Poisons, Pathogens, And Parasitoids: The Immunophysiological Network Of *Manduca Sexta* Explored Through Trade-Offs, Microbiome Interactions And Exploitation By The Wasp *Cotesia Congregata*

Laura Elizabeth McMillan

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2022

DEDICATION PAGE

This work is dedicated to the memory of my father, G. Eric McMillan, who unequivocally believed in my ability to succeed, whether it be at optimally planning garage sail routes, or obtaining my PhD. I owe so much of my drive and enthusiasm to him. I know this achievement would make him proud.

This thesis is also dedicated to the memory of my friend and mentor Dr. David G. Biron. I hope to embody his enthusiasm for research throughout my career, and one day inspire a student the way he inspired me.

TABLE OF CONTENTS

LIST OF TABLES v

LIST OF FIGURES vii

ABSTRACT x

LIST OF ABBREVIATIONS USED xi

ACKNOWLEDGEMENTS xii

CHAPTER 1 - GENERAL INTRODUCTION 1

1.1 INSECT IMMUNITY 1

1.2 THE INTEGRATED DEFENSE SYSTEM 7

1.3 MANDUCA SEXTA 9

1.4 EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR MANDUCA SEXTA 10

1.5 FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR MANDUCA SEXTA 10

1.6 PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA CATERPILLARS PARASITIZED BY WASP COTESIA CONGREGATA 11

CHAPTER 2 -EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR, MANDUCA SEXTA 14

2.1 PUBLICATION INFORMATION 14

2.2 ABSTRACT 14

2.3 INTRODUCTION 15

2.4 METHODS 16

2.5 RESULTS 18

2.6 DISCUSSION 19

2.7 REFERENCES 21

2.8 SUPPLEMENTAL INFORMATION 23

2.10 REVISIONS 28

CHAPTER 3- FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR MANDUCA SEXTA 32

3.1 PUBLICATION INFORMATION 32

3.2 ABSTRACT 32

3.3 INTRODUCTION 33

3.4 METHODS 34

3.5 RESULTS 36

3.6 DISCUSSION 39

3.7 REFERENCES 41

3.8 SUPPLEMENTAL INFORMATION 43

3.9 REVISIONS 45

CHAPTER 4-PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA CATERPILLARS PARASITIZED BY WASP COTESIA CONGREGATA 46

4.1 PUBLICATION INFORMATION 46

4.2 ABSTRACT 46

4.3 INTRODUCTION 47

4.4 METHODS 55

4.5 RESULTS 59

4.6 DISCUSSION 91

4.7 REFERENCES 101

CHAPTER 5 -GENERAL DISCUSSION 109

5.1 EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR MANDUCA SEXTA 109

5.2 FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR MANDUCA SEXTA 111

5.3 PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF MANDUCA SEXTA CATERPILLARS PARASITIZED BY WASP COTESIA CONGREGATA 112

5.4 CONCLUDING REMARKS 114

BIBLIOGRAPHY 115

LIST OF TABLES

Chapter 2

Table S1. Forward and reverse primer sequences for target immune-related genes, detoxification genes, and reference genes..... 23

Table S2. A priori ranking of GSH rescue groups with Bonferroni correction..... 23

Chapter 3

Table 1. Effect of systemic and oral challenges on *Manduca sexta* caterpillars 4h post-treatment..... 35

Table 2. Effect of systemic and oral challenges on *Manduca sexta caterpillars* 24h post-treatment..... 35

Table 3. Standardized discriminant function coefficients for the 4h time point..... 36

Table 4. Standardized discriminant function coefficients for the 24h time point..... 37

Table S1. Forward and reverse gene primer sequences for target immune-related genes and reference genes..... 42

Chapter 4

Table 4.1 Forward and reverse gene primer sequences for target bracovirus genes and reference genes..... 55

Table 4.2 Total protein in the brain regions of *Manduca sexta* in controls and at different timepoints during parasitism..... 57

Table 4.3 Total RNA and cDNA in the supraesophageal ganglion of *Manduca sexta* in controls and at different timepoints during parasitism..... 58

Table 4.4 Total number of proteins detected in the supraesophageal and subesophageal ganglion in *Manduca sexta*. Percentage of proteins altered during parasitism..... 59

Table 4.5 Supraesophageal Ganglion: protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and presence at different timepoints during parasitism. During at least one timepoint each protein below was not found in detectable amounts in control caterpillars, but was detectable in all three parasitism samples. 61

Table 4.6 Top three most increased proteins present in the supraesophageal ganglion of parasitized caterpillars..... 64

Table 4.7 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon..... 65

Table 4.8 Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and absence at different timepoints during parasitism..... 66

Table 4.9 Top three most reduced proteins present in the supraesophageal ganglion of parasitized caterpillars..... 74

Table 4.10 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon..... 75

Table 4.11 Subesophageal ganglion: Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and presence at different timepoints during parasitism..... 77

Table 4.12 Top three biggest increases in proteins present in the subesophageal ganglion of parasitized caterpillars. 80

Table 4.13 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon..... 81

Table 4.14 Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and absence at different timepoints during parasitism..... 82

Table 4.15 Top three decreases in proteins present in the subesophageal ganglion of parasitized caterpillars. 86

Table 4.16 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon..... 87

Table 4.17 Comparison of altered proteins within the CNS in five different parasite behavioural changing interactions..... 96

LIST OF FIGURES

Chapter 1

Figure 1. Schematic representations of insect toll (a) Imd (b) and JAK-STAT (c) pathways. Adapted from (Manniello et al., 2021)..... 5

Chapter 2

Figure 1. Effect of immune and toxin challenges on the survival of Manduca sexta caterpillars. (A) Effect on survival of a sub-lethal dose of permethrin(toxin; N=41), a live Serratia marcescens infection (N=40), and a combined permethrin and live S. marcescens challenge (N=42). There was no mortality in the control or toxin group. (B) Effect on survival of an LD_{50} of permethrin (N=30) when combined with heat-killed (HK) S. marcescens (N=32) or inert bead immune activation (N=34). There was no mortality in the control, heat-killed S. marcescens or beads group 18

Figure 2. Effect of immune and toxin challenge on glutathione (GSH) concentration in the hemolymph of Manduca sexta caterpillars. Bars represent first and third quartiles, internal bar represents median, and error bars represent the maximum and minimum result. Asterisk represent groups that are significantly different from the control ($P<0.05$). N=10 for all groups..... 19

Figure 3. Effect of supplemental GSH on the survival of Manduca sexta caterpillars exposed to immune (i.e. heat-killed S. marcescens) and toxin challenges. Effect on survival of a sub-lehtal dose of permethrin, with and without additional GSH (toxin, toxin+GSH), a dual challenge of heat-killed immune challenge of Serratia marcescens with and without additional GSH. N=20 for all groups..... 19

Figure 4. Gene expression after single and dual immune-toxin challenges in the caterpillar Manduca sexta. (A) Expression of immune-related genes in response to immune and toxin challenge. Bars with an asterisk represent those that are upregulated when compared with control animals. Error bars represent SEM (B) Expression of detoxification-related genes in response to immune and toxin challenge. Bars with an asterisk represent those that are upregulated when compared with the control. Bars with two asterisks represent those that are downregulated when compared with the toxin challenge. Error bars represent s.e.m. N=13 for all groups..... 20

Figure S1. Effects of varying infections on the feeding behaviour of Manduca sexta. (A) Effect of varying immune challenges on weight gain of Manduca sexta 5^{th} instars. (B) Effect of various immune challenges on M. sexta 5^{th} instars represented by weight of food left uneaten (proxy of amount of food consumed). Error bars represent SEM. Control (N=48), 24

Sham (N=45), S. marcescens (N=14), Beads (N=16), B. cereus (N=16), Starved (N=20).....

Figure S2. Effect of differing treatments on gut transit time of high nutrition diet in *Manduca sexta.* Error bars represent SEM. For all groups (N=31)..... 25

Figure S3. Effect of quinine concentration (M) on feeding behaviour of *Manduca sexta* caterpillars. (A) Effect of increasing concentration of quinine (M) on bites taken of food during a 3 minute trial interval. Error bars represent SEM. (B) Effect of increasing quinine concentration (M) on latency to begin feeding once food has been contacted during a 3 minute trial interval. Error bars represent SEM. Control (N=62), Sham (N=63), Immune-challenge (N=60)..... 26

Chapter 3

Figure 1. Bacterial colony forming units cultured from frass collected at different time points. Frass was collected and plated on high nutrient agar from Manduca sexta caterpillars prior to oral inoculation of bacteria (Pre) and at several time points after (Post). The number of colony forming units (CFUs) increased at each time point up to 24 h post-oral inoculation before decreasing again at 72 h post-oral inoculation For all groups, n=20. Different letters indicate significant differences between time points (P<0.05). Data points represent means and error bars represent the standard deviation..... 34

Figure 2. Canonical variates from the 4 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.0001). (C) Canonical variate 3. Challenge groups with different letters are significantly different from each other (P<0.00005). (D) Individual representation of significant canonical variates calculated from canonical coefficient factor loadings. Bars represent first and third quartiles, internal bar represents the median, and error bars represent the maximum and minimum result..... 36

Figure 3. Canonical variates from the 24 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.0001). (C) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.00005). (D) Individual representation of significant canonical variates calculated from canonical coefficient factor loadings. Bars represent first and third quartiles, internal bar represent the median, and error bars represent the maximum and minimum result..... 37

Figure S1. Culturable bacteria from the midgut contents (A) and frass (B) of M. sexta, 4 hours post feeding. Oral challenge group were given a food cube that had been injected with a solution containing ~10_6 bacterial cells (50:50 M. luteus: E. aerogenes). Independent samples T-test from midgut contents indicate a significantly greater amount of bacteria in the oral challenge (N=10, M=57.9, SD=24.7) group relative to the control group (N=10, M=29.1,SD=17.5); t(18)=-3.00, p=0.008. Independent samples Ttest from plated frass similarly indicates a significantly greater amount of bacteria in oral challenge (N=10, M=147.5, SD=11.31) compared to control (N=10, M=47.0, SD=23.2);t(18)=-7.5, p<0.0001 41

Chapter 4

Figure 4.1 Differential presence of proteins within the supraesophageal ganglion of *Manduca sexta* caterpillars that have been parasitized by *Cotesia congregata*. The data show the number of proteins found to be in different concentrations when compared to uninfected control caterpillars..... 60

Figure 4.2 Differential presence of proteins within the subesophageal ganglion of *Manduca sexta* caterpillars that have been parsitized by *Cotesia congregata*. The data show the number of proteins found to be in different concentrations when compared to uninfected control caterpillars..... 76

Figure 4.3 Uncharacterized proteins that are present in differing amounts in parasitized caterpillars when compared to control caterpillars..... 88

Figure 4.4 Heatmap of bracovirus proteins detected at the different timepoints of parasitism in both the supraesophageal ganglion and the subesophageal ganglion. Green cells (+) indicate presence of protein, pink cells (-) indicate absence of protein..... 89

Figure 4.5 Amplification of viral transcripts was present for all three primer pairs in the supraesophageal ganglion of all parasitized caterpillars, regardless of time point. No amplification was seen in the supraesophageal tissues of non-parasitized controls..... 90

ABSTRACT

Animals require sophisticated means of defense against a harsh and constantly changing environment. The immune system, detoxification system, and fight-or-flight responses are generally viewed as disparate systems and studied independently. Recently, the view that these systems are largely independent of one another has been challenged, and thinking has shifted towards the idea of an integrated defense system (IDS) that encompasses all three. Within this system, resources can be shared and borrowed. Different arms of the system can suppress others in order to best deal with the most immediate threat. With this new perspective in mind, I used poisons, pathogens and parasitoids to investigate strategies with which an insect, *Manduca sexta,* may reconfigure its internal networks in response to differing threats. In addition to the immune, detoxification and stress responses being interconnected, they are additionally networked with the central nervous system (CNS), which controls behaviour. Through investigating the sickness behaviour known as illness-induced anorexia, that is coordinated by the CNS, I show that there is a molecular pinch point between the immune system and the detoxification system. Moreover, I show that this CNS-coordinated change in behaviour may lead to increased survival from an infection by decreasing the need for resources (e.g. glutathione) by the detoxification system. Additionally, the immunophysiological network between two important immune organs, the midgut and fat body, is investigated using biologically relevant stressors. The response to pathogens is time, tissue, and stressor- dependent. Finally, the CNS of *Manduca sexta* is investigated using proteomic analysis to elucidate whether the parasitic manipulator *Cotesia congregata* is able to hijack the communication network between the immune system and the CNS. In addition to finding that many different systems and pathways are altered by *C. congregata*, I also present novel evidence of parasite-coded proteins within the CNS of *Manduca sexta.* Broadly, this work provides evidence that the arms of the IDS, as well as the CNS, in *M. sexta* are deeply interconnected and may reconfigure themselves or trade-off resources to most effectively counter immediate threats; but also that this interconnectedness leaves *M. sexta* susceptible to parasitic manipulation.

LIST OF ABBREVIATIONS USED

AMP	Antimicrobial peptide
CFU	Colony forming units
CNS	Central nervous system
CPG	Central pattern generator
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
GBP	Growth blocking peptide
GSH	Glutathione
GST	Glutathione-S-Transferase
HK	Heat killed
IDS	Integrative defense system
Imd	Immune deficiency pathway
JAK-STAT	Janus kinase-signal transducer and activator of transcription pathway
LD50	Lethal Dose 50%
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular patterns
PO	Phenoloxidase
PRR	Pattern recognition receptor
PSP	Plasmatocyte spreading peptide
PTTH	Prothoracicotropic hormone
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species

ACKNOWLEDGEMENTS

Thank you to Dr. Shelley Adamo for being a wonderful, inspiring supervisor. I felt throughout the course of my PhD that you always had my best interests in mind, and never ceased to provide me with opportunities to grow as a scientist and a person.

My thanks to my supervisory committee Dr. Tamara Franklin and Dr. Ian Weaver for their advice and support as my project inevitably evolved over the course of my PhD. I would also like to thank Dr. Michel Cusson for taking the time to be my external reviewer on this thesis.

I am grateful to all of the Adamo lab members I had the pleasure of working with over the course of my PhD (there have been a lot!) but I would be remiss if I did not single out a fellow PhD student, Dylan Miller. Dylan, thank you for the discussions. Whether it be video games, DnD podcasts, movies or (heaven forbid) work, you were always an energetic and engaging debate partner/co-conspirator.

I would also like to thank my coven, Susan and Aimée McMillan. The love and encouragement you give me feeds my soul and truly allows me to grow. Even living provinces away, in the middle of a global pandemic, I never felt alone. I could not ask for a better support system.

Finally, my partner Lyam. Thank you Lyam for being the person who kept me sane throughout this degree. Possibly the most fortuitous part of coming to Dalhousie was the opportunity to meet you. None of this would have been possible without you. From our morning teas to playing video games with me while I studied in France. Thank you for literally everything. May our shenanigans never cease.

CHAPTER 1 - GENERAL INTRODUCTION

Immune systems are vital to life. Nevertheless, the same challenge can produce qualitatively and quantitatively different immune responses, depending on internal and external conditions. For example, the production of fight-or-flight behaviour depresses immune function in animals across phyla (Adamo, 2008). Just as behaviour can alter immune function, the immune system can alter behaviour (e.g. sickness behaviours, Hart, 1988). Immune systems are interconnected with other physiological systems in the body, forming part of an integrated defense system (Adamo, 2022). Physiological trade-offs and other constraints across interconnected systems help explain why immune system responses are often context dependent. In this thesis, I explore immunophysiological interactions using the caterpillar *Manduca sexta,* an important model system in insect biology. To begin I will review insect immune systems, followed by a discussion of how the immune system is interconnected with other defense systems.

1.1 INSECT IMMUNITY

Insects, in their natural environments, are constantly under threat of attack from pathogens, parasites, and predators. Being able to survive, thrive and reproduce successfully in these conditions requires a constantly evolving suite of life history traits. One of the most important of these life history traits is the immune system, which can be considered as an insect's last line of defense against pathogens.

When faced with pathogens, an insect has three major avenues of defense: behaviour, physical barriers, and immunity. Behaviour is the first, and potentially least costly, means of defense: an insect may avoid pathogens by, for example, choosing not to mate with unhealthy conspecifics. Such behaviours, however, are not sufficient to avoid all pathogens. Microorganisms exist everywhere, e.g. the soil, and on the surface of plants. It would be impossible, therefore, for an insect to avoid all potential pathogens by behavioural means alone. The second line of defense insects have against pathogens is physical barriers (Siva-Jothy et al., 2005). Most insects possess two physical barriers: the outer cuticle which forms a solid barrier around their external surfaces, and the peritrophic membrane which lines the alimentary canal (Siva-Jothy et al., 2005). The outer cuticle of most insects is a layered fibrous accumulation of chitin, protein and lipid which is secreted by a layer of epidermal cells located just beneath the

cuticle (Vincent and Wegst, 2004). This makes the body surface of most insects waxy and impermeable to water, and therefore difficult for organisms to pass through (Vincent and Wegst, 2004). For many insects, the alimentary canal is the site of many invasion opportunities for pathogens and parasites (Han et al., 2000). In particular, the midgut is constantly exposed to foreign substances while at the same time being the least-well defended by a physical barrier (Chapman, 2013). However, even though the midgut is the most permeable area of the digestive system, it is still protected by a physical barrier called the peritrophic membrane (Peters, 2012). The peritrophic membrane is chitin- and protein-based, and is smooth and continuous throughout the midgut, in theory providing no gaps or holes through which a microbe or viral particle may cross (Brandt et al., 1978). While these properties of the peritrophic membrane do confer considerable protection against pathogens and parasites targeting the midgut, it has its limitations. For example, the peritrophic membrane has been found to be susceptible to alterations, such as the reduction of structural glycoproteins (rendering it more brittle) when the insect is infected either with bacterial or viral infection (Derksen and Granados, 1988).

When behavioural avoidance and physical barriers fail, the final line of defense insects possess is their robust immune system. Their immune system is innate, lacking the equivalent of the vertebrate acquired immune system (Siva-Jothy et al., 2005). The innate immune system possesses effector mechanisms, such as hemocytes that can perform phagocytosis and melanization, that are highly efficient and functionally sophisticated for sequestering and destroying invading pathogens (Hillyer, 2016; Kurtz, 2004).

Insect immunity is typically divided into two categories: cellular immunity, which involves hemocytes (i.e. insect blood cells), and humoral immunity (non-cellular humoral components such as antimicrobial peptides (AMPS) reviewed in Buchon et al. (2014). Insect immunity can be further divided on the basis of when responses are synthesized; in this respect, immune responses may be considered constitutive or inducible. Constitutive responses are constantly present, typically in an inactive form, while inducible responses are only produced upon detection of a threat, wound or infection.

Although these divisions are useful when describing the immune system, like many other physiological traits, it is important to acknowledge that the immune system forms an intricate network with cellular and humoral immunity working in concert, not as discrete systems. Moreover, the physiological and environmental situations that an insect may find itself in at any given time will determine whether an aspect of immunity is

expressed constitutively or inducibly. For example, in the face of chronic food limitation some insects will constitutively produce certain AMPs that would be synthesized inducibly in a food abundant environment (Adamo et al., 2016a). This type of immunity can be thought of as “soldiers at the gate”, and is generally regarded as more costly in terms of energy and resource expenditure compared to inducible mechanisms (Hamilton et al., 2008). Constitutive components of cellular immunity include circulating hemocytes such as granulocytes and plasmatocytes. Both granulocytes and plasmatocytes are capable of detecting and phagocytosing smaller foreign substances encountered within the hemocoel (Elrod-Erickson et al., 2000; Hoffmann, 2003; Jutras and Desjardins, 2005). When larger foreign bodies are detected hemocytes can agglutinate in layers, systemically surrounding and encapsulating the invader (Jiravanichpaisal et al., 2006; Lavine and Strand, 2002). This particular cellular defense comes in two sizes. To deal with microorganisms and bacteria, this process forms smaller agglutination events called nodules. In the case of large invaders, such as parasitoid eggs, this process occurs on a much larger scale and is known as encapsulation (Jiravanichpaisal et al., 2006; Lavine and Strand, 2002).

The primary constitutive component of humoral immunity is the phenoloxidase cascade (Cerenius and Söderhäll, 2004; Chase et al., 2000; Gorman and Paskewitz, 2001). Phenoloxidase is an enzyme that catalyzes the initial step in the production of the biopolymer melanin (Sugumaran et al., 2000) which is critical for neutralizing foreign substances following nodulation and encapsulation. The insect innate immune system uses a suite of pattern recognition receptors (PRRs) secreted into both the hemolymph and transmembrane bound (Wang et al., 2019) to recognise a range of non-self motifs from pathogen cell surface molecules referred to as pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Medzhitov, 2009; Theopold et al., 1999). Once a PAMP has been detected, serine protease cascades are initiated which transforms prophenoloxidase into phenoloxidase (Cerenius and Söderhäll, 2004). Once phenoloxidase has been initiated, it in turn promotes the production of melanin. As an example of how both cellular and humoral immune components work together, after hemocytes have agglutinated during nodulation and encapsulation, they will apoptose (Lavine and Strand, 2002). Activity of the phenoloxidase cascade initiated by these events will result in melanization of the cell mass, forming a barrier completely isolating the melanized mass or nodule from the hemocoel (Jiravanichpaisal et al., 2006; Siva-Jothy et al., 2005).

Inducible immunity involves cellular and humoral responses that are synthesized only in the presence of a pathogen. These tend to be more specific to the threat, such as AMPs that are active specifically against Gram + or Gram - bacteria, but also are slower to activate (Siva-Jothy et al., 2005). Once an inducible immune response has been elicited (i.e. an upregulation of antimicrobial gene expression and production), the immune response may remain elevated for weeks (Schmid-Hempel and Ebert, 2003). This chronic elevation can aid in preventing reinfection (Mowlds et al., 2010). Some environmental stressors, such as low nutrition, can also induce the chronic expression of some immune responses, effectively shifting a previously inducible response towards becoming a constitutive response (Adamo et al., 2016a). Inducible cellular immunity differs for different insect taxa. Insects such as Drosophila possess an additional inducible hemocyte called a lamellocyte that aids in encapsulation (Tokusumi et al., 2018). Other insects such as lepidoptera do not produce these cells, but are able to induce a hemocyte storage organ, called the hematopoietic organ, to release additional hemocytes in response to a detected threat (Nardi et al., 2003).

