titin, chromosome 6*	**2.83**	-0.26	-0.147	-0.002
LOC115444612	*titin, chromosome 5*	**1.92**	-0.08	0.027	-0.006
LOC115451171	*tropomyosin-1*	**5.55**	-0.07	-0.041	-0.002
LOC115455845	*troponin C, chromosome 10*	**4.93**	-0.11	-0.074	-0.003
LOC115443453	*troponin C, chromosome 17*	**4.39**	-0.04	-0.022	-0.003
LOC115446640	*troponin I*	**4.94**	-0.06	-0.035	-0.002
LOC115442031	*troponin T, skeletal muscle*	**4.49**	-0.08	-0.052	-0.003
LOC115453668	*tubulin beta chain*	**1.79**	-0.01	0.070	0.008
LOC115453764	*trichohyalin*	**1.00**	**1.65**	**1.101**	**1.296**
LOC115445019	*dynein heavy chain 16*	0.39	**1.58**	**1.072**	**1.154**
LOC115449724	*phosphatidylinositol 4-kinase beta*	0.01	**1.03**	**1.401**	0.043
LOC115445604	*dynein heavy chain 1*	-0.01	**1.91**	0.096	1.565
LOC115440787	*titin, chromosome 7*	0.02	**1.86**	0.146	1.640
LOC115439868	*hemicentin-2*	-0.14	**-1.89**	**-1.613**	-0.807
LOC115446957	*nidogen*	0.02	**-2.14**	**-0.22**	0.01
LOC115456281	*integrin beta-6*	0.12	0.08	**1.281**	0.060
LOC115441482	*atlastin*	0.03	-0.12	**-1.724**	-0.065
LOC115450390	*cytoplasmic dynein 2 intermediate chain 1*	0.06	-0.12	**-3.361**	-0.011
LOC115447429	*hemicentin-1*	0.00	-0.04	**-1.504**	-0.027

Table 6. Cytoskeleton and Extracellular Matrix (ECM) related mRNA transcripts from the brain of *M. sexta* identified as having significantly altered abundance at a minimum of one time point during parasitism. Highlighted in red indicates an upregulation at that timepoint, highlighted in blue indicates a downregulation at that timepoint. To be considered significant a p-value < 0.05 must have been achieved, and a Fold change of at least 2. p-values can be found in Table S3. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline.

LOC#	Protein	Pre-emergence	Fold Change (log$_2$) Emergent	1-Day Post	3-Days Post
LOC115442365	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	**2.59**	**-2.24**	**2.63**	0.41
LOC115449101	Protein rogdi	**[3/0]**	**[0/3]**	0.04	**[0/3]**
LOC115441249	GABA neurotransmitter transporter	**1.83**	-0.97	**-2.29**	-0.98
LOC115444961	Ankyrin repeat domain-containing protein 49	**[0/3]**	[2/0]	**[3/0]**	[2/0]
LOC115442623	TWiK family of potassium channels protein 7	[0/3]	[2/0]	[1/3]	[0/0]
LOC115446162	Calcineurin subunit B type 2	[3/2]	[2/3]	**1.96**	[2/3]
LOC119191016	Esterase E4-like	-0.013	-0.14	-0.18	**1.96**
LOC115443168	Tyrosine decarboxylase	[2/3]	-0.11	**[0/3]**	[2/3]

Table 7. Proteins that are involved in neural function identified as having significantly altered abundance during at least one time point during parasitism in the brain of *M. sexta*. Highlighted in red indicates an increased abundance at that timepoint, highlighted in blue indicates a reduced abundance at that timepoint. To be considered significant the Fold change must have been a minimum of 2 and a p-value of less than 0.001 must have been achieved. P-values can be found in table S4. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline. [#/#] Indicate incidents in which a protein was below detection limit in either the experimental group (numerator), or the baseline group (denominator), e.g. 0/3 in the pre-emergence column means that 0 of the 3 biological replicates from pre-emergent caterpillars had detectable amounts of a particular protein, but 3/3 of the control samples did.