The humoral response makes up the large portion of an insect's inducible immunity through the production of antimicrobial peptides (AMPs) and immune proteins. Once activated, inducible molecules can in turn augment constitutive aspects of the immune system such as increasing PO (Haine et al., 2008). Antimicrobial peptides (AMPs) are a diverse group of small immune effectors that are quite effective at neutralizing Gram + and Gram - bacteria, as well as fungi (Siva-Jothy et al., 2005). The main tissues that can produce AMPs are the fat body, the midgut and certain hemocytes, called granulocytes (Al Souhail et al., 2016; Russell and Dunn, 1991; Siva-Jothy et al., 2005; Zhu et al., 2003). Production of AMPs usually begins with the recognition of a PAMP that leads to activation of Toll dorso-central signaling pathway, the immune deficiency pathway (Imd) and/or the Janus kinase-signal transducer and activator of transcription pathway (JAK-STAT) (Figure 1.1).

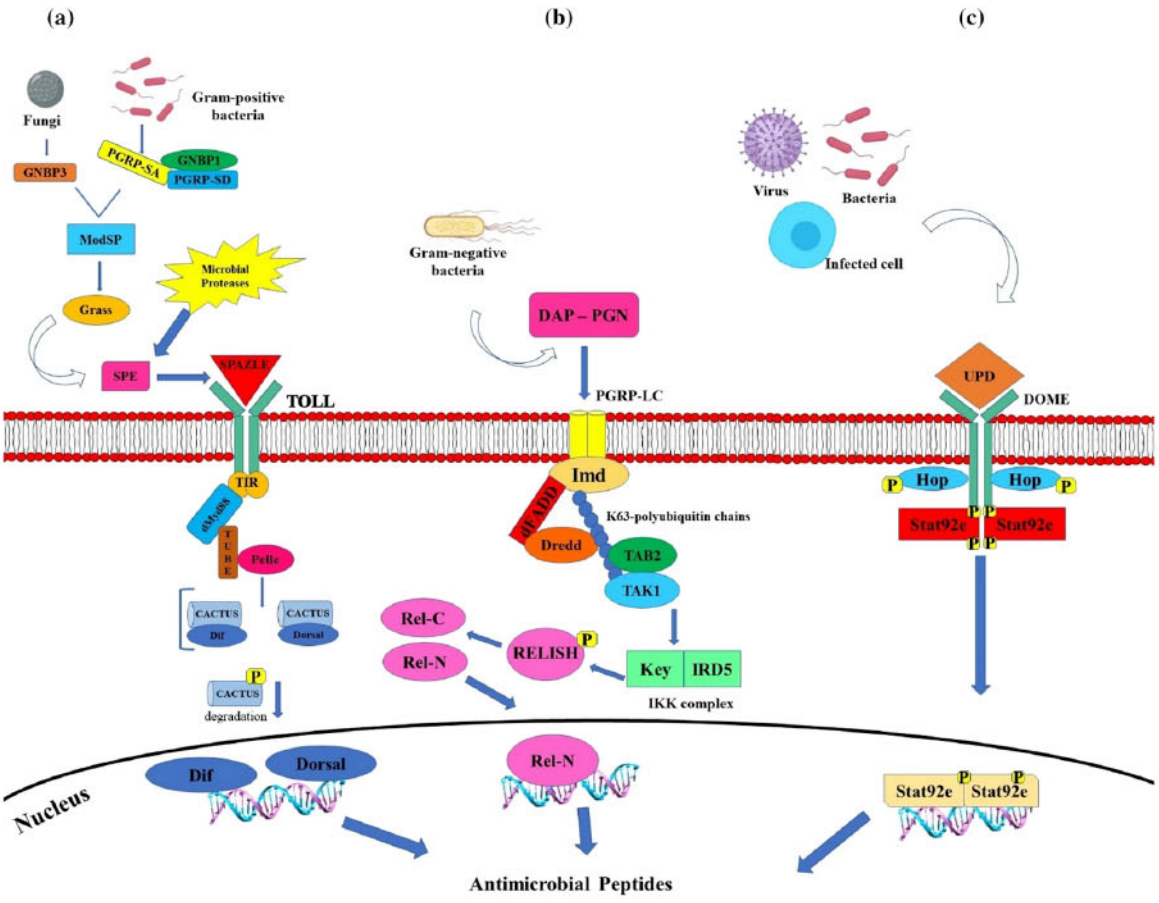

Figure 1. Schematic representations of insect toll (a) Imd (b) and JAK-STAT (c) pathways. Adapted from (Manniello et al., 2021).

Although distinct pathways, there is evidence that cross-talk occurs between them, and in some cases more than one of these pathways need to be activated for successful production of AMPs (Hoffmann and Reichhart, 2002; Kaneko et al., 2008; Lemaitre et al., 1997).

The Toll, Imd, and JAK-STAT pathways are cell-based transduction pathways, so named for their mediating proteins (Hultmark, 2003). The Toll pathway is activated when a PAMP from Gram + bacteria or fungi is detected, and also by certain viruses (Ao et al., 2008; Imler and Zheng, 2004; Michel et al., 2001; Zambon et al., 2005). Identification of the PAMP leads to activation of a cytokine called Spätzle which will bind with Toll receptors on the cell surface after being activated by an enzyme (Ao et al., 2008; Zhong et al., 2012). The toll receptor activates a cascade within the cytoplasm which results in the transcription factors Dif and Dorsal relocating to the nucleus and activating the transcription of Toll regulated genes (Hoffmann and Reichhart, 2002; Zhong et al., 2012). Examples of AMPs regulated by the Toll pathway are attacin, which is effective against Gram - bacteria such as *Escherichia coli* (Carlsson et al., 1998), and drosomycin, which is an anti-fungal that also has anti-parasitic and anti-yeast properties (Zhang and Zhu, 2009). The Imd pathway is another cascade that is activated in response to Gram - bacteria, fungus, parasites, and even some viruses (Avadhanula et al., 2009; Costa et al., 2009; Hultmark, 2003; Meister et al., 2005). When an invader signal is detected by a membrane bound pattern recognition receptor (PRR) a cascade begins in the cytoplasm which results in translocation of the transcription factor Relish into the nucleus, activating the transcription of Imd regulated AMPs. AMPs regulated by the Imd pathway include cecropin, defensins and gloverin. Finally, the JAK-STAT pathway is responsible for certain anti-viral defenses (Kingsolver et al., 2013). In some insects, viral dsRNA will activate an RNAi pathway which in turn will both reduce viral RNA replication and also activate the JAK-STAT pathway leading to production of effector molecules that can decrease the replication of viruses (Kingsolver et al., 2013).

Of the three pathways outlined above, only the Imd pathway is completely dedicated to immunity. The Toll pathway also plays a role in insect development in addition to immunity (Viljakainen, 2015). The JAK-STAT pathway is involved in embryonic development, fate mapping, cell proliferation, differentiation and apoptosis (Harrison, 2012). In addition to this, the Toll and Imd pathway work synergistically, and depending on the location within the insect (i.e. fat body vs midgut), may regulate the same AMPs and other humoral immune factors such as lysozymes (Tanji et al., 2007;

Viljakainen, 2015; Yokoi et al., 2012). There is even some evidence of cross-over between the Toll pathway and cellular immunity, whereby activation of the Toll pathway may result in further activation of hemocytes (Qiu et al., 1998). The interconnection of different pathways within the insect's innate immunity forms a complex network wherein no singular response—cellular or humoral; constitutive or inducible—is solely responsible for the reaction to a threat.

1.2 THE INTEGRATED DEFENSE SYSTEM

In recent years, research has shifted towards understanding the immune system as part of a larger physiological network (Dolezal et al., 2019). The insect immune system is a complex and interconnected network (Tanji et al., 2007; Viljakainen, 2015), and it is also part of a larger physiological network generally referred to as the Integrated Defense System (IDS) (Adamo, 2022). Insects face threats in the form of predation, infection from microorganisms, as well as toxins (or, as was nicely alliterized by Adamo, 2022: Predators, pathogens, and poisons). The responses to all of these threats require resource expenditure. It is likely that an insect will encounter more than one of these insults simultaneously, which will ultimately require the reconfiguration of responses to best ensure survival (Adamo, 2017; Adamo et al., 2017).

The IDS encompasses all of the molecular cascades, tissues and signaling molecules involved in the fight-flight-freeze response, the immune system, and detoxification systems, as well as any crosstalk molecules used as signals between them (Adamo, 2022). Considering these systems together, and the interplay between them, offers a more holistic perspective on an insect’s responses to threats, whether they be parasitic, pathogenic or predatory.

Adamo (2022) postulates that viewing immune, detoxification and stress responses as interconnected systems is logical based on the fact that there are extensive connections between the three systems on a cellular and molecular level. The first argument in favor of viewing these systems as constituent parts of the IDS is that all three lead to increased lipid and carbohydrate in the hemolymph using overlapping neurohormonal signals (Adamo et al., 2008; Mullen et al., 2004; Singh, 1986). Secondly, these three systems share some of the same molecular signaling systems and hormones such as octopamine (Adamo, 2017; Adamo, 2022; Miyashita and Adamo, 2020). Thirdly the components of the IDS also share effector molecules. For instance, phenoloxidase (PO), which is a major commodity for constitutive immunity, also plays an

important role in detoxification (Liu et al., 2009). Fourthly, activation of any one of the three component systems of the IDS results in changes in expression of effector genes in the other two systems (summarized in (Adamo, 2022). For example, stress responses caused by non-consumptive predation simulations (Adamo et al., 2017) and exposure to toxins (Gao et al., 2021) both lead to an increase in AMPs. Although seemingly off-target for these types of responses, these changes may be part of an adaptive reconfiguration. For example, there is evidence that intense flight, such as from a predator, reduces the antimicrobial properties of hemolymph, thereby reducing the insect's ability to defend themselves against bacteria (Adamo et al., 2008). Conversely, an increase in the synthesis of AMPs, a different immune response, during fight-or-flight stress has been shown to increase pathogen resistance to some pathogens (Mowlds and Kavanagh, 2008). This shift in AMP production to a more constitutive pattern may help maintain immune protection while some immune resources are being shifted towards fight-or-flight behaviour.

The immune system is also interconnected with the nervous system. This is shown by the fact an insect can change its behaviour in response to an immune insult. Sickness behaviours, such as behavioural fever, lethargy and illness induced anorexia, increase an animal's probability of survival during an immune challenge and are pervasive across insects (Hart, 1988; Sullivan et al., 2016). In both insects and vertebrates, sickness behaviours are induced by pro-inflammatory cytokines that are released from immune cells and bind to receptors on nervous tissue (Dantzer, 2004; Dantzer et al., 2008). The cytokines most likely implicated in immune-neural connections are the plasmatocyte-spreading-peptide (PSP) and growth-blocking peptide (GBP) (reviewed in Adamo, 2011). PSP is a proinflammatory cytokine that plays a role in cellular immunity, as mentioned previously. It is expressed in both the fat body and the CNS, although its role within the CNS is not clear at this point (Lavine and Strand, 2002).

In the absence of stressors, most resources are devoted to growth and reproduction. However, when a stressor such as predators, pathogens or toxins are present, resource trade-offs occur, with negative impacts on both growth and reproduction; such trade-offs have been demonstrated following fight-or-flight stressors (Hermann and Landis, 2017), detoxification stressors (Freeman et al., 2021), and immune stressors (Schwenke et al., 2016). The shifting of resources across the different defense systems can have negative impacts on the defense systems donating the resources. This perspective helps to explain the negative impact of immune responses

on fight-or-flight behaviour. Insects facing an immune challenge show a reduction in anti- predator behaviour (Otti et al., 2012), in part due to the oxidative stress generated by the immune response (Janssens and Stoks, 2014).

1.3 *MANDUCA SEXTA*

Manduca sexta is a lepidopteran native to Canada, the United States of America and Mexico, and was first described by Linnaeus in 1763 in his *Centuria Insectorum*. The larval form, or caterpillar, of this insect feeds on solanaceous plants such as tobacco, but can be reared successfully on a lab made diet (Ahmad et al., 1989; Bernays and Woods, 2000). *M.sexta* caterpillars are quite large, reaching masses of up to 5 grams (g) before pupating (Nijhout and Williams, 1974), with a robust immune response to pathogens (Jiang et al., 2010; Kanost et al., 2004). The caterpillar's large size makes it an ideal model organism for studying immunophysiological networks because of the ease with which several tissues can be dissected, including the main immune organ (the fat body), the main detoxification organ (the midgut), as well as the central nervous system (CNS). *M. sexta* caterpillars have been used successfully in immunity research for over 20 years (reviewed in Jiang et al., 2010). There is a wealth of knowledge to support research into the immunophysiological network of *M.sexta* caterpillars including an annotated genome (Gershman et al., 2021; Kanost et al., 2016), an immunotranscriptome (Gunaratna and Jiang, 2013), transcriptomes from the fat body (Zhang et al., 2011) and from hemocytes (Zhang et al., 2011; Zou et al., 2008) and even from the midgut (Pauchet et al., 2010). This abundance of foundational research allows for a deeper dive into the complex networks at play within the *M. sexta* immunophysiological network.

M. sexta caterpillars exhibit both constitutive and inducible cellular and humoral responses. The caterpillars possess hemocytes that are capable of phagocytosis, nodulation and encapsulation of fungi, bacteria and parasitoids (Horohov and Dunn, 1983; Lavine and Beckage, 1996; Lord et al., 2002). They have a suite of mobile and membrane bound PRRs that are capable of recognizing PAMPs such as lipopolysaccharide, peptidoglycan, β-1,3 glucans another others (Yu et al., 2002) which then activate the Toll, Imd and JAK-STAT pathways (Cao et al., 2015; Zhong et al., 2012). *M. sexta* caterpillars express a number of AMPs such as attacin, gloverin, and moricin from fat body and hemocytes (Rao and Yu, 2010; Xu et al., 2012). In addition,

they have a robust PO response as well as other humoral immune components such as lysozyme (Hall et al., 1995; Russell and Dunn, 1991).

1.4 EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR *MANDUCA SEXTA*

In the first study of this work, I investigate how two important defense systems, the immune and detoxification systems, interact. Previous work in *M. sexta* caterpillars has shown reconfiguration and sharing of resources between fight-or-flight stress systems and the immune system (Adamo, 2017). I set out to investigate whether illness-induced anorexia, a self generated sickness behaviour, may be an adaptive ramification of a detoxification/immune response interaction.

Illness-induced anorexia is a ubiquitous (Sullivan et al., 2016) but poorly understood response. It seems maladaptive, mounting an immune response requires substantial energy and resources (Ardia et al., 2012; Freitak et al., 2003). Despite the need for resources, immune challenged animals stop eating, even though eating would help supply the resources they need. In *M. sexta*, illness-induced anorexia carries additional costs. A reduction in eating leads to slower growth rates, prolonging the larval stage of the caterpillar (Bernays and Woods, 2000). However, prolonging this developmental stage increases the risk of predation (Bernays, 1997).

In this chapter, I propose the hypothesis that the CNS-controlled sickness behaviour of illness-induced anorexia is present to reduce competition for limited resources between the immune response and detoxification response.

1.5 FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR *MANDUCA SEXTA*

In the second study of this thesis I look at the interaction between the microbiome and immune system of *M. sexta* caterpillars. The midgut is an interesting tissue to examine from the perspective of an immunophysiological network. Due to its role in nutrient absorption and food digestion it is the system least protected by physical barriers (Chapman, 2013). That being said, it is also quite immunologically active and has the ability to produce antimicrobial peptides and proteins (Pauchet et al., 2010). Nonetheless, the midgut is the main route of infection for most insects (Buchon et al., 2014; Vallet-Gely et al., 2008). When faced with a severe systemic infection, the

peritrophic membrane within the midgut can be damaged by immunopathogenesis and delamination of enterocytes (which secrete the peritrophic membrane) can occur (Buchon et al., 2014). This in turn can increase the amount of pathogens, such as bacteria, that have access to the hemocoel of the caterpillar (Steinhaus, 1959).

Unlike most animals, *M. sexta* do not seem to benefit from a resident gut microbiome (Hammer et al., 2017). While there are bacteria and fungi present in their gut, it is transient and not as phylogenetically diverse as is seen in other animals. Moreover, sterilizing their gut with antibiotics has no impact on rate of growth or time to pupation (Hammer et al., 2017). That being said, there are still transient microbiota that are obtained from the leaf microbiome of their host plant (Zhou et al., 2020). Properly managing a microbiome to keep it from becoming pathogenic is resource intensive (Zhai et al., 2018). Previous research in the caterpillar, *Galleria mellonella*, showed that in the presence of a microbiome, baseline constitutive expression of some AMPs is increased (Krams et al., 2017). Although the transient microbiome of *M. sexta* does not appear to benefit the caterpillars, there is always risk of harm should it grow from benign to pathogenic.

I investigate the impacts of a systemic infection on the regulation of the transient gut microbiome of *M. sexta* caterpillars. I examine the immunophysiological network that exists between the two main immune tissues (the fat body and the midgut), quantify changes in immune expression in these tissues, and also look at the impact of systemic challenge on the gut microbiome itself. I pose three alternative hypotheses: that in the face of a severe immune challenge, immune reconfiguration would sterilize the midgut completely, leaving more resources available to fight the systemic infection; that there would be major trade-offs between the two immune tissues, or that rather than complete trade-offs smaller reconfigurations may be occurring.

1.6 PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA* CATERPILLARS PARASITIZED BY WASP *COTESIA CONGREGATA*

It has been established that arms of the IDS, such as the immune system, can alter behaviour to increase chances of survival of an immediate threat (Dantzer, 2004; Hart, 1988). While these behavioural changes are generally advantageous to the insect, parasites have adapted to hijack these communication networks in order to control the behaviour of their hosts (Moore, 2002). One such parasite is the braconid wasp, *Cotesia congregata*, which is an obligate parasitoid of the caterpillar *M. sexta*. *C. congregata*

injects young *M. sexta* caterpillars with her eggs, a venom and a domesticated virus (polydnavirus) that facilitates parasitism success. This injected cocktail enables the wasp to modulate both the immune system of *M. sexta*, reducing the likelihood of her eggs being encapsulated or killed by the immune response (Lavine and Beckage, 1996), and later, the hosts' self generated behaviours that could result in pupal death (see Adamo et al., 2016b). Specifically, it has been hypothesized that *C. congregata* manipulates immune-neural connections to suppress feeding in its host (Adamo et al., 2016b).

In the final study of this work, I build on existing knowledge about the *M. sexta* genome (Gershman et al., 2021; Kanost et al., 2016) and immunotranscriptome (Gunaratna and Jiang, 2013) as well as the wealth of knowledge on the parasitoid manipulator *C. congregata* (see Adamo et al., 2016b; Chevignon et al., 2014; Chevignon et al., 2015; Gelman et al., 1998). In so doing I conduct a complete proteomic analysis of the change of protein profiles present in two important components of *M. sexta* CNS, namely the supraesophageal ganglion and the subesophageal ganglion, in order to characterize the possible mechanisms by which *C. congregata* manipulates host immunity and behaviour. The supraesophageal ganglion, which is located in the head capsule of *M. sexta* caterpillars, houses the neural circuits for self generated behaviours such as walking initiation and feeding motivation (Emanuel et al., 2020). The supraesophageal ganglion houses the central complex, an area that is targeted by the venom of another parasitoid, the jewel wasp, to abolish self-generated behaviours in the cockroach (Kaiser and Libersat, 2015). Mushroom bodies, bilateral neuropils composed of densely packed neuronal processes and glia, also within the supraesophageal ganglion, have been shown to be a critical site for inducing feeding through appetitive memory in Drosophila (Keene et al., 2006; Krashes and Waddell, 2008; Schwaerzel et al., 2003). For these reasons, I believe that the supraesophageal ganglion is an excellent candidate to explore for possible correlates of behaviour manipulation by *C. congregata*. The subesophageal ganglion, also located in the head capsule, controls the physical movement of insect mouthparts (Blaney and Simmonds, 1987) as well as housing the central pattern generations (CPGs) necessary to coordinate feeding (Altman and Kien, 1987; Rohrbacher, 1994). Because of its role in mouthpart function and coordination, I also chose to examine this ganglion for proteomic changes that may indicate parasitic manipulation.

By examining three different time points of late stage parasitism (over the course of two days prior to wasp emergence, to three days post wasp emergence) I

demonstrate dynamic alterations to a variety of proteins involved in diverse pathways and cascades both before and after behaviour change in the caterpillar, in both of the CNS regions examined. In addition to this, I demonstrate the presence of several virally encoded proteins being expressed within the CNS of *Manduca sexta* that both precede and coincide with the change in behaviour.

CHAPTER 2 -EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR, *MANDUCA SEXTA*

2.1 PUBLICATION INFORMATION

McMillan, Laura E., Dylan W. Miller, and Shelley A. Adamo. "Eating when ill is risky: immune defense impairs food detoxification in the caterpillar Manduca sexta." *Journal of Experimental Biology* 221.3 (2018): jeb173336.

2.2 ABSTRACT

Mounting an immune response consumes resources, which should lead to increased feeding. However, activating the immune system reduces feeding (i.e. illness-induced anorexia) in both vertebrates and invertebrates, suggesting that it may be beneficial. We suggest that illness-induced anorexia may be an adaptive response to conflicts between immune defense and food detoxification. We found that activating an immune response in the caterpillar *Manduca sexta* increased its susceptibility to the toxin permethrin. Conversely, a sublethal dose of permethrin reduced resistance to the bacterium *Serratia marcescens*, demonstrating a negative interaction between detoxification and immune defense. Immune system activation and toxin challenge each depleted the amount of glutathione in the hemolymph. Increasing glutathione concentration in the hemolymph increased survival for both toxin and immune + toxin challenged groups. The results of this rescue experiment suggest that decreased glutathione availability, such as occurs during an immune response, impairs detoxification. We also found that the expression of some detoxification genes were not upregulated during a combined immune-toxin challenge, although they were when animals received a toxin challenge alone. These results suggest that immune defense reduces food detoxification capacity.
Illness-induced anorexia may protect animals by decreasing exposure to food toxins when detoxification is impaired.