LOC#	Gene	Fold Change (Log2)			
		Pre-emergence	Emergent	1-Day Post	3-Days Post
LOC115445366	*sodium/potassium-transporting ATPase subunit beta-1*	**-1.03**	**-1.83**	**-1.125**	-0.04
LOC115442260	*synaptic vesicle glycoprotein 2B*	0.00	-0.16	**-1.730**	-0.04
LOC115455538	*protein henna*	0.02	**3.26**	**3.271**	**1.51**
LOC119193692	*acetylcholinesterase-1-like*	0.03	**2.92**	**2.479**	**2.60**
LOC115450343	*acetylcholinesterase*	0.03	**2.73**	**2.709**	**3.28**
LOC119191009	*esterase E4-like*	0.01	**3.18**	**3.725**	0.01
LOC119191016	*esterase E4-like*	0.80	**1.52**	**1.475**	0.90
LOC115452938	*G-protein coupled receptor moody*	-0.01	**4.21**	0.148	**2.66**
LOC115450344	*esterase E4*	0.05	**1.45**	0.112	**2.48**
LOC115443415	*probable G-protein coupled receptor Mth-like 3*	0.01	**2.60**	0.138	0.01
LOC115440368	*pH-sensitive chloride channel 2*	-0.04	**1.56**	0.171	0.04
LOC115439876	*ankyrin repeat and zinc finger domain-containing protein 1*	0.06	0.40	**1.273**	0.47

Table 8. mRNA transcripts from the brain of *M. sexta* involved in neural function identified as having significantly altered abundance at a minimum of one time point during parasitism. Highlighted in red indicates an upregulation at that timepoint, highlighted in blue indicates a downregulation at that timepoint. To be considered significant a p-value of less than 0.05 must have been achieved, and a Fold change of at least 2. P-values can be found in table S3. Pre-emergence groups were contrasted with unparasitized caterpillars as a baseline, whereas Emergent, 1-Day post, and 3-Days Post, were contrasted with the pre-emergence condition as baseline.

build-up of these neuropeptides[12]. Similarly, at least one biogenic amine (octopamine) increases in abundance in the brain and CNS after wasp emergence[77] as would be expected if release was reduced. We also found a decline in neural activity descending from the brain (both spontaneous and evoked activity) in caterpillars after wasp emergence ($n=7$) compared to controls ($n=8$) or to parasitized caterpillars prior to wasp emergence (Fig. 3, spontaneous: $F(2, 16)=6.8$, $p=0.007$. Tukey's multiple comparison test, Control vs. Pre-emergent caterpillars ($n=4$), $p=0.72$; Control vs. Post-emergent caterpillars, $p=0.006$; Evoked: $F(2, 18)=5.89$, $p=0.01$; Control ($n=8$) vs. Pre-Emergent caterpillars ($n=6$), $p=0.91$; Control vs. Post-Emergent caterpillars ($n=8$), $p=0.01$, Pre-Emergent vs. Post-emergent, $p=0.04$). These results are consistent with a reduction in synaptic release in post-emergent caterpillars (i.e. during the "bodyguard" phase); however, more studies are required to determine whether synaptic transmission is actually reduced. Further studies are also required to determine whether neuroinflammation, bracoviral effects, and/or other factors are causally linked to thes decline in descending neural activity.

A decline in synaptic transmission throughout the CNS could produce the "bodyguard" phenotype. Neural circuits mediating defensive behaviours tend to have few synaptic connections (e.g. *M. sexta*[78][79]) and would probably be minimally affected by a small decline in synaptic transmission. However, motivated behaviours, that rely on more complex multisynaptic circuits[80], would be disproportionately reduced. This differential impact on neural circuits could produce a caterpillar with robust defensive reflexes, but one that lacks motivated behaviours – i.e. a "bodyguard". This hypothesis will provide the basis for future studies.

Limitations
Changes in host behaviour can reflect parasitic manipulation or host responses (e.g. see discussion Bernardo and Singer[81]). The large number of changes that occur within the host during parasitism make it difficult to determine which of our reported changes are directly caused by the parasite (e.g. via CcBV) and whether any of these changes are directly responsible for producing the "bodyguard" phenotype. Future studies (e.g. suppressing the production of CcBV proteins BV7-1 and CcPL4.001 at the time of wasp emergence) are needed. A further difficulty is that our data likely underestimate the number of molecular changes occurring during host behavioural change.

Conclusions
Proteomics and transcriptomics are complementary techniques[82]. Changes in immune, cytoskeleton and ECM molecules were found in both the proteomics and transcriptomics results using two different methods of assessing network connections (e.g. Tables 1, 2, 3, 4, 5, 6, 7 and 8, and S5), giving us confidence that these networks are altered during parasitism.

As predicted, we found that: (1) systemic immune activity increased immune activity in the brain of *M. sexta*, and (2) immune activity within the brain increased dramatically with the change in host behaviour. These results support the hypothesis that enhanced immune activity in the caterpillar brain is playing a role in the change in host behaviour. (3) Changes in CcBV gene expression and protein abundance correlated with the appearance of the "bodyguard" phenotype. This correlation suggests that polydnaviruses may play a role in parasitic manipulation of host behaviour. (4) Changes in genes and proteins important for intracellular transport and the ECM suggested that synaptic transmission could be reduced in "bodyguards". This hypothesis was consistent with our finding of a reduction in descending neural activity from the brain after wasp emergence.

Controlling the ECM and cytoskeleton may be important for parasitic manipulators. The ECM[83] and cytoskeleton[84] contribute to neuronal homeostasis. Manipulating the ECM and neuronal cytoskeleton may be
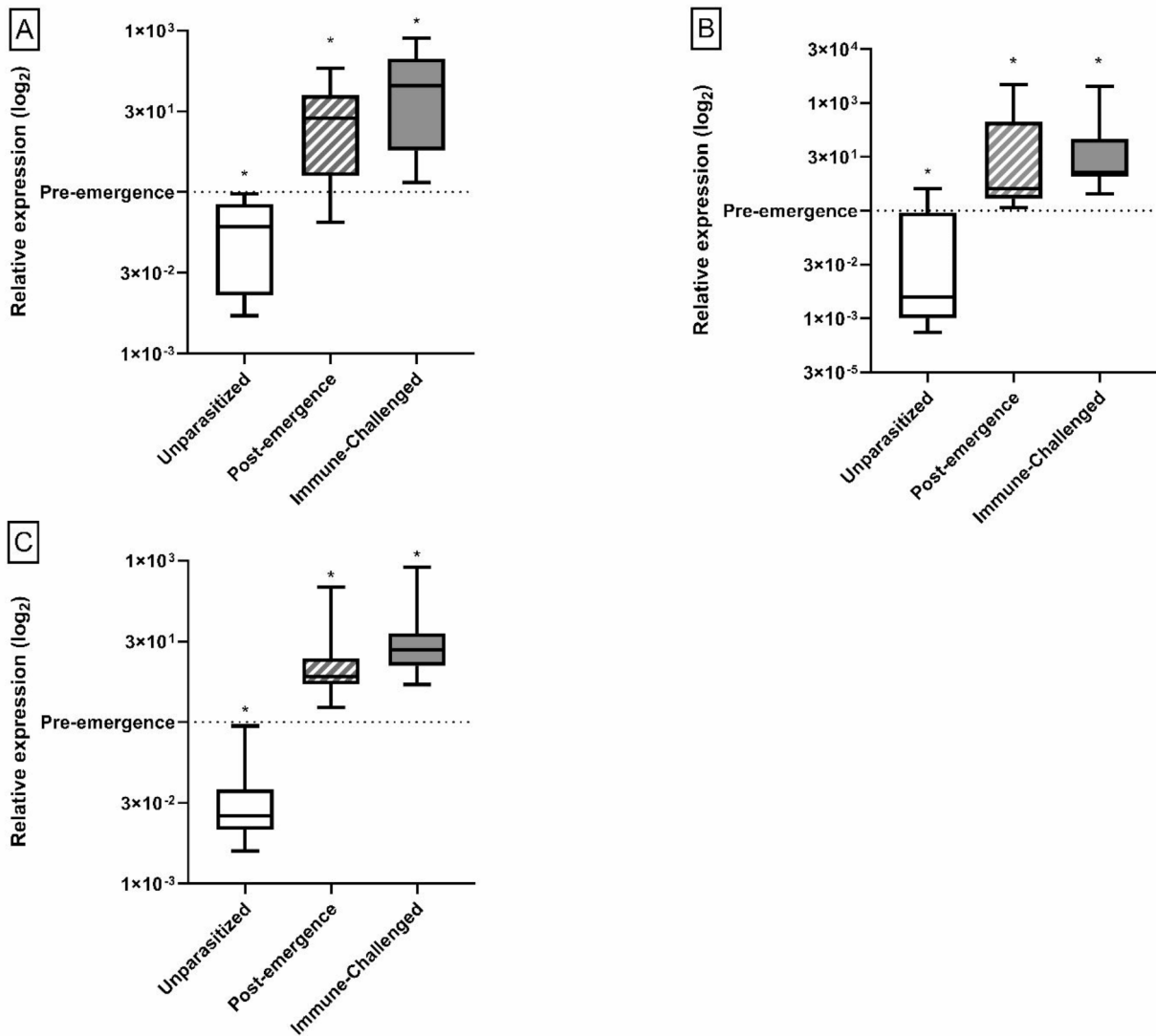
Fig. 2. Targeted qPCR for immune gene expression in the brains of unparasitized, Post-emergence parasitized, and Immune challenged *M. sexta* compared to Pre-emergence parasitized caterpillars. (A) Relative expression of *attacin-1* (B) Relative expression of *gloverin* (C) Relative expression of *hemolin*. All groups compared to the pre-emergence group whose expression has been normalized to 1 (Indicated by dashed line). Immune challenged *M. sexta* have been injected with an inert (heat-killed) challenge. * Denotes a significant change $p < 0.001$.