© 2018. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2018) 221, jeb173336. doi:10.1242/jeb.173336

RESEARCH ARTICLE

Eating when ill is risky: immune defense impairs food detoxification in the caterpillar *Manduca sexta*

Laura E. McMillan*, Dylan W. Miller and Shelley A. Adamo

ABSTRACT

Mounting an immune response consumes resources, which should lead to increased feeding. However, activating the immune system reduces feeding (i.e. illness-induced anorexia) in both vertebrates and invertebrates, suggesting that it may be beneficial. We suggest that illness-induced anorexia may be an adaptive response to conflicts between immune defense and food detoxification. We found that activating an immune response in the caterpillar *Manduca sexta* increased its susceptibility to the toxin permethrin. Conversely, a sublethal dose of permethrin reduced resistance to the bacterium *Serratia marcescens*, demonstrating a negative interaction between detoxification and immune defense. Immune system activation and toxin challenge each depleted the amount of glutathione in the hemolymph. Increasing glutathione concentration in the hemolymph increased survival for both toxin- and immune+toxin-challenged groups. The results of this rescue experiment suggest that decreased glutathione availability, such as occurs during an immune response, impairs detoxification. We also found that the expression of some detoxification genes were not upregulated during a combined immune–toxin challenge, although they were when animals received a toxin challenge alone. These results suggest that immune defense reduces food detoxification capacity. Illness-induced anorexia may protect animals by decreasing exposure to food toxins when detoxification is impaired.

KEY WORDS: Illness-induced anorexia, Psychoneuroimmunology, Sickness behavior, Ecoimmunology, Pesticide

INTRODUCTION

Animals typically lose their appetite when their immune systems are activated (i.e. illness-induced anorexia; Sullivan et al., 2016). However, immune defense is energetically costly (Demas et al., 1997; Freitak et al., 2003); therefore, animals would be expected to increase, not decrease, their food intake when mounting an immune response. Nevertheless, illness-induced anorexia can enhance recovery in both vertebrates (Murray and Murray, 1979) and invertebrates (Adamo et al., 2010). Moreover, illness-induced anorexia is induced by signaling molecules (e.g. cytokines) released by the host's immune system that bind with receptors within the host's brain (Dantzer, 2004). The existence of these signaling pathways supports the hypothesis that illness-induced anorexia benefits the host. However, how loss of appetite is beneficial remains a puzzle. A number of non-mutually exclusive hypotheses have been put forward to explain the adaptive function of illness-induced anorexia (Kyriazakis et al., 1998); however, these hypotheses do not fully explain the phenomenon, especially for some animal groups (Adamo et al., 2007). We present a novel explanation for its presence in immune-challenged animals.

We postulate that illness-induced anorexia protects animals from the effects of a competition for molecular resources between digestion-related processes and immune defense (Adamo et al., 2010, 2007). For example, immune defense and food detoxification require some of the same molecular resources and occur within the same organ [e.g. liver in vertebrates (Hill et al., 2016), fat body in insects (Chapman, 2013)]. Such sharing can lead to reductions in function if both systems are activated simultaneously (Adamo, 2017). To test this hypothesis, we examine interactions between the immune system and detoxification pathways in the last larval instar of *Manduca sexta* (Linnaeus 1763). *Manduca sexta* larvae (i.e. caterpillars) are specialist herbivores that spend their larval stage on their food source (Bernays and Woods, 2000). Illness-induced anorexia is a general response to immune activation in *M. sexta* (Adamo et al., 2007; also see Fig. S1). However, it is a costly behavior for this insect, suggesting that it should be selected against unless it supplies some benefit. Illness-induced anorexia interrupts mass gain (Diamond and Kingsolver, 2011), and decreased mass gain increases the time spent in the larval stage (Diamond and Kingsolver, 2010). Lingering in the larval stage increases the risk of predation (Bernays, 1997; Kingsolver et al., 2012). Moreover, reduction in feeding also results in smaller adults, and that leads to lower fecundity (Kingsolver et al., 2012). The typical host plant of *M. sexta* contains toxic compounds (e.g. nicotine), and, therefore, food detoxification is crucial for this species (Snyder and Glendinning, 1996). Therefore, this species is a good model system in which to search for conflicts between food detoxification and immune defense.

Glutathione (GSH) is a tripeptide important for both disease resistance (Stahlschmidt et al., 2015) and food detoxification (Jeschke et al., 2016) in insects. GSH is an antioxidant that helps to reduce self-harm from the reactive oxygen species (ROS) that are created by immune functions such as the phenoloxidase (PO) cascade (González-Santoyo and Córdoba-Aguilar, 2012). Reducing the presence of ROS is important because their production by PO leads to Malpighian tubule damage (Khan et al., 2017; Sadd and Siva-Jothy, 2006). GSH is also important for detoxification pathways. It is the substrate for glutathione-s-transferases (GSTs), enzymes that enhance the disposal of xenobiotics (Habig et al., 1974). Toxins in food have been shown to reduce glutathione concentration in *M. sexta* (Guillet et al., 2000) and other insects (Clark et al., 2010) as well. Insects may have insufficient GSH to supply both detoxification and immune defense when both are co-activated. GSH contains the amino acid cysteine, a limited resource in most insects (Barbehenn et al., 2013; Jeschke et al.,

Department of Psychology and Neuroscience, Dalhousie University, Halifax, NS, Canada, B3H4R2.

*Author for correspondence (Laura.McMillan@dal.ca)

L.E.M., 0000-0002-0023-7011; D.W.M., 0000-0003-1673-4060; S.A.A., 0000-0002-5973-571X

Received 31 October 2017; Accepted 30 November 2017

Journal of Experimental Biology

Journal of Experimental Biology (2018) 221, jeb173336. doi:10.1242/jeb.173336

2016). Moreover, in some caterpillars, 20% of the animal's total cysteine is invested in GSH (Barbehenn et al., 2013). Given this high baseline level, it may be difficult to increase this amount significantly. For example, during toxin exposure, the caterpillar *Spodoptera littoralis* depletes hemolymph GSH and must catabolize proteins to supply cysteine for continued GSH production (Jeschke et al., 2016). We tested the impact of a toxin challenge, an immune challenge and a combined toxin and immune challenge on hemolymph GSH concentration. We predicted that GSH resources in the caterpillar are insufficient to meet the demands of both food detoxification and an immune challenge.

To remain healthy, insects rely on both constitutive immune defenses (i.e. defenses that are maintained prior to an immune challenge, such as the PO pathway) and inducible defenses (e.g. antimicrobial peptides) that are produced when pathogens are present (Schmid-Hempel, 2011). We assessed how activation of detoxification pathways impacted gene expression of key components of both inducible and constitutive immunity. Similarly, we examined how an immune challenge impacted the expression of key genes in the detoxification pathway, including *Glutathione-S-transferase-1* (*GST1*). *GST1* codes for the enzyme GST1 (Snyder et al., 1995). GST1 requires GSH as a co-factor (Enayati et al., 2005), and GST1 gene expression is upregulated by toxins in *M. sexta* (Snyder et al., 1995).

We make five predictions: (1) activation of an immune response will reduce resistance to toxins, and toxin exposure will reduce resistance to pathogens; (2) GSH hemolymph concentration will be reduced in immune-challenged and toxin-challenged caterpillars; (3) a combined immune and toxin challenge will reduce GSH concentration, but by less than expected given the decline caused by each challenge given singly; (4) giving supplemental GSH will increase the survival of dual-challenged animals; and (5) an immune challenge will depress detoxification gene expression, especially those genes that are involved in the expression of GSH-consuming proteins.

MATERIALS AND METHODS

Chemicals

All chemicals and Sephadex beads were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted. Bacteria were MicroKwik cultures from Carolina Biological Supply Company (Burlington, NC, USA). Heat-killed pathogens were used during immune challenges because they induce an immune response (Adamo, 2004), but no pathogenesis.

Animals

All studies were performed on fifth instar larvae of *M. sexta* obtained from our colony. The colony was derived from eggs supplied by Great Lakes Hornworm (Romeo, MI, USA), and was maintained as previously described (Adamo et al., 2016). Trial caterpillars were weighed after their molt to the last larval instar (fifth instar–Day 0). Caterpillars were allotted into groups by mass, such that there were no initial mass differences across groups. 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.

Is illness-induced anorexia pathogen specific?

Fifth instar–Day 2 caterpillars were assigned to one of six groups: (1) controls; (2) sham (sterile poke through cuticle with a pin to mimic an injection); (3) Gram-negative immune challenge [injected with heat-killed Gram-negative bacteria *Serretia marcescens*, adjusted to a dose equivalent to 1/10 the half maximal lethal dose (LD_{50}) of live bacteria, or approximately 2×10^4 cells 20 μl^{-1}]; (4) Gram-positive immune challenge (injected with heat-killed Gram-positive bacteria *Bacillus cereus*, adjusted to a dose equivalent to 1/10 the LD_{50} of live bacteria, or approximately 2×10^3 cells 20 μl^{-1}); (5) abiotic material challenge (injected with 20 µl of A-25 Sephadex bead suspension of a ratio of 0.01 g 200 μl^{-1} ddH_2O); and (6) starved. Beads were injected using a procedure modified from Lavine and Beckage (1996). All injections were made using a 50 µl Hamilton syringe (Hamilton Company, Reno, NV, USA). With the exception of the starved group, all caterpillars were returned to their individual containers after treatment and given a pre-weighed food cube of high-nutrition diet measuring 2 cm^3. Three control cubes of the same dimension were used to control for water loss of the food. Change in body mass and food mass over the next 3, 6 and 24 h was recorded.

Does illness-induced anorexia occur in the context of gut emptying?

Caterpillars were weighed at fifth instar–Day 2 and assigned to either the (1) control or (2) Gram-negative immune challenge group. The injected group were given heat-killed Gram-negative bacteria *S. marcescens*, adjusted to a dose equivalent to 1/10 the LD_{50} of live bacteria (or approximately 2×10^4 cells). All caterpillars were individually housed and allowed to consume high-nutrition food *ad libitum* for 24 h. Caterpillars had all food and fecal pellets removed 12 h prior to manipulation. Small high-nutrition food cubes (5 mm^3) were dyed using 0.4 ml food coloring (ClubHouse, London, ON, Canada) and given to each caterpillar. All caterpillars were given 1 h to consume the food cube. The immune challenge group was then given a 20 µl injection of heat-killed *S. marcescens*. The time until the first colored fecal pellet appeared was recorded.

Are caterpillars more likely to avoid food that requires detoxfication during an immune challenge?

Fifth instar–Day 2 caterpillars had their food removed 12 h prior to the study. Frass pellets were removed from their individual containers. Caterpillars were weighed and sorted into one of nine experimental groups: (1–3) controls: given food with (1) no quinine (0.00 mol l^{-1}), (2) low quinine (0.008 mol l^{-1}) or (3) high quinine (0.03 mol l^{-1}) concentrations; (4–6) sham: caterpillars were given a sterile poke with an insect pin followed by one of the three quinine conditions; or (7–9) immune challenged: caterpillars were injected with 2×10^4 cells 20 μl^{-1} of heated-killed *S. marcescens* followed by one of the three quinine diets. At the start of the food trial, caterpillars were placed directly on food, ensuring chemosensory hairs were in contact with the food cube. Baseline trials were conducted on all animals prior to treatment. The latency to eat, and number of bites in 3 min were recorded. After the baseline trial, all caterpillars were given their treatments and had additional feeding tests at 1, 3 and 20 h post exposure.

Does activation of detoxification pathways reduce resistance to *S. marcescens* infection?

Fifth instar–Day 2 caterpillars were weighed and assorted into four groups: (1) control caterpillars were given a 2.5 mm^3 food cube injected with 1 µl of ddH_2O; (2) toxin-challenged caterpillars were given a 2.5 mm^3 food cube injected with permethrin (1 µl, 0.1 µg μl^{-1}; ChemFree Insectigone); (3) immune-challenged caterpillars received an injection of live *S. marcescens* (LD_{50} 2×10^5 cells; Adamo et al., 2016), and water-injected food; and (4) dual-challenged caterpillars received an injection of live

S. marcescens (as above) and received food injected with permethrin (as above). Each group was given 1 h to fully consume the 2.5 mm^3 food cube. Caterpillars were checked daily for mortality. Data were censored at 7 days as control caterpillars typically enter metamorphosis (i.e. dorsal vessel exposure) on day 7.

Does an immune challenge reduce resistance to permethrin?

Fifth instar–Day 2 caterpillars had their food removed for 1 h prior to manipulation. Caterpillars were weighed and sorted into six groups: (1) control (unmanipulated) caterpillars were given water-treated food; (2) immune-challenged caterpillars received a 1/10 LD_{50} dose of heat-killed *S. marcescens* with water-treated food (as above); (3) bead-challenged caterpillars were injected with A25 Sephadex beads (see above), with water-treated food; (4) toxin-challenged caterpillars were provided with permethrin-treated food (LD_{50} dose 1 µg $µl^{-1}$; Adamo et al., 2016); (5) combined bead- and toxin-challenged caterpillars were injected with A25 Sephadex beads (as above), with permethrin-treated food (LD_{50} dose 1 µg $µl^{-1}$); and (6) immune+toxin-challenged caterpillars were injected with a 1/10 LD_{50} dose of heat-killed *S. marcescens*, with permethrin-treated food (LD_{50} dose 1 µg $µl^{-1}$). Each group was given a 2.5 mm^3 food cube as described above and given 1 h to fully consume it. Caterpillars were checked daily for mortality. Data were censored at 7 days as control caterpillars typically enter metamorphosis (i.e. dorsal vessel exposure) on day 7.

Do immune and toxin challenges impact circulating GSH levels?

Fifth instar–Day 2 caterpillars were weighed and assigned to one of three groups: (1) control, (2) immune challenged or (3) toxin challenged (see above). Hemolymph was deproteinated immediately after collection by centrifuging at 18,845 ***g*** for 10 min at 4°C and then adding the supernatant to an equal amount of metaphosphoric acid (0.1 g ml^{-1}, Sigma-Aldrich). After incubating at room temperature for 5 min, the samples were spun at 3350 ***g*** for 3 min. The supernatant was stored at −80°C. The deproteinated samples were thawed and processed according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Absorbance was measured at 405 nm. GSH hemolymph concentration was assessed by measuring both reduced and oxidized GSH species (GSH/GSSG) following the manufacturer's instructions (Cayman Chemical).

Can additional GSH rescue dual-challenged animals?

We tested whether supplemental GSH could rescue doubly challenged caterpillars. Toxin and immune (heat-killed *S. marcescens*) challenges were delivered as described in the previous sections. Supplemental GSH (20 µl of a 10 mmol l^{-1} solution) was delivered via injection after the toxin or immune challenge. Caterpillars were weighed at fifth instar–Day 0 and split into one of eight different groups: (1) control, (2) control+GSH, (3) toxin challenged, (4) toxin challenged+GSH, (5) toxin challenged +sham (given a sterile poke), (6) toxin and immune dual-challenged (as above)+GSH, or (7) toxin and immune dual-challenged (as above) with no supplemental GSH. In addition, one group (8) received the toxin challenge as well as an injection of 10 mmol l^{-1} glycine (BDH Chemicals LDT, UK). Caterpillars were monitored daily for mortality and censored at 21 days. A longer trial period was used in this experiment to test whether GSH had late acting effects (e.g. whether it first needed to be broken down before being used). Caterpillars are considered to have survived if they reached metamorphosis. Control *M. sexta* data end at day 7 because all had entered metamorphosis by that time. Immune challenge consisted of an injection of 20 µl mixture of heat-killed *S. marcescens* (Gram-negative bacterium, Microkwik culture, Carolina Biological, 1/10 LD_{50}), *Bacillus cereus* (Gram-positive bacterium, Microkwik culture, Carolina Biological, 1/10 LD_{50}) and *Beauveria bassiana* (strain GHA, fungus, 1/10 LD_{50}, BotaniGard 22WP; Laverlam, Butte, MT, USA). Toxin challenge was administered via food cube treated with permethrin (LD_{50}, ChemFree, Insectigone 0.25% permethrin in water) and paraquat [2.5 mg 100 $µl^{-1}$ paraquat dichloride hydrate (Fluka, Germany) in ddH_2O].

Does an immune challenge interfere with upregulation of detoxification-related genes?

Fat body was harvested from the seventh abdominal segment (A7) of fifth instar–Day 2 caterpillars 24 h post-manipulation. Fat body was excised from five different groups: (1) control; (2) sham (given a sterile poke); (3) immune challenged: injected with 20 µl mixture of heat-killed *S. marcescens* (Gram-negative bacterium, Microkwik culture, Carolina Biological, 1/10 LD_{50}), *Bacillus cereus* (Gram-positive bacterium, Microkwik culture, Carolina Biological, 1/10 LD_{50}) and *Beauveria bassiana* (strain GHA, fungus, 1/10 LD_{50}, BotaniGard 22WP; Laverlam); (4) toxin challenged: given a food cube treated with permethrin (1/10 LD_{50}, ChemFree, Insectigone 0.25% permethrin in water) and paraquat [2.5 mg 100 $µl^{-1}$ paraquat dichloride hydrate (Fluka, Germany) in ddH_2O] and (5) double-challenged: received both the same immune challenge as outlined above as well as the toxin challenge outlined above.

RNA extraction was performed using the 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 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 an Implen Nanophotometer P360 (Westlake, CA, USA) as well as a Qubit Fluorometer (Q32857, Invitrogen, CA, USA). Only samples with an A260/280 ratio greater than 1.8 were used. cDNA was synthesized using iScript (Bio-Rad, Hercules, CA, USA) and samples were stored at −20°C.

Primers were purchased from integrated DNA technologies (http://www.idtdna.com/site) and stored at −20°C at a working stock of 10 µmol l^{-1}. Each biological sample was diluted to a set concentration of 100 ng $µl^{-1}$ using the Qubit Fluorometer (Q32857, Invitrogen). For each biological sample and gene, a 25 µl reaction mixture was prepared containing 1 µl of sample cDNA, 12.5 µl SYBR Green Supermix (Bio-Rad), 1 µl of forward primer (10 µmol l^{-1}), 1 µl of reverse primer (10 µmol l^{-1}) and 9.5 µl RNase-free ddH_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). After the qPCR, a melt curve analysis was run to assess the specificity of the qPCR product. Quantitative cycle (C_q) values for each sample and gene target were calculated in CFX Manager (Bio-Rad).

For reference gene assessment, we selected the most stable of six candidate reference genes used in a previous study in *M. sexta* (Adamo et al., 2016): *Rp17A*, *actin* (*MSA*), *ribosomal protein S3* (*MsS3*), *ubiquitin*, *beta FTZ-F1* and *glycerol-3-phosphate dehydrogenase* (*G3PDH*). We used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes

Journal of Experimental Biology (2018) 221, jeb173336. doi:10.1242/jeb.173336

(Andersen et al., 2004) (i.e. *Rp17A* and *ubiquitin*), using the C_q values of five biological samples for each candidate reference gene, for each treatment. The qPCR efficiency (E) and correlation coefficient (R^2) for primer sets were estimated from a standard curve generated with 10-fold dilutions of mixed cDNA samples and are given in Table S1.

For reference gene assessment, we used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes (i.e. *Rp17A* and *ubiquitin*) chosen from a suite of six, using the C_q values of five biological samples for each candidate reference gene, for each treatment (Andersen et al., 2004). See Table S1 for details.

Statistics

Data were analyzed using SPSS (version 22) and GraphPad Prism (version 7.0). The qPCR data were analyzed using CFX Manager v. 3.1 (Bio-Rad) and the REST program (2009; http://rest.gene-quantification.info). Data were found to be normally distributed using a Shapiro–Wilk test. When multiple tests were performed on the same dataset, the alpha criterion was corrected (Benjamini and Hochberg, 1995) (see Table S2). Sample sizes were determined based on effect sizes derived from pilot data or literature values.

RESULTS

Is illness-induced anorexia pathogen specific?

Previous work has shown that illness-induced anorexia is a broad response in animals such as mammals (Hart, 1988; Sullivan et al., 2016). We sought evidence that the same could be said in insects such as *M. sexta*. Our results show that Gram-negative bacteria *S. marcescens* (N=14), Gram-positive bacteria *B. cereus* (N=16) and the injection of Sephadex beads (N=16) all resulted in smaller mass gains at three time points (3, 6, 24 h) post-manipulation when compared with control animals (N=48) (Fig. S1). In addition, the remaining food mass also differed in our three manipulations, supporting our hypothesis that illness-induced anorexia was the cause of the lack of mass gain in our manipulated animals. Even after controlling for desiccation, the remaining food mass also differed between groups over our three time points (Fig. S1).

Does illness-induced anorexia occur in the context of gut emptying?

Emptying the gut quickly could reduce contact with ingested pathogens. We tested whether immune challenge decreased the gut transit time of the high-nutrition diet (Fig. S2). However, our results indicate that this was not so. Those caterpillars undergoing an immune challenge (N=31) and expressing illness-induced anorexia did not have increased gut transit times ($F_{1,2}$=1.73, P=0.187) or increased pellet production times ($F_{1,2}$=1.53, P=0.22) compared with control (N=31) or sham (N=31) conditions.

Are caterpillars more likely to avoid food that requires detoxification during an immune challenge?

Illness-induced anorexia is a reduction in food consumption over time, not necessarily a cessation of feeding (Hart, 1988). Our hypothesis was that foods that were perceived as more toxic would induce a greater manifestation of illness-induced anorexia. Consistent with our prediction, immune-challenged caterpillars (N=60) took fewer bites of food laced with quinine than did controls (N=61) or shams (N=63) [$F_{1,2}$=36.55, P<0.0001; general linear model with repeated measures, no interaction between time and treatment (F=0.99, P=0.84) or between quinine dose and time (F=0.98, P=0.44); sham (N=63) and control groups (N=61) were not significantly different, P=0.106] (Fig. S3).

Does activation of detoxification pathways reduce resistance to *S. marcescens* infection?