a novel method of circumventing neuronal homeostasis, leading to long term changes of neural circuits. Many manipulated hosts from different parasite-host systems exhibit altered cytoskeleton and/or ECM dynamics in the host's brain (e.g. jewel wasp/cockroach[9]; trematode/crustacean[85]; gordian worm/crustacean[86]; Hairworm/grasshopper[87], cestode/ant[88]). The need to overcome homeostatic mechanisms will be especially important for parasitic manipulators that require their hosts to survive for several days in the altered state. Multi-targeted interventions appear to allow parasitic manipulators to achieve effective, predictable and long-lasting control of the host's brain.

Methods

Animals

All studies were performed on *Manduca sexta* larvae obtained from our in-house colony, and were maintained as described previously[31].

Cotesia congregata were obtained from an in-house colony. Mated adults were given 3rd instar *M. sexta* (Proteomics, transcriptomics, late-stage qPCR, and electrophysiological studies) or 4th instar (early-stage qPCR) in which to lay their eggs. Parasitized *M. sexta* were reared on lab-made high nutrition diet (Frontier Agricultural Sciences, (#F9783B Neward, DE) until tissue extraction.
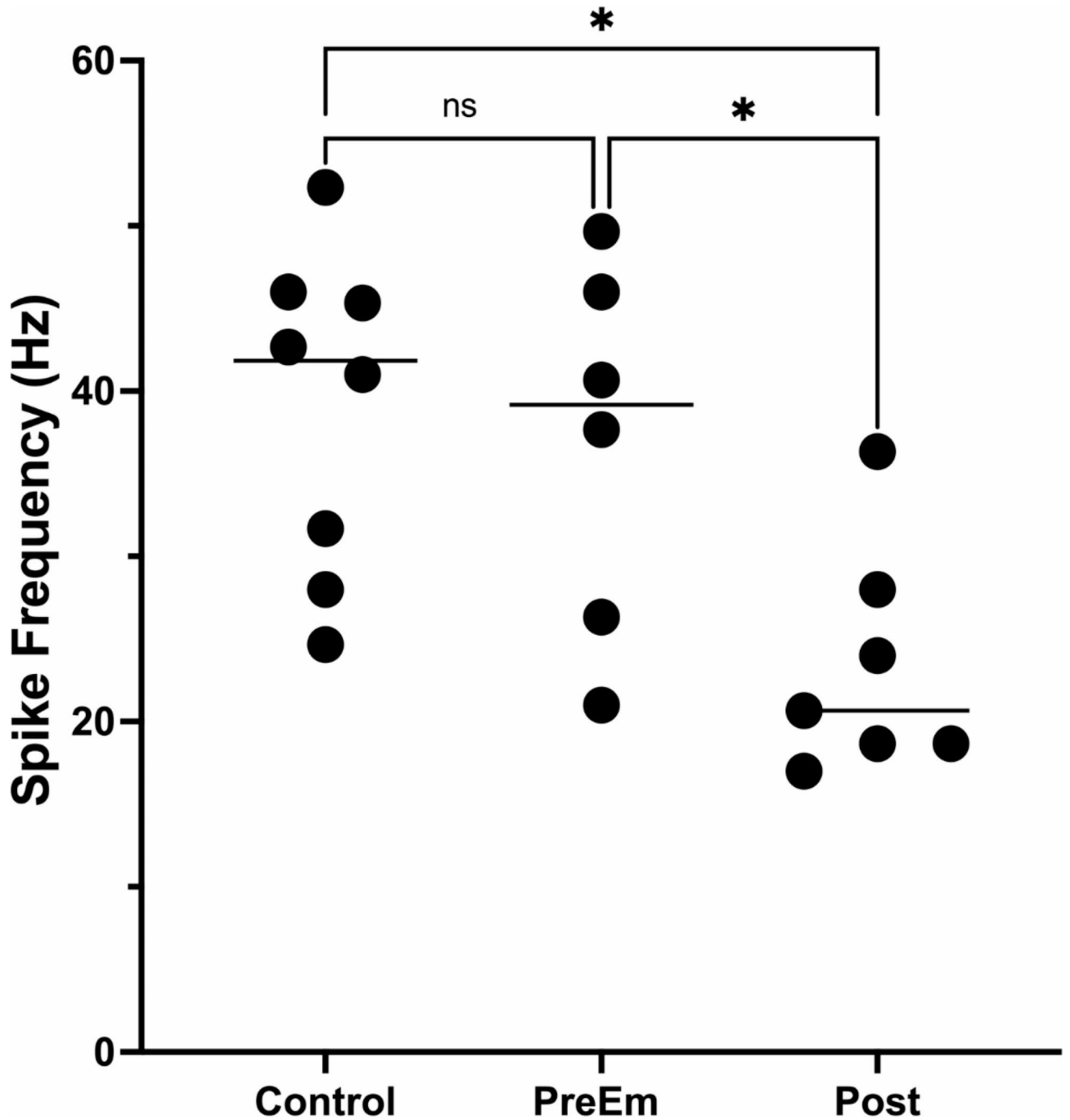
Fig. 3. Descending activity in the supraesophageal connective contralateral to the stimulus. After the wasps emerge (Post, $n = 7$) there is a decline in evoked neural activity compared with that of controls (Control, $n = 8$). A significant decline was not observed prior to wasp emergence (PreEm, $n = 6$). The horizontal lines denote the median, and each circle represents an individual data point. The asterisk denotes statistically significant differences ($p < 0.05$).

All studies were approved by the University Committee on Laboratory Animals (Dalhousie University, I-11–025) and were in accordance with the Canadian Council on Animal Care.

Sample sizes followed minimum guidelines for proteomics and transcriptomics[68].

A Benjamini-Hochberg correction was applied when multiple tests were performed on the same data set[89].

Tissue extraction

Caterpillars were cooled to induce a chill coma[90] and dissected over ice.

Methods for proteomics, transcriptomics, immune and bracovirus qPCR

Final instar (5th) *M. sexta* caterpillars were sorted into five groups based on stage of parasitism: Unparasitized (Control), Pre-emergent (3 days prior to emergence of wasp larvae), Emergent (tissue collected during the emergence of wasp larvae), 1 day post emergence (1 day after emergence of wasp larvae), and 3 days post emergence (3 days after emergence of wasp larvae).

The supraesophageal ganglion or subesophageal ganglion or ventral nerve cord (thoracic ganglia + abdominal ganglia) were extracted and washed briefly in PBS (< 10 s). For transcriptomics, the tissue was placed into an empty tube and flash frozen with liquid nitrogen. For proteomics, tissue was added to a tube containing protease inhibitor and then flash frozen using liquid nitrogen (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, Switzerland). For the qPCR assays, tissues were added to tubes containing RNA*later* (qPCR) (Invitrogen, MA, USA) and flash frozen in liquid nitrogen. All tissues were kept at −80 °C until use.

Methods for early-stage RT-qPCR

Fourth instar *M. sexta* caterpillars were sorted into two groups: Unparasitized (control), or 24 h post-parasitism (i.e. after wasp oviposition).

Tissues were collected as described above.

Proteomics

Extraction and quantification
Due to protein concentration required, 10 individuals per group were pooled to create 3 replicates per group (i.e. CNS samples from 30 individuals in total). The tissues (supraesophageal ganglia, subesophageal ganglia, or ventral nerve cords) were suspended in 30 μl of extraction buffer containing Urea 7 M, Thiourea 2 M, TrisHCl 40 mM, CHAPS 4%, DTT 1% and protease inhibitor. Samples were then mechanically homogenized on ice using a micro pestle. Samples were further disrupted using a micro-sonicator on maximum for 3 cycles of 10 s, followed by 20 s on ice. Following homogenization, samples were centrifuged at 15 800 g at 4 °C for 12 min, after which the supernatant was collected for protein quantification.

A Bradford test for total protein was conducted on the samples followed by a 1D SDS PAGE on a 10% pre-cast acrylamide gel (Biorad). Gels were stained overnight using Sypro ruby protein stain (Invitrogen, Massachusetts, USA) before being visualized under UV light.

Protein identification

Extracted protein samples were denatured and digested in a trypsin solution. The resulting peptide solution was suspended in 10 μl of a 0.1% formic acid solution and injected into an HPLC nano debit (RSLC U3000, Thermo Fisher Scientific) coupled with a nanoelectrospray mass spectrometer (Q Exactive HF, Thermo Fisher Scientific). Peptides were separated on a C18 reverse-phase capillary column (0.075 mm x 500 mm, Acclaim Pepmap 100, NanoViper, Thermo Fisher Scientific), using a gradient of 0.1% formic acid: acetonitrile, 2–40%:98−60%, at a flow rate of 300 nL/min.