Activating detoxification pathways by feeding caterpillars a sub-lethal dose of the insecticide permethrin increased susceptibility to live *S. marcescens* (Mantel–Cox, $X^2_3 = 185.2$, P<0.0001; Fig. 1A). Caterpillars that were given a toxin challenge (N=41) were more susceptible to live *S. marcescens* infection than those that were given a control diet (N=42; Mantel–Cox, $X^2_1 = 71.9$, P<0.0001, Bonferroni correction P=0.025). Caterpillars that were given a live *S. marcescens* challenge had a median survival of 6 days, whereas those that were given a toxin and live bacterial challenge had a median survival of 3 days (95% CI=1.385–2.615).

Does an immune challenge reduce resistance to permethrin?

Exposing caterpillars to heat-killed *S. marcescens* increased susceptibility to permethrin (Mantel–Cox, $X^2_5 = 98.16$, P<0.0001; Fig. 1B); caterpillars that were given an immune challenge (N=32) were more susceptible to toxin than controls (N=30; Mantel–Cox, $X^2_1 = 8.68$, P=0.003, Bonferroni correction P=0.025). Caterpillars that had been given a Sephadex bead challenge (N=34) were more susceptible to toxin than controls (Mantel–Cox, $X^2_1 = 3.4$,

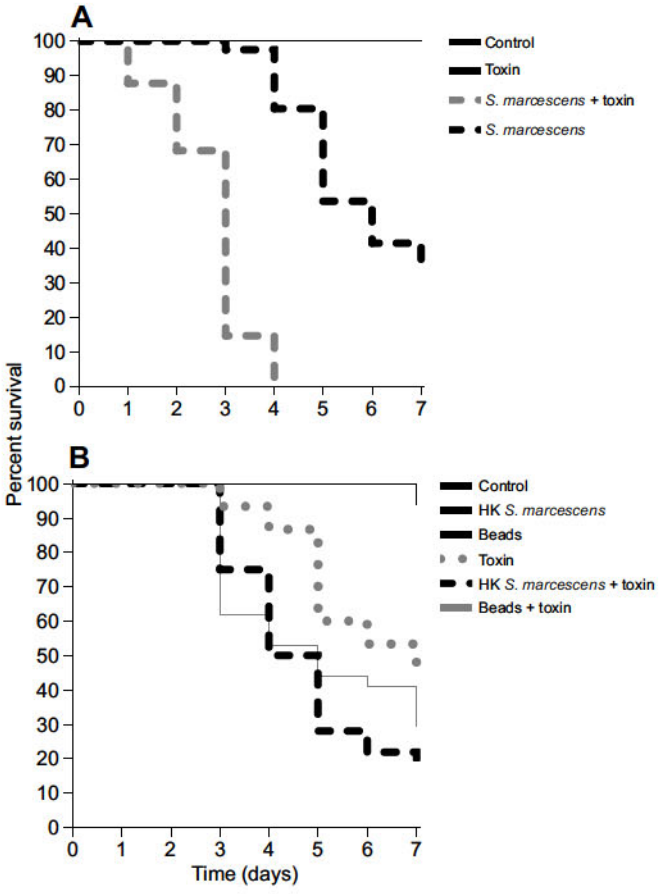

Fig. 1. Effect of immune and toxin challenges on the survival of *Manduca sexta* caterpillars. (A) Effect on survival of a sub-lethal dose of permethrin (toxin; N=41), a live *Serratia marcescens* infection (N=40), and a combined permethrin and live *S. marcescens* challenge (N=42). There was no mortality in the control or toxin group. (B) Effect on survival of an LD_{50} of permethrin (N=30) when combined with heat-killed (HK) *S. marcescens* (N=32) or inert bead immune activation (N=34). There was no mortality in the control, heat-killed *S. marcescens* or beads group.

Journal of Experimental Biology

Journal of Experimental Biology (2018) 221, jeb173336. doi:10.1242/jeb.173336

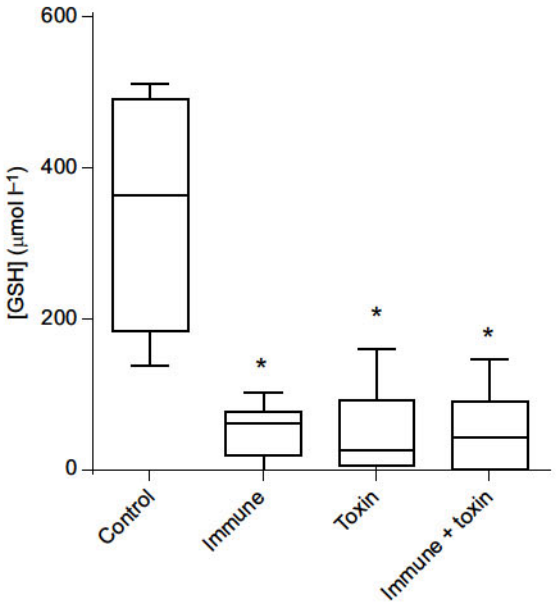

Fig. 2. Effect of immune and toxin challenge on glutathione (GSH) concentration in the hemolymph of *Manduca sexta* caterpillars. Bars represent first and third quartiles, internal bar represents median, and error bars represent the maximum and minimum result. Asterisk represent groups that are significantly different from the control (P<0.05). N=10 for all groups.

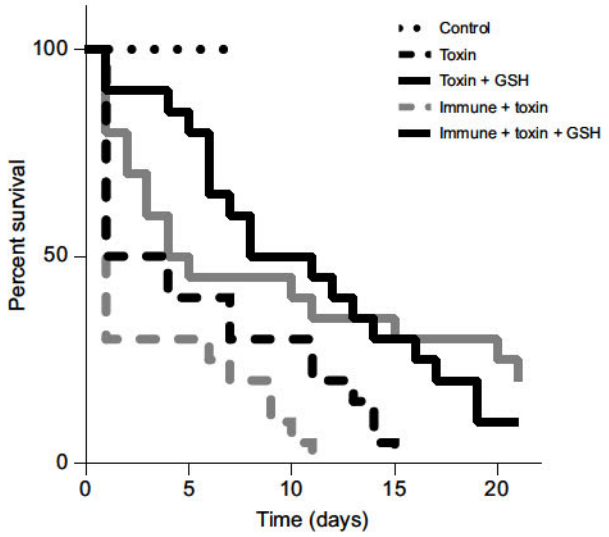

Fig. 3. Effect of supplemental GSH on the survival of *Manduca sexta* caterpillars exposed to immune (i.e. heat-killed *S. marcescens*) and toxin challenges. Effect on survival of a sub-lehtal dose of permethrin, with and without additional GSH (toxin, toxin+GSH), a dual challenge of heat-killed immune challenge of *Serratia marcescens* with and without additional GSH. N=20 for all groups.

P=0.002, Bonferroni correction P=0.0025). Caterpillars that were given the toxin challenge alone had a median survival of 7 days, whereas those given the toxin challenge and heat-killed *S. marcescens* lived 4.5 days (ratio 1.556, 95% CI=1.019–2.092), and those that had been injected with beads as well as given a toxin challenge had a median survival of 5 days (ratio 1.4, 95% CI=0.87–1.9). Caterpillars injected with heat-killed bacteria or beads alone suffered no mortality during the trial (N=60). Heat-killed bacteria activate antimicrobial defenses (Zhang et al., 2014), whereas beads activate encapsulation, a common response to multicellular parasites in insects (Lavine and Beckage, 1996).

Do immune and toxin challenges impact circulating GSH levels?

GSH hemolymph concentration was significantly reduced by (1) immune challenge (heat-killed *S. marcescens*), (2) toxin challenge and (3) combined toxin and immune challenge, compared with controls (general linear univariate analysis, $F_{1,3}$=31.05, P<0.001, N=10 for all groups; Fig. 2). GSH concentration did not differ among immune-challenged, toxin-challenged and dual-challenged caterpillars (immune versus toxin P=0.931; immune versus dual P=0.936; toxin versus dual P=0.995).

Can additional GSH rescue animals given immune and toxin challenges?

Increasing GSH concentration by injection caused a significant increase in survivorship in caterpillars given a combined immune–toxin challenge (Gehan–Breslow–Wilcoxon test, $X_1^2 = 7.08$, P=0.008; Fig. 3, Table S2) and in caterpillars given toxin challenge alone (Gehan–Breslow–Wilcoxon test, $X_1^2 = 8.99$, P=0.003; Fig. 3). The injection of glycine, another amino acid found in GSH, but which is abundant in insects (Chapman, 2013), did not increase caterpillar survival after a toxin challenge (Gehan–Breslow–Wilcoxon test, $X_1^2 = 0.005$, P=0.94).

Does an immune challenge reduce upregulation of detoxification-related genes?

Both the immune response and detoxification pathways are complex systems with many different branches and redundancies. We tested expression of key genes in each pathway (Kanost et al., 2016; Table S1). An immune challenge caused an upregulation in the immune genes *attacin-1* (3.47-fold, P=0.02), *lysozyme* (6.0-fold, P<0.0001), *PAP-3* (3.0-fold, P=0.012) and *serpin-3* (2.12-fold, P=0.015; Fig. 4A). No significant upregulation occurred in the detoxification genes *CYP4M1*, *GST1* or *thioredoxin* in response to an immune challenge. A toxin challenge (Fig. 4B) caused an upregulation in the immune genes *attacin-1* (3.28-fold, P=0.047), *lysozyme* (3.68-fold, P=0.003), *PAP-3* (5.0-fold, P=0.002) and *serpin-3* (4.5-fold, P=0.001). The toxin challenge also caused an upregulation of detoxification genes *CYP4M1* (2.9-fold, P=0.001), *GST1* (1.9-fold, P=0.029) and *thioredoxin* (1.8-fold, P=0.002) as expected. The combination of an immune-challenge and a toxin-challenge resulted in an upregulation of immune genes *attacin-1* (20.6-fold, P<0.0001), *lysozyme* (4.4-fold, P=0.004), *PAP-3* (3.9-fold, P=0.003) and *serpin-3* (2.1-fold, P=0.015). The only detoxification gene that was upregulated after a dual challenge was *CYP4M1* (3.22-fold, P=0.001). *Thioredoxin* and *GST1* were not upregulated in comparison to controls. *GST1* showed significant downregulation when compared with toxin-challenged animals (0.4-fold, P=0.004 with Bonferroni correction P=0.025).

DISCUSSION

An immune challenge reduced resistance to permethrin and a permethrin challenge reduced resistance to *S. marcescens* (Fig. 1). Both beads and heat-killed bacteria reduced resistance to permethrin (Fig. 1), suggesting that there is a broad range of immune responses that reduce toxin resistance. Caterpillars appeared to have insufficient amounts of GSH to both support an activated immune system and to detoxify food. Both activities reduced standing GSH blood concentrations (Fig. 2). However, a combined challenge caused the same level of decline as a single challenge, suggesting

Journal of Experimental Biology (2018) 221, jeb173336. doi:10.1242/jeb.173336

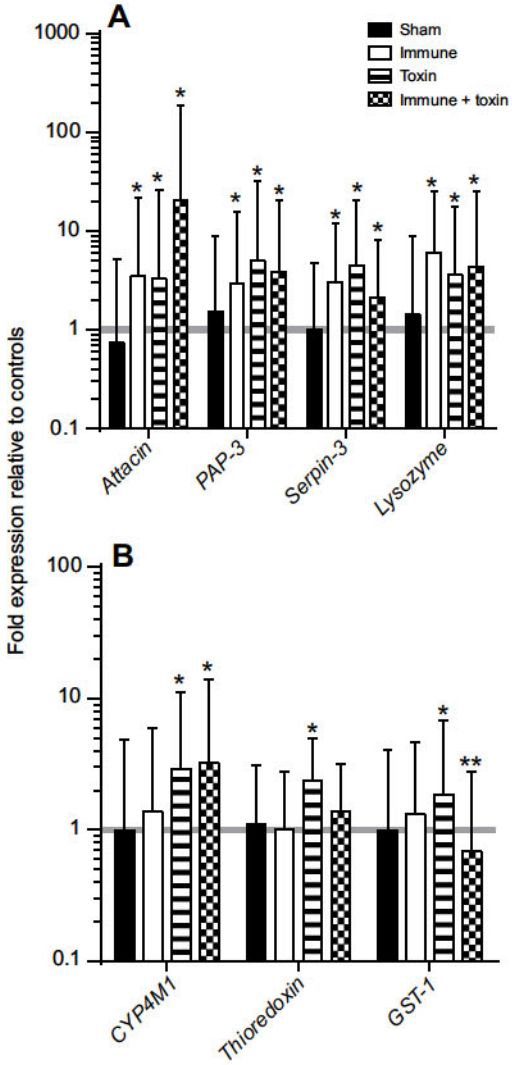

Fig. 4. Gene expression after single and dual immune-toxin challenges in the caterpillar *Manduca sexta*. (A) Expression of immune-related genes in response to immune and toxin challenge. Bars with an asterisk represent those that are upregulated when compared with control animals. Error bars represent s.e.m. (B) Expression of detoxification-related genes in response to immune and toxin challenge. Bars with an asterisk represent those that are upregulated when compared with the control. Bars with two asterisks represent those that are downregulated when compared with the toxin challenge. Error bars represent s.e.m. N=13 for all groups.

that one or both systems were consuming less GSH than when activated alone (Fig. 2). Injection of additional GSH restored toxin resistance in caterpillars that were mounting an immune response, further supporting the argument that GSH levels are limiting in dual-challenged caterpillars (Fig. 3). Furthermore, we discovered that an immune challenge reduced the expression of *GST1* and prevented the upregulation of *thioredoxin* in response to a toxin challenge (Fig. 4B). These results suggest that immune responses suppress food detoxification mechanisms. By reducing food intake, illness-induced anorexia could protect the caterpillar from food toxins when food detoxification is impaired.

A conflict between food detoxification and immune function probably exists in most animals. Inducing an immune response leads to a suppression of detoxification pathways in a variety of vertebrates (e.g. pigs, mice and chickens; Renton, 2001). In humans, this phenomenon leads to reduced drug clearance rates in patients fighting an infection (Aitken et al., 2006). Therefore, illness-induced anorexia could benefit most animals by reducing exposure to food toxins when animals are less able to detoxify them. Illness-induced anorexia is consistent with the detoxification limit hypothesis that suggests that animals limit feeding when detoxification pathways are saturated (Marsh et al., 2006). Although this hypothesis was devised with mammalian herbivores in mind, it probably applies to animals more broadly. For example, *M. sexta* caterpillars eat less nicotine-laced food when P450 detoxification systems are compromised than do controls, although both groups eat the same amount of non-toxic food (Snyder and Glendinning, 1996). Our finding that immune-challenged *M. sexta* exhibited even greater anorexia when faced with food containing quinine also supports this hypothesis (Fig. S3). Why vertebrates reduce detoxification capacity during an immune challenge remains unclear (Renton, 2001). Our research suggests this is likely to be the result of a physiological trade-off.

A negative relationship between immune function and toxin tolerance has also been noted in other insects, especially bees (Köhler et al., 2012). Bees given an immune challenge become more susceptible to toxins (Köhler et al., 2012), and even low levels of insecticides reduce disease resistance (Collison et al., 2016). The combination of novel pathogens and pesticide exposure are recognized as likely drivers of colony collapse disorder (Collison et al., 2016). The present study suggests that one possible explanation for the negative relationship is a conflict for molecular resources between immune function and food detoxification. If bees are similar to caterpillars, our study suggests that increasing GSH levels in bees could reduce some of the negative impacts of endemic infections on pesticide sensitivity.

Glycine could not replace GSH in the rescue experiment, suggesting that the GSH results were not a non-specific effect of injecting additional nutrients. The increased GSH could have promoted the removal of toxin in the dual-challenged caterpillars by increasing the amount of substrate available for extracellular GSTs. The added GSH could also have increased survival via other routes (e.g. by acting as a substrate for glutathione peroxidases, resulting in a reduction in oxidative stress). Regardless of the precise routes by which GSH promoted survival in immune+toxin challenged caterpillars, these results suggest that events that reduce GSH hemolymph concentration (e.g. an immune response) reduce resistance to toxins.

Previous studies on illness-induced anorexia may have missed its importance for reducing toxin exposure because they were conducted using laboratory animals provided with clean, high-quality and highly processed food. However, in the field, animals are exposed to food that is frequently contaminated with toxins, either plant-derived or bacterially derived. The adaptive value of sickness behaviors may be difficult to determine in the benign conditions of the laboratory. From a broader perspective, our results also suggest that failure to find trade-offs between somatic maintenance and traits such as reproduction can occur when resources are abundant (Krams et al., 2015), but also because a decline in detoxification can be silent under typical laboratory conditions. Finally, behaviors such as illness-induced anorexia may provide multiple benefits for a host (Kyriazakis et al., 1998; LeGrand and Alcock, 2012) and some of these benefits may be species and/or pathogen specific (Ayres and Schneider, 2009; Rao et al., 2017; Wang et al., 2016). More studies are needed to fully describe the complex effects of illness-induced anorexia on immune function.

Not all detoxification genes are suppressed by immune activation. In the beetle *Tenebrio molitor*, septic injury increases

p450 gene expression (Altincicek et al., 2008). And in the silkworm moth, *Bombyx mori*, viral infection increased *GST* gene expression (Gui et al., 2009). Part of this discrepancy may be due to the multifunctional nature of GSTs (Yan et al., 2012) and other enzymes. Moreover, enzymes such as GSTs are active in different physiological compartments [e.g. tissue (Yan et al., 2012) versus hemolymph enzymes (Erdem et al., 2016)]. Immune–detoxification interactions may be limited to a subset of pathways in each system.

Although we describe detoxification and immune responses as separate systems, they do have substantial overlap; for example, both infection (Dunphy and Downer, 1994) and toxins (Kodrík et al., 2015) activate a stress response in insects. The ramifications of these interconnections are poorly understood, although complex connections between intermediate metabolism and immunity may be one reason for illness-induced anorexia in mammals (Wang et al., 2016). Such interactions need to be better studied. Interactions between food detoxification and the immune response result in eating becoming riskier when fighting infection. In turn, reducing feeding when ill lowers the risk of food poisoning.

Acknowledgements
We thank G. Davies for help with data collection. We also thank L. Su Rin and S. Elsheik for help with animal care.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.E.M., S.A.A.; Methodology: L.E.M., D.W.M., S.A.A.; Validation: L.E.M., D.W.M., S.A.A.; Formal analysis: L.E.M., D.W.M., S.A.A.; Investigation: L.E.M., D.W.M., S.A.A.; Resources: S.A.A.; Writing - original draft: L.E.M., S.A.A.; Writing - review & editing: L.E.M., D.W.M., S.A.A.; Visualization: L.E.M., S.A.A.; Supervision: S.A.A.; Project administration: S.A.A.; Funding acquisition: S.A.A.

Funding
This study was supported by a grant from NSERC (Natural Sciences and Engineering Research Council of Canada) to S.A.A.

Data availability
All datasets can be accessed via Mendeley Data (McMillan et al., 2017): http://dx.doi.org/10.17632/8t2zz5c2p3.1

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.173336.supplemental

References

Adamo, S. A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J. Insect Physiol.* **50**, 209-216.
Adamo, S. A. (2017). The stress response and immune system share, borrow, and reconfigure their physiological network elements: evidence from the insects. *Horm. Behav.* **88**, 25-30.
Adamo, S. A., Fidler, T. L. and Forestell, C. A. (2007). Illness-induced anorexia and its possible function in the caterpillar, *Manduca sexta*. *Brain Behav. Immun.* **21**, 292-300.
Adamo, S. A., Bartlett, A., Le, J., Spencer, N. and Sullivan, K. (2010). Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Anim. Behav.* **79**, 3-10.
Adamo, S. A., Davies, G., Easy, R., Kovalko, I. and Turnbull, K. F. (2016). Reconfiguration of the immune system network during food limitation in the caterpillar *Manduca sexta*. *J. Exp. Biol.* **219**, 706-718.
Aitken, A. E., Richardson, T. A. and Morgan, E. T. (2006). Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu. Rev. Pharmacol. Toxicol.* **46**, 123-149.
Altincicek, B., Knorr, E. and Vilcinskas, A. (2008). Beetle immunity: identification of immune-inducible genes from the model insect *Tribolium castaneum*. *Dev. Comp. Immunol.* **32**, 585-595.
Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245-5250.
Aranda, P. S., LaJoie, D. M. and Jorcyk, C. L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* **33**, 366-369.
Ayres, J. S. and Schneider, D. S. (2009). The role of anorexia in resistance and tolerance to infections in Drosophila. *PLoS Biol.* **7**, e1000150.
Barbehenn, R. V., Kochmanski, J., Menachem, B. and Poirier, L. M. (2013). Allocation of cysteine for glutathione production in caterpillars with different antioxidant defense strategies: a comparison of *Lymantria dispar* and *Malacosoma disstria*. *Arch. Insect Biochem. Physiol.* **84**, 90-103.
Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* 57, 289-300.
Bernays, E. A. (1997). Feeding by lepidopteran larvae is dangerous. *Ecol. Entomol.* **22**, 121-123.
Bernays, E. A. and Woods, H. A. (2000). Foraging in nature by larvae of *Manduca sexta*—influenced by an endogenous oscillation. *J. Insect Physiol.* **46**, 825-836.
Chapman, R. F. (2013). *The Insects: Structure and Function*. New York, New York: Cambridge University Press.
Clark, K. D., Lu, Z. and Strand, M. R. (2010). Regulation of melanization by glutathione in the moth *Pseudoplusia includens*. *Insect Biochem. Mol. Biol.* **40**, 460-467.
Collison, E., Hird, H., Cresswell, J. and Tyler, C. (2016). Interactive effects of pesticide exposure and pathogen infection on bee health–a critical analysis. *Biol. Rev.* **91**, 1006-1019.
Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *Eur. J. Pharmacol.* **500**, 399-411.
Demas, G. E., Chefer, V., Talan, M. I. and Nelson, R. J. (1997). Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *Am. J. Physiol.* **273**, R1631-R1637.
Diamond, S. E. and Kingsolver, J. G. (2010). Fitness consequences of host plant choice: a field experiment. *Oikos* **119**, 542-550.
Diamond, S. E. and Kingsolver, J. G. (2011). Host plant quality, selection history and trade-offs shape the immune responses of *Manduca sexta*. *Proc. R. Soc. B* **278**, 289-297.
Dunphy, G. B. and Downer, R. G. H. (1994). Octopamine, a modulator of the haemocytic nodulation response of non-immune *Galleria mellonella* larvae. *J. Insect Physiol.* **40**, 267-272.
Enayati, A. A., Ranson, H. and Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol.* **14**, 3-8.
Erdem, M., Küçük, C., Büyükgüzel, E. and Büyükgüzel, K. (2016). Ingestion of the anti-bacterial agent, gemifloxacin mesylate, leads to increased gst activity and peroxidation products in hemolymph of *Galleria mellonella* (lepidoptera: pyralidae). *Arch. Insect Biochem. Physiol.* **93**, 202-209.
Freitak, D., Ots, I., Vanatoa, A. and Hörak, P. (2003). Immune response is energetically costly in white cabbage butterfly pupae. *Proc. R. Soc. Lond. Series B Biol. Sci.* **270**, S220-S222.
González-Santoyo, I. and Córdoba-Aguilar, A. (2012). Phenoloxidase: a key component of the insect immune system. *Entomol. Exp. Appl.* **142**, 1-16.
Gui, Z., Hou, C., Liu, T., Qin, G., Li, M. and Jin, B. (2009). Effects of insect viruses and pesticides on glutathione S-transferase activity and gene expression in *Bombyx mori*. *J. Econ. Entomol.* **102**, 1591-1598.
Guillet, G., Harmatha, J., Waddell, T. G., Philogène, B. J. R. and Arnason, J. T. (2000). Symposium-in-print synergistic insecticidal mode of action between sesquiterpene lactones and a phototoxin, α-terthienyl. *Photochem. Photobiol.* **71**, 111-115.
Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130-7139.
Hart, B. L. (1988). Biological basis of the behavior of sick animals. *Neurosci. Biobehav. Rev.* **12**, 123-137.
Hill, R. W., Wyse, G. A., Anderson, M. and Anderson, M. (2016). *Animal Physiology*. Sunderland, MA: Sinauer Associates.
Jeschke, V., Gershenzon, J. and Vassão, D. G. (2016). A mode of action of glucosinolate-derived isothiocyanates: detoxification depletes glutathione and cysteine levels with ramifications on protein metabolism in *Spodoptera littoralis*. *Insect Biochem. Mol. Biol.* **71**, 37-48.
Kanost, M. R., Arrese, E. L., Cao, X., Chen, Y.-R., Chellapilla, S., Goldsmith, M. R., Grosse-Wilde, E., Heckel, D. G., Herndon, N. and Jiang, H. (2016). Multifaceted biological insights from a draft genome sequence of the tobacco hornworm moth, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **76**, 118-147.
Khan, I., Agashe, D. and Rolff, J. (2017). Early-life inflammation, immune response and ageing. *Proc. R. Soc. B* 284, 20170125.
Kingsolver, J. G., Diamond, S. E., Seiter, S. A. and Higgins, J. K. (2012). Direct and indirect phenotypic selection on developmental trajectories in *Manduca sexta*. *Funct. Ecol.* **26**, 598-607.
Kodrík, D., Bednářová, A., Zemanová, M. and Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects—an update. *Int. J. Mol. Sci.* **16**, 25788-25816.