Resulting mass spectrographs were recorded using Xcalibur 4 software (Thermo Fisher Scientific). These spectrographs were then analyzed using MaxQuant v1650 and Perseus v1.6.10.43 programs using the script leading FPP v3.2. As a template for protein comparison, we created four protein databases that encompassed known proteins from the family braconidae (NCBI txid 7402), the polydnavirus genus bracoviriform (NCBI txid 2946836), the species *C. congregata* (NCBI txid51543) and the species *M. sexta*(NCBI txid 7130). These datasets have been made publicly available[91]. A MaxQuant database was also used to reduce proteins being identified due to contamination (contaminants_fpp_180320.FASTA)[92]. Protein validation was conducted with a 1% false discovery rate filter at both the peptide and the protein level.

Ratio analysis

Normalization of protein intensity signals was required to analyze the data. Individual proteins had to have been detected in all samples (all three replicates of parasitized caterpillars per group, and all three replicates of control caterpillars) to be included in the analysis. Preliminary examination of the data found that the raw intensity values were not normally distributed, therefore these values were log2 transformed prior to statistical analysis. Furthermore, the intensity values per replicate were centered using the following procedure. For each sample, the median intensity value was established taking all detectable protein intensities into account. This overall median was then subtracted from each individual protein intensity value within that sample. This process was repeated for each of the three replicates. Once the data was centered, a ratio was calculated for each protein intensity by subtracting the control group intensities from the treatment group intensities for each protein. The median ratio of the three replicates was then Z-Scored. These Z-scores were converted to p-values, and a Benjamini-Hochberg procedure was used to determine statistical significance[89].

qPCR

RNA extraction
RNA extraction was performed using a RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). All steps adhered to the manufacturer's instructions and included a DNase 1 treatment (RNase-free DNaset, Qiagen) step to remove genomic DNA. The integrity of total RNA samples was assessed using a Bioanalyzer (Agilent, California, USA). The purity of extracted RNA was determined using an EPOCH spectrophotometer (BioTek, Vermont, USA) using the 260/280 ratio. Only samples with a 260/280 between 1.8 and 2.4 were used in accordance with the MIQE guidelines[93]. The concentration of total RNA was determined using a Qubit Fluorometer (Q32857, Invitrogen, California, USA) using a HS RNA quantification kit. cDNA was synthesized using iScript Reverse

Experiment	Groups	N per group
Early parasitism	Unparasitized, 24-hours post parasitism (2 groups, total of 16 samples)	8
Bracovirus PCR	Unparasitized, Pre-emergence, emergence, post-emergence (4 groups, total of 20 samples)	5
RNA-Seq immune validation	Unparasitized, Pre-emergence, post-emergence, Immune challenged (4 groups, total of 48 samples)	12

Table 9. Sample Group information for RT-QPCR studies. Each sample consists of CNS from a single caterpillar (i.e. CNS tissue was not pooled).

	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	% Efficiency
Ubiquitin[94]	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	94
RpL17a[95]	TCCGCATCTCACTGGGTCT	CACGGCAATCACATACAGGTT	98
Cystatin-1[22]	TCGAGCGGCCGCAATGGGCAAGGAATATCGAG	TGGCGCGGCCGCTTAACAATTTTCATATTCCCAAC	98
EP-1[96]	GCGCCCGTAGTGTCATTAATG	CCCAGTACTTGATGCGCTTG	101
CcV1	ATTCCTGGGCACCTCCAAG	TGCAACGATCGATCCAGGTC	104
Hemolin[97]	CAACCAAGCAACAACACAGG	CAGCACAGGCATCTTCTCC	96
Gloverin	CCCGCAATACGCTCAGATA	TGCTGGAAGAGACCTTGGA	91
Attacin-1[97]	GCAGGCGACGACAAGAAC	ATGCGTGTTGGTAAGAGTAGC	94

Table 10. Forward and reverse gene primer sequences for target bracovirus genes, *M. Sexta* immune genes and reference genes.

Transcription Supermix for RT-qPCR (Bio-Rad, California, USA) and samples were stored in −80 °C until use. Samples sizes are given in Table 9.

Primers
Primer efficiency (E) and correlation coefficient (R^2) were estimated from a standard curve generated with 10-fold dilutions of mixed cDNA samples (Table 10).

Primer specificity was checked by running endpoint PCR products for each primer on a 1.5% acrylamide gel. The resulting bands were excited and sent out for Sanger sequencing (Genewiz, NJ, USA). The resulting sequences were put through a BLAST search on NCBI to confirm fragment identity.

Reference gene selection
Six candidate reference genes were investigated to select two of the most stable reference genes in our tissues of interest. These genes were used in previous studies in *M. sexta caterpillars: RpL17a*[95] (Rewitz et al., 2006), *actin*[98], *glycerol-3-phosphate dehydrogenase (G3PDH)*[99], *beta-FTZ-F1*[100], *ubiquitin*[94], and *ribosomal protein S3*[101]. We used NormFinder for R (http://moma.dk/normfinder-software) to determine the stability of pairs of reference genes (Andersen et al., 2004), using C_q values of five biological samples per treatment for each candidate reference gene. *RpL17a* and *ubiquitin* were found to be the most stable across treatment groups and were used as reference genes in further qPCR analysis.

RT-qPCR
RT-qPCR was run using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 real-time system (Bio-rad) with the following parameters: 40 cycles of (95 °C for 10 s; 55 °C for 30 s; 60 °C for 45 s) followed by a final extension of 60 °C for 10 min. After the qPCR a melt curve analysis was run to assess the specificity of the qPCR product. For each biological sample, qPCR reactions were performed in duplicate, and for each gene no-template controls were run. C_q values for each sample and gene target were calculated in CFX Maestro (BioRad). The qPCR data were analysed using the REST program[102] (http://rest.genequantification.info).

Transcriptomics
Sample Group Information   For the transcriptomic analysis there were five groups of interest: Unparasitized caterpillars, pre-emergent caterpillars, emergent caterpillars, 1-day post emergence, and 3-days post emergence (see Tissue Extraction for more details). There were 6 biological replicates per group.

RNA extraction
RNA extraction was performed using a RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). All steps adhered to the manufacturer's instructions and included a DNase 1 treatment (RNase-free DNaset, Qiagen) step to remove genomic DNA. The integrity of total RNA samples was assessed using a Bioanalyzer (Agilent, California, USA). The purity of extracted RNA was determined using an EPOCH spectrophotometer (BioTek, Vermont, USA) using the 260/280 ratio. Only samples with a 260/280 between 1.8 and 2.4 were used in accordance with the MIQE guidelines[93]. The concentration of total RNA was determined using a Qubit Fluorometer (Q32857, Invitrogen, California, USA) using a HS RNA quantification kit. All RNA samples were stored at −80 °C until library preparation.

Library preparation and transcriptome sequencing

Library construction and sequencing were performed at Farncombe Metagenomics Facility (McMaster University, ON, Canada). The RNA-Seq libraries were prepared using Illumina TruSeq RNA sample preparation kit (Illumina, CA, USA). RNA-Seq libraries were sequenced on an Illumina NextSeq for 2×50 bp paired-end reads.

Trimming, mapping of sequences, and differential gene analysis

Raw sequencing reads were trimmed using Trimmomatic software[103](v0.39), removing low quality leading and tailing bases (Q < 3) and reads below 30 bases long. Trimmed reads were aligned using STAR (v.2.7.5a)[104], to either the *M. sexta* genome[105] (JHU_Msex_v1.0), the *C. congregata* genome[106], or the *Bracoviriform congregatae* genome[107](ViralMultiSegProj14556. Uniquely mapped reads with a maximum of four mismatches were counted to genes using FeatureCounts from the Rsubread R package[108] (v2.4.2). First, reads were counted to features in the *M. sexta* genome, which resulted in an average 32 million counted reads per sample. Next, reads were counted to features in the *C. congregata* genome. For the *C. congregata* analysis, some *M. sexta* reads mapped to the *C. congregata* genome, aligning to a handful of genes in controls (e.g. LOCUS4576). To exclude these reads, *C. congregata* was aligned simultaneously with *M. sexta* and then separated during counting. We effectively found no *C. congregata* reads in control samples (an average of 66 counted reads), and in parasitized samples there was an average of 420,000 reads. Finally, we counted reads to features in the *Bracovirus* genomes, where control samples had on average 0 counted reads, and parasitized samples had on average 9000 counted reads. Genes were pre-filtered before differential expression analysis by removing genes with < 10 counts across all samples. Differential expression analysis was performed using the R package DESeq2 (v1.40.2)[109], in 4 pairwise comparisons, either using the pre-emergent condition as the reference level (Pre-emergent vs. Emergent, Pre-emergent vs. 1-Day Post, and Pre-emergent vs. 3-Days Post), or the control (Pre-emergent vs. Control). Statistical significance was determined using a Wald test and corrected using a Benjamini–Hochberg correction for multiple comparisons[89]. Differentially expressed genes were considered for further analysis with a cut-off of an FDR adjusted p-value < 0.05 and a fold change in expression > 2.0[110].