Köhler, A., Pirk, C. W. W. and Nicolson, S. W. (2012). Simultaneous stressors: Interactive effects of an immune challenge and dietary toxin can be detrimental to honeybees. *J. Insect Physiol.* **58**, 918-923.
Krams, I. A., Krama, T., Moore, F. R., Rantala, M. J., Mänd, R., Mierauskas, P. and Mänd, M. (2015). Resource availability as a proxy for terminal investment in a beetle. *Oecologia* **178**, 339-345.
Kyriazakis, I., Tolkamp, B. J. and Hutchings, M. R. (1998). Towards a functional explanation for the occurrence of anorexia during parasitic infections. *Anim. Behav.* **56**, 265-274.
Lavine, M. D. and Beckage, N. E. (1996). Temporal pattern of parasitism-induced immunosuppression in *Manduca sexta* larvae parasitized by *Cotesia congregata*. *J. Insect Physiol.* **42**, 41-51.
LeGrand, E. K. and Alcock, J. (2012). Turning up the heat: immune brinksmanship in the acute-phase response. *Q Rev. Biol.* **87**, 3-18.
Marsh, K. J., Wallis, I. R., Andrew, R. L. and Foley, W. J. (2006). The detoxification limitation hypothesis: where did it come from and where is it going? *J. Chem. Ecol.* **32**, 1247-1266.
McMillan, L., Miller, D. and Adamo, S. (2017). Eating when ill is risky: Immune defense impairs food detoxification in the caterpillar, *Manduca sexta*. Mendeley Data, v1. http://dx.doi.org/10.17632/8t2zz5c2p3.1.
Murray, M. and Murray, A. (1979). Anorexia of infection as a mechanism of host defense. *Am. J. Clin. Nutr.* **32**, 593-596.
Rao, S., Schieber, A. M. P., O'Connor, C. P., Leblanc, M., Michel, D. and Ayres, J. S. (2017). Pathogen-mediated inhibition of anorexia promotes host survival and transmission. *Cell* **168**, 503-516.e12.
Renton, K. W. (2001). Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacol. Ther.* **92**, 147-163.
Sadd, B. M. and Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proc. R. Soc. B* **273**, 2571-2574.
Schmid-Hempel, P. (2011). *Evolutionary Parasitology: The Integrated Study of Infections, Immunology. Ecology and Genetics*. Oxford: Oxford University Press.
Snyder, M. J. and Glendinning, J. I. (1996). Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A* **179**, 255-261.
Snyder, M. J., Walding, J. K. and Feyereisen, R. (1995). Glutathione S-transferases from larval Manduca sexta midgut: Sequence of two cdnas and enzyme induction. *Insect Biochem. Mol. Biol.* **25**, 455-465.
Stahlschmidt, Z. R., Acker, M., Kovalko, I. and Adamo, S. A. (2015). The double-edged sword of immune defence and damage control: do food availability and immune challenge alter the balance? *Funct. Ecol.* **29**, 1445-1452.
Sullivan, K., Fairn, E. and Adamo, S. A. (2016). Sickness behaviour in the cricket *Gryllus texensis*: comparison with animals across phyla. *Behav. Process.* **128**, 134-143.
Wang, A., Huen, S. C., Luan, H. H., Yu, S., Zhang, C., Gallezot, J.-D., Booth, C. J. and Medzhitov, R. (2016). Opposing effects of fasting metabolism on tissue tolerance in bacterial and viral inflammation. *Cell* **166**, 1512-1525. e12.
Yan, H., Meng, F., Jia, H., Guo, X. and Xu, B. (2012). The identification and oxidative stress response of a zeta class glutathione S-transferase (GSTZ1) gene from *Apis cerana cerana*. *J. Insect Physiol.* **58**, 782-791.
Zhang, S., Cao, X., He, Y., Hartson, S. and Jiang, H. (2014). Semi-quantitative analysis of changes in the plasma peptidome of *Manduca sexta* larvae and their correlation with the transcriptome variations upon immune challenge. *Insect Biochem. Mol. Biol.* **47**, 46-54.

SUPPLEMENTAL INFORMATION

Table S1. Forward and reverse primer sequences for target immune-related genes, detoxification-related genes, and reference genes

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	R^2	Efficiency	Reference
Attacin-1	GCAGGCGACGACAAGAAC	ATGCGTGTTGGTAAGAGTAGC	0.98	1.0	[1]
Lysozyme	GTGTGCCTCGTGGAGAATG	ATGCCTTGGTGATGTCGTC	0.99	1.0	[1]
PAP-3	ATTAAGCTGTTGTGTGGTG	CGGGTGCGGTATTGTCTTC	0.99	0.98	[2]
Serpin-3	GATTCCTCGCGATTCGATGC	CATTTACGTCATTAAGTTTCATG	0.99	0.97	[3]
RpL17A	TCCGCATCTCACTGGGTCT	CACGGCAATCACATACAGGTT	0.99	0.96	[4]
Ubiquitin	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	0.99	1.0	[5]
GST-1*	AAGTACCCGTTCCAGCTGAA	TGGGTTGGACAGGACAGTTT	0.98	1.0	[6]
Thioredoxin*	ATCAGACGACCTGAAGATGA	GACCTTCATAACGACGATAG	0.99	0.97	[7]
CYP4M1*	GATGCGGTATTTGGAGAGAT	CTCAGGTAAGAATCGGTCAG	0.99	1.0	[8]

*Primers created from mRNA sequences published by reference

Table S2. A priori ranking of GSH rescue groups with Bonferroni correction

A priori ranking	Comparison	Corrected α (Bonferroni)	p value
1	Immune-Toxin vs Immune-Toxin GSH	0.05	0.0027
2	Toxin vs Toxin GSH	0.05	0.0078
3	Toxin vs Toxin Glycine	0.025	0.9437
4	Toxin-GSH vs Toxin-sham-GSH	0.025	0.291
5	Toxin vs Immune-Toxin	0.017	0.1416
6	Control vs Control GSH	0.05	1

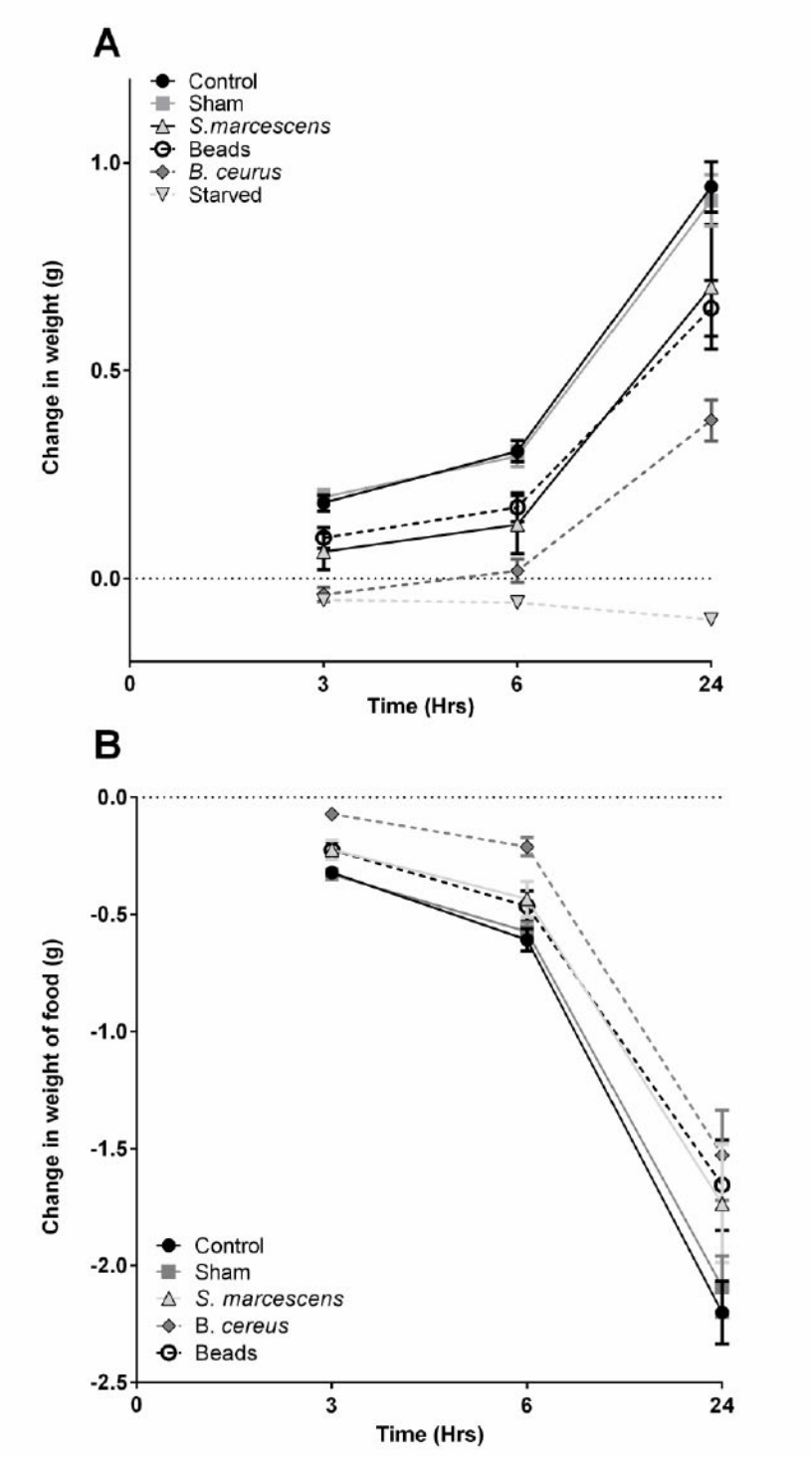

FIGURE SI1. **Effects of varying infections on the feeding behaviour of *Manduca sexta.*** (A) Effect of varying immune challenges on weight gain of *Manduca sexta* 5th instars. (B) Effect of various immune challenges on *M. sexta* 5th instars represented by weight of food left uneaten (proxy of amount of food consumed). Error bars represent SEM. Control (N=48), Sham (N=45), *S. marcescens* (N=14), Beads (N=16), *B. cereus* (N=16), Starved (N=20)

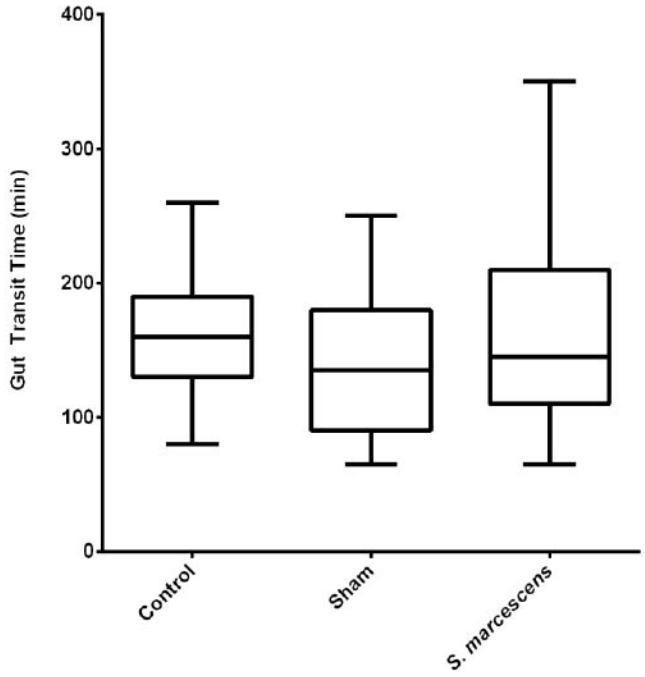

FIGURE SI2. **Effect of differing treatments on gut transit time of high nutrition diet in *Manduca sexta***. Error bars represent SEM. For all groups (N=31).

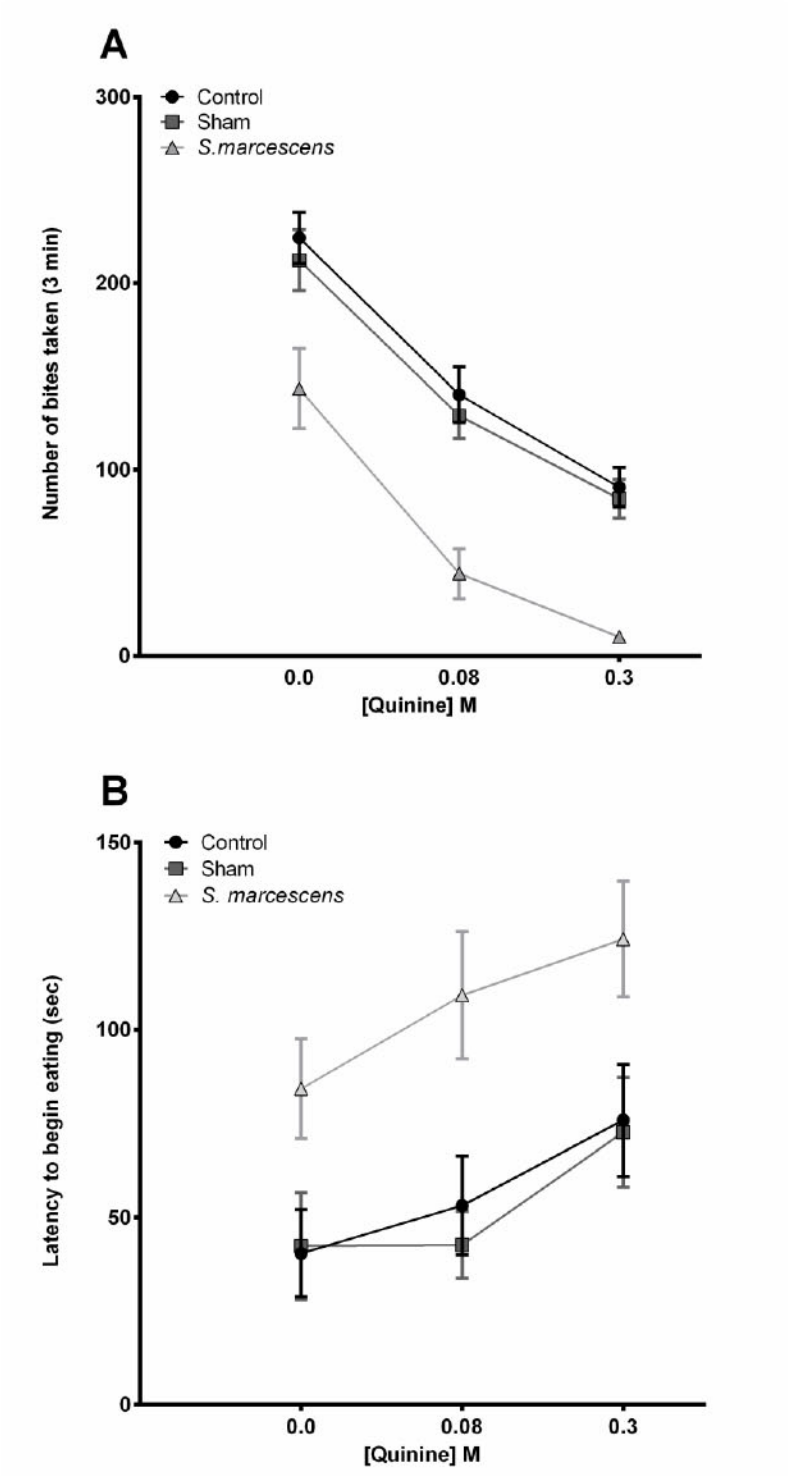

FIGURE SI3. **Effect of quinine concentration (M) on feeding behaviour of *Manduca sexta* caterpillars.** (A) Effect of increasing concentration of quinine (M) on bites taken of food during a 3 minute trial interval. Error bars represent SEM. (B) Effect of increasing quinine concentration (M) on latency to begin feeding once food has be contacted during a 3 minute trial interval. Error bars represent SEM. Control (N=62), Shams (N=63), Immune-challenged (N=60).

2.10 REVISIONS

Methods

Are caterpillars more likely to avoid food that requires detoxification during an immune challenge

Fifth instar-Day 2 caterpillars had their food removed 12 h prior to the study. Frasss pellets were removed from their individual containers. Caterpillars were weighed and sorted into one of nine experimental groups: (1-3) controls: given food with (1) no quinine (0.00 mol 1^{-1}), (2) low quinine (0.008 mol 1^{-1}) or (3) high quinine (0.03 mol 1^{-1}) concentrations; (4-6) sham: caterpillars were given a sterile poke with an insect pin followed by one of the three quinine conditions; or (7-9) immune challenged: caterpillars were injected with $2x10^{-4}$ cells 20 μl^{-1} of heat-killed *S. marcescens* followed by one of the quinine diets. At the start of the food trial, caterpillars were placed directly on food, ensuring chemosensory hairs were in contact with the food cube. Baseline trials were conducted on all animals prior to treatment in which caterpillars had 3 minutes to fully consume a cube of control high nutrition diet. The latency to eat, and number of bites were recorded. After the baseline trial, all caterpillars were given their treatments and had additional feeding tests at 1, 3 and 20 h post exposure.

Do immune and toxin challenges impact circulating GSH levels

Fifth instar-Day 2 caterpillars were weighed and assigned to one of three groups: (1) control, (2) immune challenged or (3) toxin challenged (see above). Twenty-four hours post treatment (injection, consumption of toxin or both), 50 µl of hemolymph was collected and immediately deproteinated by centrifuging at 18, 845 g for 10 min at 4°C and then adding the supernatant to an equal amount of metaphosphoric acid (0.1 g ml^{-1}, Sigma-Aldrich). After incubating at room temperature for 5 min, the samples were spun at 3350 g for 3 min. The supernatant was stored at -80°C.The deproteinated samples were thawed and processed according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Absorbance was measured at 405 nm. GSH hemolymph concentration was assessed by measuring both reduced and oxidized GSH species (GSH/GSSG) following the manufacturer's instructions (Cayman Chemical).

Results

Does illness-induced anorexia occur in the context of gut emptying?

Emptying the gut quickly could reduce contact with ingested pathogens. We tested whether an immune challenge increased the gut transit time of the high-nutrition diet (Fig. S2). However, our results indicate that this was not so. Those caterpillars undergoing an immune challenge (N=31) and expressing illness-induced anorexia did not have increased gut transit times ($F_{1,2}$=1.73, P=0.187) or increased pellet production times ($F_{1,2}$=1.53, P=0.22) compared with control (N=31) or sham (N=31) conditions.