To test the robustness of the results for the both the proteomics and transcriptomics analyses, we performed a second analysis using the STRING database (https://string-db.org) to identify significantly enriched terms within the protein-protein interaction network[111] (Yuan et al., 2021).

Validation of gene expression profiles using RT-qPCR of select immune genes

RT-qPCR was used to validate the expression profiles of select immune genes from RNA-seq results. For more information, please see RT-qPCR section. An additional group was added "Immune Challenge". This group allowed us to test whether a systemic infection also increased gene expression for antimicrobial peptides (AMPs) in the brain. This group was reared on high nutrition diet and were unparasitized. On day 1 of their fifth instar this group was given a 40 *µl* injection of heat-killed mix of *Serratia marcescens* (Gram-negative bacterium, Microkwik culture, Carolina Biological, 1/10 LD50), *Bacillus cereus* (Gram-positive bacterium, Microkwik culture, Carolina Biological, 1/10 LD50), *Beauveria bassiana* (strain GHA, fungus, 1/10 LD50, BotaniGard 22WP; Laverlam, Butte, MT, USA) into the hemocoel. Post injection, these caterpillars had their food removed for 24 h to mimic the large systemic immune response and lack of feeding observed in Post 1 caterpillars[28,31]. The supraesophageal ganglia was collected 24 h after the injection using the same methods listed above.

The Effect of Parasitism on the Descending Neural Activity from the Supraesophageal Ganglion.

Caterpillars were cooled for approximately 10 min to induce chill coma and then the gut and a section of the body wall (between the second and fifth abdominal ganglia) was removed without disturbing the ventral nerve cord. The physiological saline for *M. sexta* was modified from Miyzaki[112] by Trimmer and Weeks[78] and contained 140 mM NaCl, 5 mM KCl, 4 mM CaCl2, 29 mM glucose and 5 mM HEPES adjusted to pH 7.4 using NaOH (all chemicals were from Sigma-Aldrich (St Louis, MO). The posterior and anterior ends of the caterpillar were pinned to an elastomer-covered dish. A suction electrode (Bipolar Suction Electrode, A-M Systems, Carlsberg, WA) was connected to a differential amplifier (A-M Systems, Model 3000), and digitized using a PowerLab SP4 (ADInstruments, Colorado Springs, CO). The output was collected and analyzed using Lab Chart (ver. 7.3.8, ADInstruments). The suction electrode was used to make en passant recordings of the connective between the subesophageal ganglion and the supraesophageal ganglion. Spontaneous activity was collected for 3 min and then the posterior end of the animal was electrically stimulated by a Grass S9 stimulator. Low voltage was applied to the dorsal nerve root of the sixth abdominal ganglion that was ipsilateral to the suction electrode. The voltage was increased until evoked potentials were visible in the en passant recordings. The suction electrode was then moved to the opposite supraesophageal – subesophageal connective. The connective was cut near the subesophageal ganglion, and recordings of the descending activity in the contralateral connective recorded for 3 min using the suction electrode. The stimulus to the dorsal root of the contralateral sixth abdominal ganglion was re-applied as described above. If no evoked potentials were observed, the voltage was increased until evoked potentials were visible. Recordings were made from control caterpillars (5th instar day 2 to day 3, *n* = 8), pre-emergent caterpillars (estimated as 3 days prior to emergence, *n* = 4–6, see Results and Discussion section); and post emergent caterpillars (1 to 3 days after wasp emergence, *n* = 7)). The number of potentials at least two times above noise were counted during spontaneous recordings, as well as after the stimulus artifact. Using this threshold meant that only the largest spikes were counted (i.e. greater than 30 µV in amplitude and 2 ms in duration). We used the response of control caterpillars to determine the time frame for counting the evoked potentials (i.e. for 30 s after the stimulus artifact).

Data Availability

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Author contributions

L.M. Study design, Data collection, Data curation, Analysis, Writing of manuscript; R.H. Analysis; Data collection, Manuscript review; D.B. Conceptualization of the study, Study Design, Analysis; A.B. Data collection, Manuscript review; D.M. Analysis, Manuscript review; NR: Study design, Analysis, Manuscript Review; S.A.: Conceptualization of the study, Analysis, Interpretation of Results, Writing of manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information