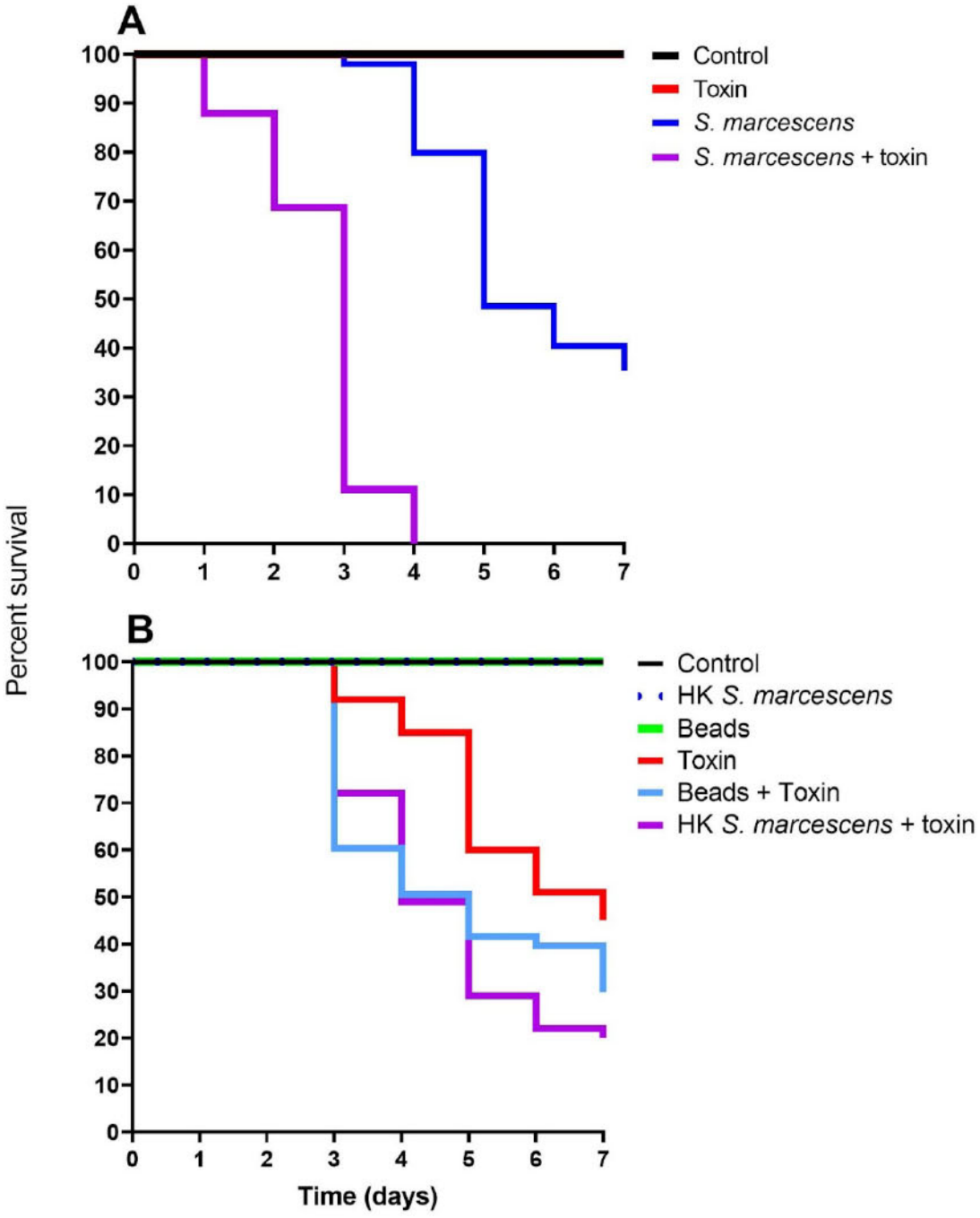

Fig. 1.1. Effects of immune and toxin challenges on the survival of *Manduca sexta* caterpillars. (A) Effect on survival of a sub-lethal dose of permethrin (toxin; N=41), a live *Serratia marcescens* infection (N=40), and a combined permethrin and live *S. marcescens* challenge (N=42). There was no mortality in the control or toxin group. (B) Effect on survival of an LD_{50} of permethrin (N=30) when combined with heat-killed (HK) *S. marcescens* (N=32) or inert bead immune activation (N=34). There was no mortality in the control, heat-killed *S. marcescens*, or bead group.

CHAPTER 3- FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR MANDUCA SEXTA

3.1 PUBLICATION INFORMATION

McMillan, Laura E., and Shelley A. Adamo. "Friend or Foe? Effects of Host Immune Activation on the Gut Microbiome in the Caterpillar Manduca Sexta." *Journal of Experimental Biology* 223, no. 19 (October 12, 2020): jeb226662.

3.2 ABSTRACT

For many animals the gut microbiome plays an essential role in immunity and digestion. However, certain animals, such as the caterpillar *Manduca sexta,* do not have a resident gut microbiome. Although these animals do have bacteria that pass through their gut from their natural environment, the absence of such bacteria does not reduce growth or survival. We hypothesized that *Manduca sexta* would sterilize their gut as a protective measure against secondary infection when faced with a gut infection, or exposure to heat-killed bacteria in the blood (hemolymph). However, we found that gut sterilization did not occur during either type of immune challenge, i.e. bacterial numbers did not decrease. By examining the pattern of immune-related gene expression, gut pH, live bacterial counts, and weight change (as a measure of sickness behaviour), we found evidence for physiological trade-offs between regulating the microbiome and defending against systemic infections. Caterpillars exposed to both gut pathogens and a systemic immune challenge had higher numbers of bacteria in their gut than caterpillars exposed to a single challenge. Following a multivariate analysis of variance, we found that the response patterns following an oral challenge, systemic challenge or dual challenge were unique. Our results suggest that the immune response for each challenge resulted in a different configuration of the immunophysiological network. We hypothesize that these different configurations represent different resolutions of physiological trade-offs based on the immune responses needed to best protect against the present immune challenges.

© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb226662. doi:10.1242/jeb.226662

RESEARCH ARTICLE

Friend or foe? Effects of host immune activation on the gut microbiome in the caterpillar *Manduca sexta*

Laura E. McMillan* and Shelley A. Adamo

ABSTRACT

For many animals, the gut microbiome plays an essential role in immunity and digestion. However, certain animals, such as the caterpillar *Manduca sexta*, do not have a resident gut microbiome. Although these animals do have bacteria that pass through their gut from their natural environment, the absence of such bacteria does not reduce growth or survival. We hypothesized that *M. sexta* would sterilize their gut as a protective measure against secondary infection when faced with a gut infection or exposure to heat-killed bacteria in the blood (haemolymph). However, we found that gut sterilization did not occur during either type of immune challenge, i.e. bacterial numbers did not decrease. By examining the pattern of immune-related gene expression, gut pH, live bacterial counts and mass change (as a measure of sickness behaviour), we found evidence for physiological trade-offs between regulating the microbiome and defending against systemic infections. Caterpillars exposed to both gut pathogens and a systemic immune challenge had higher numbers of bacteria in their gut than caterpillars exposed to a single challenge. Following a multivariate analysis of variance, we found that the response patterns following an oral challenge, systemic challenge or dual challenge were unique. Our results suggest that the immune response for each challenge resulted in a different configuration of the immunophysiological network. We hypothesize that these different configurations represent different resolutions of physiological trade-offs based on the immune responses needed to best protect the animal against the present immune challenges.

KEY WORDS: Ecoimmunology, Microbiome regulation, Physiological networks, Lepidopteran

INTRODUCTION

The gut microbiome is vital for many animals, both vertebrate (Montalban-Arques et al., 2015; Youngblut et al., 2019) and invertebrate (Fraune and Bosch, 2010; Weiss and Aksoy, 2011). The gut microbiome in insects is involved in: immune priming (Contreras-Garduño et al., 2016), supplying nutrients lacking in the diet (Brune, 2014), and protecting hosts against parasites (Weiss and Aksoy, 2011) and harsh environmental conditions (Ferguson et al., 2018). However, these very same bacteria can become pathogenic if they are not regulated (e.g. if they reproduce without control and/or are allowed to cross into the blood (Buchon et al., 2014). Additionally, it has been shown in some insects (e.g. the greater wax moth, *Galleria mellonella*) that the presence of bacteria in the gut results in an increase of baseline antimicrobial peptides (AMPs), which in turn has its own costs (Krams et al., 2017). During a systemic illness (i.e. an infection in the blood/haemolymph), the ability to control the gut microbiome wanes (Krieg, 1987). This decline in control increases the risk of a secondary infection via the gut (Krieg, 1987). Given this risk, how should animals deal with their gut microbiome during a systemic illness? The answer will depend, in part, on the role of the gut microbiome in a given species.

Although the gut microbiome is essential for survival in some species (e.g. termites; Brune, 2014), in others it appears to be optional. For example, in the caterpillar stage of *Manduca sexta*, *Danaus chrysippus* and *Ariadne merione*, destroying the gut microbiome with antibiotics has no effect on growth or development (Hammer et al., 2017; Phalnikar et al., 2019). In *Drosophila*, too, flies can grow without a microbiome, but only if provided with a rich food source (Buchon et al., 2014). However, even when they are fed a natural, relatively low quality, food source (i.e. *Datura wrightii*; Ojeda-Avila et al., 2003), growth and survival are normal in *M. sexta* without a microbiome (Hammer et al., 2017). These results suggest that larval *M. sexta* do not require a gut microbiome.

Although gut bacteria do not appear to be necessary in *M. sexta*, they can still pose a threat. The gut is the main route of infection for most insects (Buchon et al., 2014; Vallet-Gely et al., 2008). Reducing the gut microbiome during gut infections may be important for survival. During severe immune responses within the gut, immunopathological damage can result in tissue injury, as well as the delamination of enterocytes (Buchon et al., 2014). Damage to the gut increases the ability of gut bacteria to cross over into the haemocoel and cause lethal sepsis (Steinhaus, 1959).

Another potential reason to remove the microbiome is that regulating it requires immune resources in insects (Zhai et al., 2018). These costs could be problematic when *M. sexta* is facing multiple stressors simultaneously. Tackling a combination of predators, toxins or pathogens leads to a significantly increased likelihood of mortality than if each challenge was confronted alone (McMillan et al., 2018). The increased mortality is due, in part, to physiological trade-offs between different defence systems (e.g. the immune system and stress response; Adamo et al., 2017) created by limited molecular resources (Adamo et al., 2016, 2017; McMillan et al., 2018). Therefore, in this species, it may benefit caterpillars to destroy their microbiome during a systemic infection to reduce the cost of maintaining it and reduce the risk of secondary infection. Sterilizing the gut is not without precedent in this species. *Manduca sexta* caterpillars sterilize their gut prior to metamorphosis (Russell and Dunn, 1996). During metamorphosis, the gut is compromised, raising the real risk of attack by bacteria within the caterpillar's system (Russell and Dunn, 1996). *Manduca sexta* sterilize their gut during metamorphosis by secreting large amounts of AMPs and proteins into the gut lumen (Dunn et al., 1994; Russell and Dunn, 1996, 1991). AMPs, such as lysozyme, accumulate in the gut epithelium. When the epithelium starts to slough off, the AMPs are

Department of Psychology and Neuroscience, Dalhousie University, Halifax, NS, Canada, B3H4R2.

*Author for correspondence (Laura.McMillan@dal.ca)

L.E.M., 0000-0002-0023-7011; S.A.A., 0000-0002-5973-571X

Received 9 April 2020; Accepted 12 August 2020

Journal of Experimental Biology

released into the lumen, destroying the caterpillar's microbiome (Russell and Dunn, 1996).

In this study, we examined first whether a systemic infection leads to a loss of the transient microbiome, by measuring the bacterial content in the frass (faecal pellets) after a systemic immune challenge. We tested whether systemic infection induces an upregulation of AMP gene expression in the midgut, similar to that observed during metamorphosis. We also tested whether gut sterilization may be aided by an increase in gut pH. *Manduca sexta* typically have a highly alkaline gut with a pH ranging from 10 to 11 (Dow, 1992). An increase in pH could help in reducing the number of bacteria in the gut by making the surrounding environment inhospitable.

Despite the potential advantages of removing the transient microbiome during a systemic immune challenge, there are also disadvantages. Sterilizing the gut is likely to be resource intensive. Activating a systemic immune response requires resources, and has been found to be energetically costly in insects and other animals (Ardia et al., 2012; Bajgar et al., 2015; Lochmiller and Deerenberg, 2000), leading to physiological trade-offs (Krams et al., 2017; McMillan et al., 2018; Sheldon and Verhulst, 1996). Therefore, we also examined a second hypothesis that systemic immune activation will induce physiological trade-offs with microbiome regulation, leading to a reduction in midgut AMP gene expression and a reduction in gut alkalinity. Such trade-offs would be expected to produce an increase or maintenance of the number of bacteria in the gut during systemic immune activation, despite the risks.

A third possibility is that resource limitation may produce a reconfiguration of physiological networks as opposed to straightforward trade-offs. In some situations, *M. sexta* larvae adopt an alternative network configuration when faced with dual challenges (Adamo et al., 2016, 2017; McMillan et al., 2018). These alternative network strategies take the pressure off molecular pinch points (i.e. limiting resources that are used in multiple processes) and increase survival (Adamo et al., 2016, 2017; McMillan et al., 2018). One possible alternative strategy in terms of microbiome regulation during an immune challenge would be to increase mechanisms of infection tolerance within the gut. Infection tolerance increases the ability of organisms to avoid damage due to pathogens, but it does not result in a reduction in pathogen load (Ayres and Schneider, 2009). Mechanisms of infection tolerance are less well studied than those of resistance, but include antioxidants and detoxification pathways (Soares et al., 2014, 2017). Reducing the chance of microbe-induced gut damage may help prevent gut bacteria from reaching the haemocoel. We examined whether systemically challenged caterpillars increase the expression of genes such as that encoding gluthatione *S*-transferase (GST1). GST1 helps to detoxify compounds such as bacterial lipids (Snyder et al., 1995).

A fourth possible response is that *M. sexta* may use the microbiome to help it survive a systemic infection. Hammer et al.'s (2017) study showed that *M. sexta* grew normally without their gut microbiome, but this study did not assess whether the animals had a normal immune response. In *Drosophila*, there is evidence that the microbiota can participate in protecting against pathogens (Buchon et al., 2014). If *M. sexta* have similar mechanisms, than caterpillars may attempt to retain their microbiome during illness. In that case, during a systemic infection, the number of gut microbes should stay the same, and possibly even increase. We would not expect an increase in immune gene expression in the midgut.

To examine the likelihood of these four hypotheses, we exposed caterpillars to one of five treatments. Two of the groups underwent a single challenge, either oral inoculation with live bacteria (increasing the number and type of organisms in the microbiome) or injection of heat-killed pathogens (activating a systemic immune response). The other two treatment groups received dual challenges of oral inoculation of live bacteria combined with a sterile wound, or oral inoculation of live bacteria combined with an injection of heat-killed pathogens. The final group was an unmanipulated control. We measured gene expression in the midgut and fat body, as well as gut pH and microbial load.

Although *M. sexta* is unusual in not requiring a gut microbiome, the issue of how to deal with the gut microbiome during infection is a dilemma for all animals. This study will help us examine the costs and benefits of different microbiome control strategies in an animal in which the data may be easier to interpret than they would be in other, more complex, systems.

MATERIALS AND METHODS

Animals

All studies were performed on 5th instar larvae of *Manduca sexta* (Linnaeus 1763) obtained from our colony. The colony was derived from eggs supplied by Great Lakes Hornworms (Romeo, MI, USA), and was maintained as previously described (Adamo et al., 2016). Caterpillars were isolated upon hatching and fed on a high nutrition wheat germ and soy flour based diet (Great Lakes Hornworm). Trial caterpillars were weighed after their moult to the last larval instar (5th instar–day 0). Caterpillars were allotted into groups by mass, such that there were no initial mass differences across groups. 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.

Bacterial inoculation

Fifth instar–day 0 caterpillars were weighed and sorted into five different treatment groups at two time points (4 and 24 h): (1) control, (2) oral inoculation, (3) oral inoculation+wounding challenge (control for systemic inoculation), (4) systemic inoculation and (5) oral+systemic inoculation.

Bacteria for oral inoculation, *Micrococcus luteus* (Microkwik culture, Carolina Biologica, Burlington, NC, USA) and *Enterobacter aerogenes* (Microkwik culture, Carolina Biologica) were chosen based on the types of mildly pathogenic bacteria previously found in field populations of lepidopterans (Hammer et al., 2017; Paniagua Voirol et al., 2018). Dosage was based on concentrations of gut bacteria found in wild-caught caterpillars (Hammer et al., 2017; Paniagua Voirol et al., 2018) as well as a dose–response pilot conducted in the lab. Bacteria for systemic inoculation were a mixture of heat-killed *Serratia marcescens* (Gram-negative bacterium, Microkwik culture, Carolina Biological, 1/10 LD50), *Bacillus cereus* (Gram-positive bacterium, Microkwik culture, Carolina Biological, 1/10 LD50) and *Beauveria bassiana* (strain GHA, fungus, 1/10 LD50, BotaniGard 22WP; Laverlam, Butte, MT, USA; triple mix).

All 5th instar–day 0 caterpillars were given a 2.5 mm^3 cube of high nutrition diet that had been dyed green with food colouring (ClubHouse, London, ON, Canada). For all groups except the control and systemic inoculation groups, the cube had been injected with a solution containing ~10^6 bacterial cells (50:50 *M. luteus*:*E. aerogenes*). Bacteria numbers were estimated by optical density measurements 600 nm and confirmed by plating on high nutrition agar. The remaining groups received a green food cube with the same dimensions but without the bacterial solution. Each caterpillar was given 1 h to fully consume the cube. Any caterpillars that failed to consume the full cube were excluded from the study. Following

this, all groups were given uncoloured high nutrition diet *ad libitum* for the following 23 h. On 5th instar–day 1, all caterpillars had their food removed for 1 h prior to manipulation and were weighed. The feeding protocol was then repeated. After the consumption of the green food cube was completed, control and oral inoculation were placed on uncoloured high nutrition diet for 4 or 24 h depending on the group. Oral inoculation+wounding challenge caterpillars were given a sterile poke between the 6th and 7th abdominal segments before being placed on uncoloured high nutrition diet. Systemic inoculation and systemic+oral inoculation groups were given an injection of 20 µl of heat-killed triple mix (see above) between the 6th and 7th abdominal segments before being placed on uncoloured high nutrition diet.

Oral inoculation and bacterial colonies

In a pilot study, we found that oral inoculation of bacteria significantly increased the culturable bacteria in both midgut contents (N=10) and frass (N=10) compared with controls (N=10 per contrast; Fig. S1). Using faecal samples as a proxy method for a non-invasive gut microbiome has been previously established in Lepidoptera as well as in *Drosophila* (Fink et al., 2013; Schwarz et al., 2018 preprint).

Frass pellets from an orally inoculated group as well as a control group were collected, suspended in phosphate-buffered saline (PBS) and plated on nutrient agar (Sigma-Aldrich cat. no. 70148: by weight: 54% agar, 3% meat extract, 18% peptone, 18% NaCl, 7% yeast extract). The gut transit time of the green coloured food bolus was found to be ∼2 h regardless of the presence of inoculated bacteria, which is consistent with previous studies (McMillan et al., 2018). Frass samples from all groups were collected and plated in duplicate at the following time points post-ingestion: 1, 2, 6, 24 and 72 h. Agar plates were kept isolated at 23°C and colonies were counted 48 h post-plating.

Tissue sampling and RNA extraction

Caterpillars were killed and dissected for midgut and fatbody tissue collection either 4 or 24 h post-manipulation for 5th instar–day 2 animals. Caterpillars were chilled to induce a cold coma and decapitated. The midgut was removed from the caterpillar, and the midgut contents and peritrophic membrane were placed in a microcentrifuge tube. The midgut tissue was washed 3 times using ice-cold PBS before being placed in RNA*later* solution (Invitrogen, Carlsbad, CA, USA). Fatbody was extracted from the 7th abdominal segment and placed in RNA*later* solution. Samples were kept at −80°C until processed.

RNA extraction was performed using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). All steps adhered to the manufacturer's instructions and included a DNase I treatment (RNase-Free DNase Set, 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 were determined using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA) as well as a Qubit Fluorometer (Q32857, Invitrogen, CA, USA). Only samples with an $A_{260/280}$ ratio greater than 1.8 were used. cDNA was synthesized using iScript (Bio-Rad, Hercules, CA, USA) and samples were stored at −20°C. Primers were purchased from integrated DNA technologies (http://www.idtdna.com/site) and stored at −20°C at a working stock of 10 µmol l^{-1}. For primer sequences and efficiencies, please see Table S1.

Prior to qPCR, each sample was diluted to a set concentration of 100 ng μl^{-1} using the Qubit Fluorometer (Q32857, Invitrogen). For each biological sample and gene, a 16 µl reaction mixture was prepared containing 4 µl of sample cDNA, 10 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.6 µl of forward primer (10 µmol l^{-1}), 0.6 µl of reverse primer (10 µmol l^{-1}) and 0.8 µl RNase-free ddH_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: 10 s), annealing and extension (60°C: 45 s). After the qPCR, a melt curve analysis was run to assess the specificity of the qPCR product. Quantitative cycle (ΔΔCq) values for each sample and gene target were calculated in CFX Maestro (Bio-Rad).

For reference gene assessment, we selected the most stable of six candidate reference genes used in a previous study in *M. sexta* (Adamo et al., 2016): *Rp17A*, actin (*MSA*), ribosomal protein S3 (*MsS3*), *ubiquitin*, *βFTZ-F1* and glycerol-3-phosphate dehydrogenase (*G3PDH*). We used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes (Andersen et al., 2004) (i.e. *Rp17A* and *ubiquitin*), using the Cq values of five biological samples for each candidate reference gene, for each treatment. The qPCR efficiency (Eff.) and correlation coefficient (R^2) for primer sets were estimated from a standard curve generated with 10-fold dilutions of mixed cDNA samples and are given in Table S1.

pH measurements

The food bolus removed from the midgut was vortexed briefly to homogenize the sample, then the pH was measured using an StMicro5 pH electrode (Ohaus, Parsippany, NJ, USA) attached to a Corning pH meter 430 (Corning, Corning, NY, USA). All pH measurements were taken within 5 min of removal of the bolus from caterpillar.

Bacterial colonies and treatment groups

Caterpillars had their abdominal segments surface sterilized with 70% ethanol and were placed in a disinfected container. Frass was collected immediately post-excretion for 1 h prior to tissue collection. Therefore, contamination from the outside environment should be minimal. The frass pellets were suspended in PBS and plated in duplicate on nutrient agar. The plates were isolated and allowed to grow at 23°C; 48 h post-plating, bacterial colony forming units (CFUs) were counted.

In a pilot study, frass was sent away for 16S metagenomic sequencing in order to better determine bacterial diversity, but the amount of bacterial DNA was below the detection limit for a single individual. Data per individual were required for multivariate analysis.

Statistics

Data were analysed using SPSS (v.25). Data met the assumptions for MANOVA and univariate tests. Data for mass, pH and bacterial colonies were found to be normally distributed using a Shapiro–Wilk test. The qPCR data were analysed using CFX Maestro 1.1 (Bio-Rad) and the REST program (2009; http://rest.gene-quantification.info). ΔΔCq values were log transformed prior to statistical analysis. When multiple tests were performed on the same dataset, the alpha criterion was corrected. Sample sizes were determined based on effect sizes derived from pilot data or literature values.

Three canonical variates were then established for each time point by using the raw subscale scores and multiplying them by the corresponding subscale unstandardized discriminant function coefficients. Following this, an ANOVA was performed on each of the three canonical variates (Enders, 2003). Considering the

Journal of Experimental Biology (2020) 223, jeb226662. doi:10.1242/jeb.226662

proper data handling for multivariate derived data, a conservative statistical significance level was pre-set at $P<0.001$ for canonical variate 1, $P<0.0005$ for canonical variate 2, and $P<0.00025$ for canonical variate 3 (Neufeld and Gardner, 1990).

RESULTS

Bacterial gut transit times

Gut transit times were not altered by the presence of bacteria. The gut transit times of high nutrition artificial diet inoculated with ~10^6 bacterial cells (50:50 *M. luteus*:*E. aerogenes)* was 135.5±23 min while for control animals it was 125.2±23 min (independent samples *t*-test, $n=20$, $P=0.33$). The number of CFUs in the frass was higher for caterpillars fed bacteria, and increased over time (Fig. 1; one-way ANOVA time point; $F_{4,95}=1052.86$, $P<0.0001$), with the exception of the 72 h post-inoculation group, in which the CFUs dropped to the bacterial count found between the 2 and 6 h time point (Fig. 1).

Group×time interaction

A MANOVA was conducted using challenge type (group) and terminal time point (4 or 24 h) as fixed factors. This MANOVA showed a significant effect of group as well as a group×time point interaction (Wilks' $\lambda=0.03$, $F_{52,459}=12.8$, $P<0.0001$).

4 h time point

For each treatment group, change in mass (in g), frass bacteria CFUs, gut pH and ΔΔCq values for fat body and midgut gene expression [genes encoding attacin, lysozyme, GST1, inducible nitric oxide synthase (iNOS) and transferrin] were collected. A MANOVA showed that there was a significant effect of treatment group (Wilks' $\lambda=0.002$, $F_{13,56}=15.65$, $P<0.0001$). Individual univariate analysis was then conducted on the variables with *P*-values corrected using the Bonferroni adjustment (Table 1).

Change in mass is representative of the intensity of illness-induced anorexia, and a sign of immune activation (Sullivan et al., 2016). At 4 h post-manipulation, *M. sexta* from both the systemic and oral+systemic challenge gained significantly less mass than control animals, indicating that at this time point both of these groups exhibit illness-induced anorexia. Interestingly, oral inoculation of bacteria (50:50 *M. luteus*:*E. aerogenes*) alone did not elicit this sickness behaviour until the 24 h time point (Tables 1 and 2).

The gut pH of *M. sexta* is very basic, averaging at about pH 10–11 (Dow, 1992). At 4 h post-treatment, *M. sexta* in the oral+wounding, systemic and oral+systemic challenge groups all dropped their gut pH compared with controls, making their midgut significantly less basic than that of control animals. *Manduca sexta* that had been orally inoculated alone did not have this drop in midgut pH, but did not increase pH either (Table 1).

The frass that was collected and plated 4 h post-manipulation showed a significant increase in the amount of CFUs present in *M. sexta* in the oral+wounding and oral+systemic challenge groups compared with controls. This result demonstrates that the ingested bacteria survived during the trial. The amount of CFUs present in the frass of the oral+systemic challenge group was significantly higher than that in all other groups (Table 1).

Within the fat body, attacin and lysozyme gene expression were upregulated in the oral+wounding, systemic and oral+systemic challenge groups. Oral inoculation did not elicit the production of AMPs within the fat body at this time point (Table 1). Within the midgut tissue, attacin was upregulated in the systemic and oral+systemic challenge groups. Lysozyme expression in the midgut was upregulated in all groups, relative to controls, at 4 h post-manipulation (Table 1).

The transcription levels of the mRNA of GST1 showed interesting patterns of regulation, with expression being upregulated in the oral+wounding challenge group at 4 h post-manipulation and being significantly downregulated in the oral+systemic challenge group (Table 1). Within the midgut tissue, the same downregulation occurred within the oral+systemic challenge but the upregulation seen in the oral+wounding challenge group was not present (Table 1).

iNOS was upregulated under all conditions in the fat body at this time point. However, in the midgut tissue, iNOS was not induced in any of the challenge groups (Table 1).

24 h time point

A MANOVA showed that there was a significant effect of treatment group (Wilks' $\lambda=0.002$, $F_{13,56}=16.04$, $P<0.0001$). Individual univariate analysis was then conducted on the variables, with *P*-values corrected using the Bonferroni adjustment (Table 2).

At the 24 h time point we expected to see a recovery from illness-induced anorexia. As predicted, the oral+systemic challenge group had recovered (Table 2). However, illness-induced anorexia appeared in the oral+wounding challenge, and was still measurable in the oral and systemic challenge groups (Table 2).

The pH of the oral+wounding and systemic challenge group remained less basic than that in the control group 24 h post-manipulation. However, the midgut pH of the oral+systemic challenge group was no longer different compared with the control (Table 2).

All groups that were orally inoculated with live bacteria had significantly higher CFUs in their frass than control animals (Table 2).

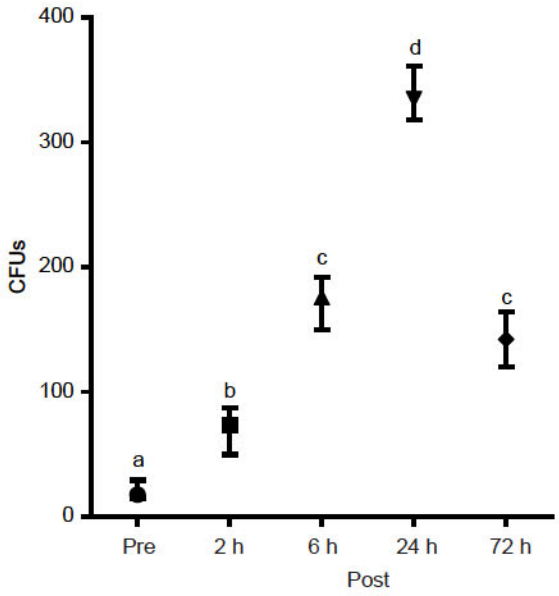

Fig. 1. Bacterial colony forming units cultured from frass collected at different time points. Frass was collected and plated on high nutrient agar from *Manduca sexta* caterpillars prior to oral inoculation of bacteria (Pre) and at several time points after (Post). The number of colony forming units (CFUs) increased at each time point up to 24 h post-oral inoculation before decreasing again at 72 h post-oral inoculation For all groups, $n=20$. Different letters indicate significant differences between time points ($P<0.05$). Data points represent means and error bars represent the standard deviation.

Journal of Experimental Biology

Table 1. Effect of systemic and oral challenges on *Manduca sexta* caterpillars 4 h post-treatment

	Control (*n*=14)	Oral+wounding (*n*=14)	Oral (*n*=14)	Systemic (*n*=14)	Oral+systemic (*n*=14)	*F*	*P*
ΔMass (g)	0.72±0.23^{4,5}	0.68±0.22^{4,5}	0.54±0.27^{5}	0.37±0.08^{1,2}	0.26±0.32^{1,2,3}	9.28	5.0×10^{-6}
CFUs	19.35±14.7^{2,5}	69.32±72.7^{1,5}	28.57±9.01^{5}	45.57±47.46^{5}	165.36±42.28^{1,2,3,4}	25.24	1.22×10^{-12}
pH	10.27±0.12^{2,4,5}	10.04±0.11^{1}	10.22±0.22^{4}	9.86±0.3^{1,3,5}	10.07±0.22^{1,4}	9.23	2.0×10^{-5}
ΔΔCq							
FB attacin	1.23±0.83^{2,4,5}	3.74±0.66^{1,3,4,5}	1.11±1.18^{2,4,5}	4.94±0.24^{1,2,3}	4.71±0.32^{1,2,3}	90.29	8.22×10^{-26}
MG attacin	0.63±1.10^{4,5}	1.13±0.79^{4,5}	0.74±0.98^{4,5}	2.30±0.18^{1,2,3}	2.23±0.26^{1,2,3}	15.70	4.86×10^{-9}
FB lysozyme	0.34±0.30^{2,4,5}	1.49±0.52^{1,3}	0.26±0.60^{2,4,5}	1.85±0.12^{1,3}	1.70±0.23^{1,3}	53.12	8.38×10^{-20}
MG lysozyme	0.59±0.75^{2,3,4,5}	2.13±0.65^{1,3}	1.28±0.90^{1,2,4,5}	2.59±0.38^{1,3}	2.76±0.37^{1,3}	28.06	1.49×10^{-13}
FB GST	−0.13±0.15^{2,5}	0.22±0.08^{1,3,4,5}	−0.20±0.20^{2}	−0.24±0.30^{2,5}	−0.36±0.26^{1,2,4}	14.46	1.70×10^{-8}
MG GST	−0.16±0.24^{5}	−0.12±0.35^{5}	−0.21±0.23	−0.22±0.27	−0.45±0.29^{1,2}	3.0	0.025
FB transferrin	0.23±0.26^{2}	0.78±0.40^{1,3,5}	−0.03±0.48^{2,4}	0.49±0.24^{3}	0.25±0.45^{2}	9.12	7.0×10^{-6}
MG transferrin	0.25±0.29	0.08±0.50	0.22±0.34	0.17±0.33	0.20±0.29	0.47	0.76
FB iNOS	−0.75±0.41^{2,3,4,5}	−0.08±0.58^{1,3}	1.11±1.18^{1,2,4,5}	0.21±0.29^{1,3}	0.38±0.50^{1,3}	14.65	1.40×10^{-8}
MG iNOS	−0.23±0.46	−0.22±0.29	−0.32±0.33	−0.18±0.19	−0.08±0.30	1.08	0.375

Univariate analysis followed by Bonferroni *post hoc* tests. Values are means±s.d. Superscript numbers indicate a significant difference from specific groups (1, control; 2, oral+wounding; 3, oral; 4, systemic; 5, oral+systemic). ΔMass, change in mass; CFUs, colony-forming units; ΔΔCq, quantitative cycle values for qPCR results on genes encoding attacin, lysozyme, glutathione *S*-transferase (GST), transferrin and inducible nitric oxide synthase (iNOS); FB, fat body; MG, midgut tissue.

The mRNA expression for the AMP attacin was upregulated in the fat body tissue in the oral+wounding, systemic and oral+systemic challenge groups. Unlike at the 4 h time point, at 24 h post-manipulation the oral challenge group also showed increased expression of attacin (Table 2). Lysozyme expression within the fat body remained upregulated for the oral+wounding, systemic and oral+systemic challenge groups (Table 2). Within the midgut tissue, gene expression of attacin was upregulated in the oral, systemic and oral+systemic challenge groups. Lysozyme was upregulated in the oral+wounding, systemic and oral+systemic challenge groups (Table 2).

At 24 h post-manipulation, GST1 gene expression was downregulated in the fat body in each of the groups when compared with that in control animals (Table 2). This same trend was not seen in the midgut tissue, where at the 24 h time point there was only downregulation in the oral challenge group (Table 2).

Gene expression of the iron-binding glycoprotein transferrin was upregulated in the fat body and midgut tissues of *M. sexta* that had undergone an oral+systemic challenge (Table 1).

The increased expression of iNOS in the fat body tissue was still visible at 24 h post-manipulation in the oral, systemic and oral+systemic challenge groups. The expression of iNOS was no longer increased in the oral challenge group when compared with controls (Table 2). In the midgut, there were no significant changes in iNOS expression at 24 h (Table 2).

Canonical correlations

A canonical correlation derived from canonical variates of the MANOVA for each time point showed that the type of immune challenge resulted in different patterns in the dependent variables (4 h; Wilks' λ=0.00058, $F_{52,207.38}$=23.3, P<0.001, 24 h; Wilks' λ=0.00098, $F_{52,207.38}$=19.9, P<0.001).

For the 4 h time point, our analysis found that the first three eigenvalues were significant (Wilks' λ; P<0.001, P<0.0005, P<0.00025, respectively; Table 3). The first eigenvalue accounted for 67% of the model variance (see Table 3 for details). Taken together, the three eigenvalues accounted for >95% of the model variance.

For canonical variate 1, the ANOVA was significant ($F_{4,65}$=350, P<0.001). This was followed by Bonferroni-adjusted (0.001/5=0.0002) *post hoc* tests. All treatments were found to be significantly different from each other (P<0.0002) with the exception of the control and oral challenge groups (P=0.01) and the systemic and oral+systemic challenge groups (P=1) (Fig. 2A).

Table 2. Effect of systemic and oral challenges on *Manduca sexta* caterpillars 24 h post-treatment

	Control (*n*=14)	Oral+wounding (*n*=14)	Oral (*n*=14)	Systemic (*n*=14)	Oral+systemic (*n*=14)	*F*	*P*
ΔMass (g)	1.83±0.40^{2,3,4}	1.33±0.57^{1}	0.91±0.50^{1}	1.05±0.32^{1}	1.41±0.53	8.00	2.60×10^{-5}
CFUs	40.43±12.13^{2,3,5}	324.57±64.47^{1,4}	277.5±69.72^{1,4,5}	76.86±28.9^{2,3,5}	349.43±50.04^{1,3,4}	116.89	6.08×10^{-29}
pH	10.30±0.13^{2,4}	9.99±0.40^{1,5}	10.15±0.10^{4,5}	9.84±0.28^{1,3,5}	10.41±0.12^{2,3,4}	13.04	7.5×10^{-8}
ΔΔCq							
FB attacin	−0.01±0.55^{2,3,4,5}	1.17±0.30^{1,3,4,5}	0.79±0.37^{1,2,4,5}	2.33±0.40^{1,2,3}	2.40±0.17^{1,2,3}	103.78	1.73×10^{-27}
MG attacin	0.25±0.45^{3,4,5}	0.79±0.57^{4,5}	0.93±0.84^{1,4,5}	1.74±0.56^{1,2,3}	1.60±0.40^{1,2,3}	15.25	7.57×10^{-9}
FB lysozyme	0.70±0.31^{2,4,5}	1.24±0.24^{1,3,4,5}	0.81±0.3^{2,4,5}	1.57±0.28^{1,2,3}	1.81±0.26^{1,2,3}	40.45	5.54×10^{-17}
MG lysozyme	0.65±0.35^{2,4,5}	1.38±0.60^{1}	1.07±0.66	1.21±0.40^{1}	1.39±0.38^{1}	5.37	0.001
FB GST	−0.10±0.20^{2,3,4,5}	−0.31±0.09^{1,3}	−0.53±0.18^{1,2}	−0.35±0.18^{1}	−0.40±0.16^{1}	12.28	1.70×10^{-7}
MG GST	−0.02±0.27^{3}	0.08±0.19^{3,4}	−0.31±0.22^{1,2}	−0.18±0.29^{2}	−0.17±0.20	5.85	4.39×10^{-4}
FB transferrin	−0.29±0.19^{4,5}	−0.10±0.25^{5}	−0.09±0.25^{5}	−0.03±0.23^{1}	0.21±0.23^{1,2,3,4}	8.43	1.50×10^{-5}
MG transferrin	−0.92±0.62^{5}	−0.67±0.36	−0.86±0.33^{5}	−0.87±0.35^{5}	−0.25±0.62^{1,3,4}	4.80	0.002
FB iNOS	−0.06±0.20^{3,4,5}	0.14±0.26	0.18±0.23^{1}	0.34±0.33^{1}	0.27±0.19^{1}	5.37	0.001
MG iNOS	−0.03±0.27	−0.13±0.21	−0.15±0.33	−0.26±0.20	−0.12±0.27	1.39	0.246

Univariate analysis followed by Bonferroni *post hoc* tests. Values are means±s.d. Superscript numbers indicate a significant difference from specific groups (1, control; 2, oral+wounding; 3, oral; 4, systemic; 5, oral+systemic). ΔMass, change in mass; CFUs, colony-forming units; ΔΔCq, quantitative cycle values for qPCR results on genes encoding attacin, lysozyme, glutathione *S*-transferase (GST), transferrin and inducible nitric oxide synthase (iNOS); FB, fat body; MG, midgut tissue.

Journal of Experimental Biology (2020) 223, jeb226662. doi:10.1242/jeb.226662

Table 3. Standardized discriminant function coefficients for the 4 h time point

Variable	Function 1	Function 2	Function 3
ΔMass	0.08	0.42	−0.21
CFUs	0.20	0.27	0.92
pH	0.01	0.42	0.53
ΔΔCq			
FB attacin	1.01	0.56	0.32
MG attacin	0.22	−0.5	−0.35
FB lysozyme	0.40	−0.69	−0.83
MG lysozyme	−0.01	−0.21	0.19
FB GST	0.17	0.39	−0.36
MG GST	0.26	−0.07	−0.3
FB transferrin	−0.39	1.43	0.44
MG transferrin	0.10	0.01	0.02
FB iNOS	−0.29	−0.63	−0.23
MG iNOS	−0.22	0.32	0.12

ΔMass, change in mass; CFUs, colony-forming units; ΔΔCq, quantitative cycle values for qPCR results on genes encoding attacin, lysozyme, glutathione *S*-transferase (GST), transferrin and inducible nitric oxide synthase (iNOS); FB, fat body; MG, midgut tissue.

For canonical variate 2, the ANOVA once again showed a significant difference between challenge types ($F_{4,65}$=92.9, P<0.0005). This ANOVA was followed by Bonferroni-adjusted (0.0005/5=0.0001) *post hoc* tests. These tests revealed that the control and oral+wounding challenge grouped together (P=0.147) and the oral, systemic and oral+systemic challenge all grouped together (P=0.001, P=0.013, P=1.0) (Fig. 2B).

For canonical variate 3, the ANOVA showed a significant difference between challenge types was present ($F_{4,65}$=43.4, P<0.00025) (Fig. 2C). The *post hoc* tests that followed this ANOVA were Bonferroni adjusted (0.00025/5=0.00005).

When the three canonical variates means for each individual were plotted against each other, unique clusters formed for each type of challenge (Fig. 2D).

For the 24 h time point, our analysis found that the first three eigenvalues were significant (Wilks' λ; P<0.001, P<0.0005, P<0.00025, respectively; Table 4). The first eigenvalue accounted for 58% of the model variance (see Table 4 for details). Taken together, the first three eigenvalues accounted for >95% of the model variance.

For canonical variate 1, the ANOVA was significant ($F_{4,65}$=264, P<0.001). This was followed by Bonferroni-adjusted (0.001/5=0.0002) *post hoc* tests. All treatments were found to be significantly different from each other (P<0.0002) with the exception of the oral and oral+wounding challenge groups (P=0.037) (Fig. 3A).

For canonical variate 2, the ANOVA once again showed a significant difference between challenge types ($F_{4,65}$=136, P<0.0005). This ANOVA was followed by Bonferroni-adjusted (0.0005/5=0.0001) *post hoc* tests. These tests revealed that the control and oral+wounding challenges grouped together (P=1), with the rest of the groups being significantly different from each other (Fig. 3B).

For canonical variate 3, the ANOVA showed a significant difference between challenge types was present ($F_{4,65}$=33, P<0.00025). The *post hoc* tests that followed this ANOVA were

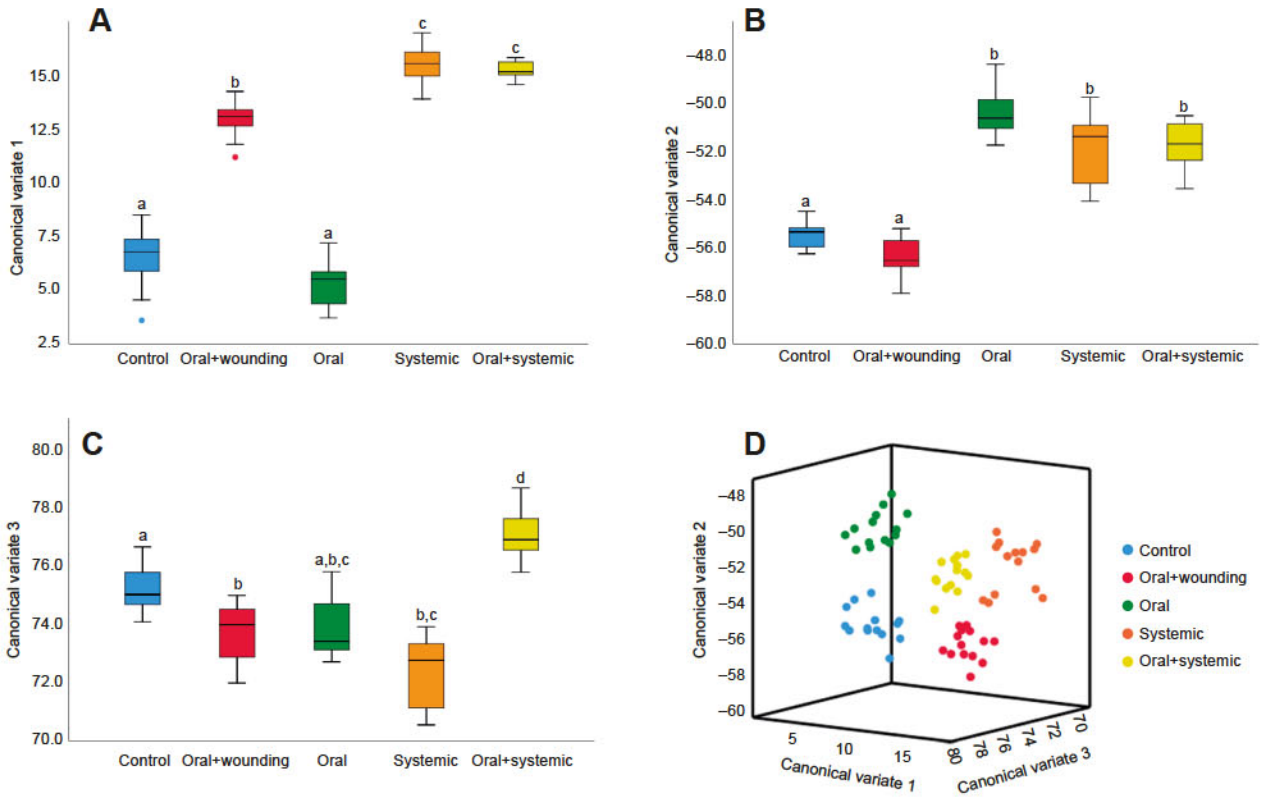

Fig. 2. Canonical variates from the 4 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.0001). (C) Canonical variate 3. Challenge groups with different letters are significantly different from each other (P<0.00005). (D) Individual representation of significant canonical variates calculated from canonical coefficient factor loadings. Bars represent first and third quartiles, internal bar represents the median, and error bars represent the maximum and minimum result.

Journal of Experimental Biology (2020) 223, jeb226662. doi:10.1242/jeb.226662

Table 4. Standardized discriminant function coefficients for the 24 h time point

Variable	Function 1	Function 2	Function 3
ΔMass	−0.02	0.18	0.28
CFUs	−0.9	0.29	−0.06
pH	0.1	0.46	0.89
ΔΔCq			
FB attacin	−0.6	−1.11	0.03
MG attacin	0.28	0.45	0.36
FB lysozyme	0.09	−0.13	0.06
MG lysozyme	0.04	−0.05	−0.28
FB GST	0.23	−0.44	0.11
MG GST	−0.1	−0.17	0.005
FB transferrin	0.04	−0.0007	0.25
MG transferrin	−0.16	0.11	0.24
FB iNOS	−0.02	−0.1	−0.04
MG iNOS	0.1	0.05	0.04

ΔMass, change in mass; CFUs, colony-forming units; ΔΔCq, quantitative cycle values for qPCR results on genes encoding attacin, lysozyme, glutathione *S*-transferase (GST), transferrin and inducible nitric oxide synthase (iNOS); FB, fat body; MG, midgut tissue.

Bonferroni adjusted (0.00025/5=0.00005). These tests revealed that the control group and the oral+systemic challenges grouped together (P=0.01). The remaining groups, oral+wounding, oral and systemic challenge, also grouped together (P=1.0) (Fig. 3C).

When the three canonical variates means for each individual were plotted against each other, unique clusters formed for each type of challenge (Fig. 3D).

Change in mass increased significantly with time ($F_{13,118}$=201.9, P<0.0001), as did the number of CFUs ($F_{13,118}$=394.0, P<0.0001). The other variables changed significantly across the two time points except for lysozyme and iNOS gene expression in the fat body, and iNOS gene expression in the midgut (P=0.21, 0.90, 0.17, respectively). The MANOVA also showed that there was a significant interaction of group and time point (Wilks' λ=0.031, $F_{52,459}$=12.81, P<0.0001) for all treatments except midgut gene expression of GST and iNOS.

DISCUSSION

We found no evidence that *M. sexta* sterilizes its gut during a systemic, oral or dual immune challenge. Bacteria remained in the gut (as estimated from counting bacteria CFUs in the frass) during both systemic and oral challenges. In fact, there was no evidence that the number of gut bacteria even declined during these challenges (Tables 1 and 2). A decline in gut bacteria numbers was expected in treatments with systemic immune challenges because the microbiome of *M. sexta* comes from its food (Hammer et al., 2017), and caterpillars eat less during an immune challenge (i.e. illness-induced anorexia). However, we saw no such decline. Although immune-challenged caterpillars eat less, their gut transit time remains unchanged (McMillan et al., 2018). Therefore, the number of culturable bacteria in the frass was not artificially elevated by a decline in the movement of material through the gut. We also know that both the oral and systemic challenges produced robust immune responses in the caterpillars because of the expression of illness-induced anorexia and the increased expression of immune-related

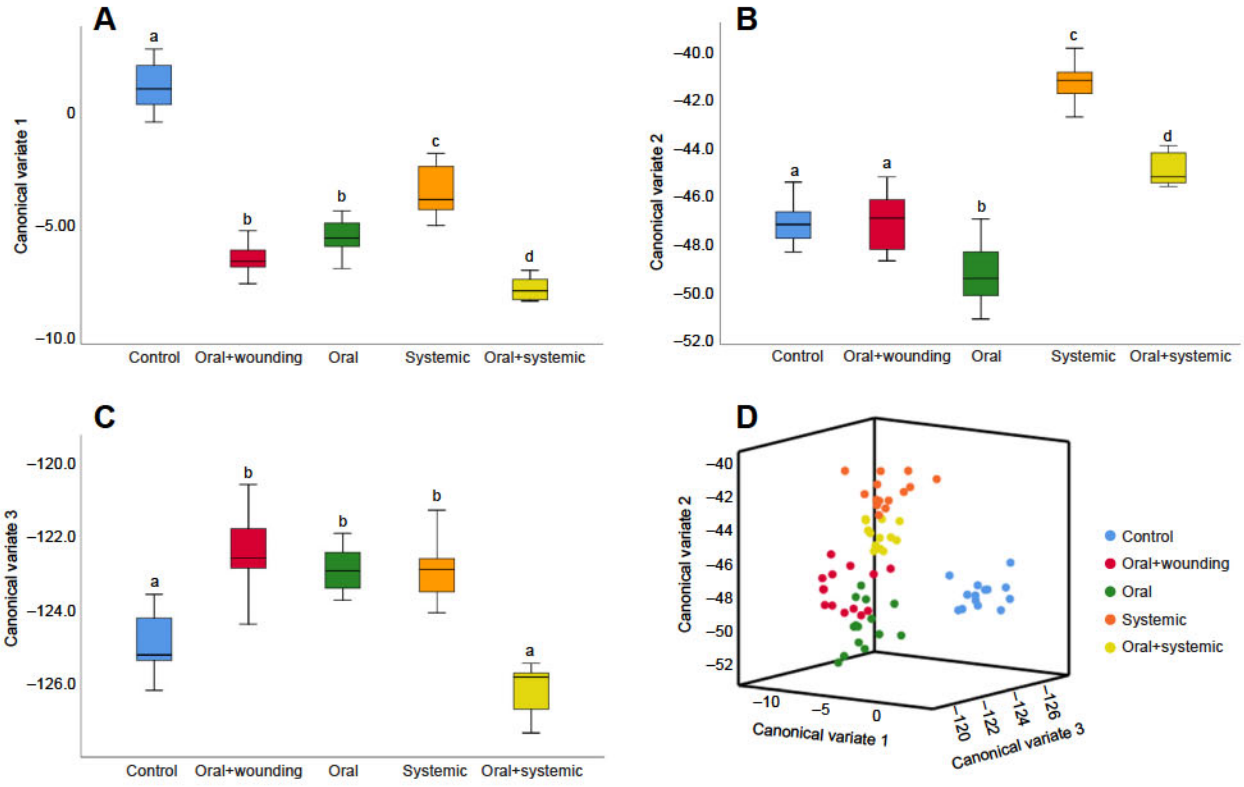

Fig. 3. Canonical variates from the 24 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.0001). (C) Canonical variate 2. Challenge groups with different letters are significantly different from each other (P<0.00005). (D) Individual representation of significant canonical variates calculated from canonical coefficient factor loadings. Bars represent first and third quartiles, internal bar represents the median, and error bars represent the maximum and minimum result.

genes such as the attacin gene (Tables 1 and 2). Therefore, the lack of gut sterilization was not due to the lack of an immune response. Finally, the immunological evidence that caterpillars attempted to sterilize their gut was equivocal. Although challenged caterpillars did demonstrate an increase in AMP production in both the midgut and fat body, there was no increase in gut alkalinity (i.e. increase in gut pH). Instead of an increase in gut alkalinity, the systemic, oral+wounding and oral+systemic challenge groups dropped their gut pH compared with controls (Tables 1 and 2). A less alkaline gut would make the gut environment more hospitable to bacteria such as *E. aerogenes* and *M. luteus* that function optimally at a more neutral pH (Kung and Wang, 1977; Tanisho, 1998). However, the decline in pH was modest (approximately 0.4 pH units). Whether this change would significantly enhance bacterial survival is uncertain. These results show that gut sterilization is not a host defence mechanism for pathogen threats in *M. sexta*, at least not for the immune challenges used in this study.

During severe sepsis, *M. sexta* produce watery faeces, suggesting that they are attempting to flush their gut of pathogens (Dunn et al., 1994). We have only observed this in *M. sexta* close to death (L.E.M. and S.A.A., personal observation). It is possible that removing bacteria from the gut remains an option for *M. sexta*, when all other defences have failed.

Our data are more consistent with the three other hypotheses we explored. The first alternative hypothesis was that gut sterilization may not occur because of physiological trade-offs between systemic immune defence and the resources needed for sterilization. Trade-offs may even cause a reduction in the mechanisms usually used to keep the microbiome in check, leading to an increase in the number of bacteria in the gut. But, in caterpillars given a systemic immune challenge, we saw no evidence for an increase in the number of culturable bacteria in the gut compared with controls. However, it is possible that there was a change in the number of bacteria that do not grow on nutrient agar. Nevertheless, minimally we can conclude that there was no increase in some types of bacteria, suggesting that there was no decline in at least some aspects of immune defence in the gut. Nevertheless, there was evidence for a trade-off between systemic defence and bacterial numbers in the gut during a dual challenge. Bacterial numbers in the gut were higher during a dual challenge than during an oral challenge. However, we did not see evidence of a decline in the production of two important AMPs in the midgut (attacin and lysozyme) during a dual challenge, as might be expected if there were physiological trade-offs involving these molecules. In fact, gene expression of these AMPs was sometimes higher during the dual challenge compared with expression during a single challenge (Table 2). It is possible that trade-offs were occurring in unmeasured immune components.

The second alternative hypothesis was that *M. sexta* may maintain its transient gut microbiome because it confers benefits in terms of immunity (Fraune and Bosch, 2010). Although *M. sexta* do not appear to possess a resident gut microbiome, they do acquire a microbiome from their food and environment (Hammer et al., 2017) that could be beneficial even if it is transient. Our results showed that the transient gut microbiome is retained during both systemic and gut bacterial infections. Determining whether it is advantageous will require further study.

The third alternative hypothesis was that caterpillars may also alter (i.e. reconfigure) their immune response when faced with a dual challenge as opposed to a single challenge. Insects have been shown to reconfigure their immune response when an immune challenge co-occurs with environmental stressors such as food availability and predators (Adamo et al., 2008, 2016, 2017). One possible reconfiguration would be a shift in emphasis from resistance mechanisms to infection tolerance mechanisms in the midgut. During a dual challenge, we predicted that the caterpillar would prioritize protection over resistance as resources for resistance mechanisms may be in short supply. Infection tolerance mechanisms may be less expensive than resistance mechanisms, and less damaging (Soares et al., 2014).

GSTs are important detoxification enzymes that perform a dual role. They are involved in detoxification of xenobiotics via catalysis of the conjugation of the toxin with glutathione (Eaton and Bammler, 1999; Snyder et al., 1995), as well as conferring protection against self-harm induced by oxidative stress pathways (Kim et al., 2011). However, GSTs are a large family of enzymes, with 40 unique gene transcripts identified in *M. sexta* (Koenig et al., 2015). The transcripts of identified GST genes have been shown to cluster in a tissue-specific manner, with some being found exclusively in the fatbody or midgut, while others such as GST1 are expressed in multiple tissues (Koenig et al., 2015; Snyder et al., 1995). GST1 was selected for this study as it has been identified in the literature as being present in the midgut and shows variable expression based on diet type and presence of bacteria (Snyder et al., 1995). We predicted an increase in GST1 production in the midgut during a dual challenge, because such an increase should enhance protection against bacterial toxins (Snyder et al., 1995). However, at the 4 h time point we saw a steep reduction in GST1 expression in both the fat body and midgut. Midgut GST1 expression had recovered by 24 h. This pattern is not found in all insects. Cabbage loopers (*Trichoplusia ni*) show no change in GST1 expression in the midgut after being fed bacteria, although a decline is found in other tissues (Freitak et al., 2009). It is difficult to make a definitive conclusion because we did not assess the activity of all GST genes active in the midgut. However, the ability of an immune challenge to induce a steep decline in a GST gene (i.e. GST1) that plays a major role in detoxification (Snyder et al., 1995) may point to a physiological trade-off between detoxification and immune defence (McMillan et al., 2018).

Transferrin is a protein that sequesters free iron as part of a tolerance strategy called nutritional immunity (Soares and Weiss, 2015), and is another potential mechanism of infection tolerance (Brummett et al., 2017). *Manduca sexta* show increased expression of mRNA for transferrin in the fatbody 24 h after a bacterial injection into the haemocoel (Brummett et al., 2017; He et al., 2015). Our results support these findings. We found an increase of transferrin in the fatbody 24 h post-treatment in both the systemic and oral+systemic challenge group. Interestingly, ingesting bacteria did not increase transferrin expression in the midgut, although a dual challenge did (Table 2). This finding may indicate that a shift towards tolerance mechanisms in the midgut may only be activated during a widespread or massive immune challenge, e.g. during a combined systemic and gut infection. It is also possible that ingesting a more virulent bacteria could have induced transferrin expression.

Although we found some evidence for an increase in tolerance mechanisms in the gut during immune challenges, we assessed only a small number of possible mechanisms. Unfortunately, tolerance mechanisms are poorly characterized (Ayres and Schneider, 2009). In the future, measurements of damage (e.g. signs of oxidative stress; Costantini, 2019) may provide the best estimate of the activity of tolerance mechanisms.

However, our results do suggest an increase in resistance mechanisms in the midgut. We observed an increase in gene expression for the AMPs attacin and lysozyme, even during a systemic immune challenge (i.e. when there was no increase in bacteria in the gut). It is possible that the midgut is recruited to support the

systemic immune system; there is evidence that AMPs secreted by the midgut can end up in the haemolymph (Freitak et al., 2007).

Another resistance mechanism used extensively in the insect gut is the production of reactive molecules (e.g. reactive oxygen species, ROS) to destroy pathogens. Buchon et al. (2014) showed that *Drosophila* increase the amount of ROS in their gut in response to natural microbiota by activating dual oxidase (DUOX) and iNOS. Similarly, Eleftherianos et al. (2009) demonstrated in *M. sexta* that when a pathogen (*Photorhabdus luminescens*) was ingested, increased gene expression of iNOS was restricted to the gut. In contrast, iNOS was increased in the fat body when pathogens were injected into the haemocoel (Eleftherianos et al., 2009). However, in our study, we found that iNOS mRNA expression increased significantly in the fatbody in the oral, systemic and oral+systemic challenge groups at both 4 and 24 h post-treatment. We did not, however, see an increase in the expression of iNOS transcripts in the midgut tissues at either time point. It is possible that these differences are due to our using a primer designed for a specific iNOS which would not capture the whole picture if other iNOS genes are involved. Moreover, we used bacteria that were mildly pathogenic as the oral challenge, and we injected heat-killed, not live, bacteria into the haemocoel for the systemic challenge. These differences may account for the differences in the expression pattern.

Interpreting the results of the MANOVA by examining the canonical coefficients also supports the hypothesis of immune reconfiguration. At the 4 h time point, canonical variate 1, which encompasses the majority of the variability (67%), was primarily influenced by AMP production in the immune tissues as well as bacterial CFUs cultured from the frass (Table 3). We hypothesize that variate 1 may reflect the strength of the immune response of each group. The immune response increases with a greater number of gut bacteria, and is reflected by strong expression of AMPs both systemically (as demonstrated by increased expression in the fat body tissue) and in the midgut. Compared with control animals, we did not see immune activation in terms of AMP production in the oral challenge group. This evidence could be interpreted as indicating that increased bacterial gut load alone does not strongly influence the factors that contribute most to this canonical variate, i.e. AMP production. If this is the case, it is not surprising to see the systemic and the oral+systemic challenge group clustering together for this variable, as it appears to be the systemic challenge of injecting heat-killed bacteria that is eliciting this response.

Canonical variate 2 revealed a different global pattern whereby transferrin and iNOS in the immune tissues influenced grouping. We also saw the influence of AMPs in the immune tissues shaping this canonical variate. In this case, change in mass (representing illness-induced anorexia) is acting on this variable in the opposite direction from AMP expression in the immune tissues. This could be due to an increase in cytokines caused by AMP expression in the tissue leading to a decrease in mass gain, as a result of the animal exhibiting illness-induced anorexia.

Canonical variate 1 for the 24 h time point was similar to that of the 4 h time point in that the production of AMPs in the immune tissues primarily influenced grouping. In addition to this, however, the CFUs produced in the frass also contributed heavily. The ANOVA for this variate shows that the control group was isolated from all other groups most likely because it is not producing increased AMPs in the immune tissues, nor is it producing large amounts of CFUs. The oral and oral+wounding challenge group clustered together, indicating an increase in both of these factors. The systemic challenge group was distinct, however, most likely because, while it exhibited higher production of AMPs than the control, it produced fewer CFUs.

Canonical variate 2 for 24 h was primarily influenced by AMP production in the immune tissues. Unlike canonical variate 2 at the 4 h time point, the change in mass ceased to be a strong driving force. This is most likely because the effects of illness-induced anorexia are starting to subside at this time point.

Regarding the global view of all three significant canonical variates taken together, at the 4 h time point all five treatment groups formed unique clusters in space (Fig. 2D). Within these variables, the systemic and oral+systemic challenges clustered closest together, most likely as a result of similar expression of AMPs within the immune tissues. The global view at the 24 h time point revealed that although there was clear clustering of individual treatment groups, certain groups such as the oral and oral+wounding challenge began to collapse into each other (Fig. 3D). We started to see recovery at 24 h post-challenge from behavioural changes such as illness-induced anorexia, even though AMP production continued to be elevated.

These results support the hypothesis that the immunophysiological response to a challenge depends on the nature and context of the challenge (Adamo et al., 2017). The immune system responds differently depending on, for example, the number of simultaneous immune challenges (Fig. 2). It is not uncommon for animals to be exposed to multiple pathogens (e.g. insects; Boucias and Pendland, 2012). Therefore, natural selection would favour animals that could produce an optimal response to concurrent gut and systemic pathogen challenges. Optimally responding to different types and numbers of pathogens requires partitioning scarce resources among different immune components and other physiological systems. In animals with microbiomes, their regulation during an infection is likely to vary depending on the number and type of concurrent immune challenges.

Acknowledgements
We thank L. Puddicombe and B. Chiang for help with animal care. We thank D. Miller for help with experimental design.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.E.M., S.A.A.; Methodology: L.E.M., S.A.A.; Validation: L.E.M.; Formal analysis: L.E.M., S.A.A.; Investigation: L.E.M.; Resources: S.A.A.; Writing - original draft: L.E.M., S.A.A.; Writing - review & editing: L.E.M., S.A.A.; Visualization: S.A.A.; Supervision: S.A.A.; Project administration: S.A.A.; Funding acquisition: S.A.A.

Funding
This study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to S.A.A.

Data availability
All datasets are available from Mendeley (McMillan, 2020): http://dx.doi.org/10.17632/zkv9mjjm53.2

Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.226662.supplemental

References

Adamo, S. A., Roberts, J. L., Easy, R. H. and Ross, N. W. (2008). Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *J. Exp. Biol.* **211**, 531-538. doi:10.1242/jeb.013136

Adamo, S. A., Davies, G., Easy, R., Kovalko, I. and Turnbull, K. F. (2016). Reconfiguration of the immune system network during food limitation in the caterpillar *Manduca sexta*. *J. Exp. Biol.* **219**, 706-718. doi:10.1242/jeb.132936

Adamo, S. A., Easy, R. H., Kovalko, I., MacDonald, J., McKeen, A., Swanburg, T., Turnbull, K. F. and Reeve, C. (2017). Predator exposure-induced immunosuppression: trade-off, immune redistribution or immune reconfiguration? *J. Exp. Biol.* **220**, 868-875. https://jeb.biologists.org/content/220/5/868

Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245-5250. doi:10.1158/0008-5472.CAN-04-0496

Aranda, P. S., LaJoie, D. M. and Jorcyk, C. L. (2012). Bleach gel: A simple agarose gel for analyzing RNA quality. *Electrophoresis* **33**, 366-369. doi:10.1002/elps.201100335

Ardia, D. R., Gantz, J. E., Brent, C. and Strebel, S. (2012). Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Funct. Ecol.* **26**, 732-739. doi:10.1111/j.1365-2435.2012.01989.x

Ayres, J. S. and Schneider, D. S. (2009). The role of anorexia in resistance and tolerance to infections in Drosophila. *PLoS Biol.* **7**, e1000150. doi:10.1371/journal.pbio.1000150

Bajgar, A., Kucerova, K., Jonatova, L., Tomcala, A., Schneedorferova, I., Okrouhlik, J. and Dolezal, T. (2015). Extracellular adenosine mediates a systemic metabolic switch during immune response. *PLoS Biol.* **13**, e1002135. doi:10.1371/journal.pbio.1002135

Boucias, D. G. and Pendland, J. C. (2012). *Principles of Insect Pathology*: Springer Science & Business Media.

Brummett, L. M., Kanost, M. R. and Gorman, M. J. (2017). The immune properties of Manduca sexta transferrin. *Insect Biochem. Mol. Biol.* **81**, 1-9. doi:10.1016/j.ibmb.2016.12.006

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev. Microbiol.* **12**, 168. doi:10.1038/nrmicro3182

Buchon, N., Silverman, N. and Cherry, S. (2014). Immunity in Drosophila melanogaster—from microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* **14**, 796-810. doi:10.1038/nri3763

Contreras-Garduño, J., Lanz-Mendoza, H., Franco, B., Nava, A., Pedraza-Reyes, M. and Canales-Lazcano, J. (2016). Insect immune priming: ecology and experimental evidences. *Ecol. Entomol.* **41**, 351-366. doi:10.1111/een.12300

Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. *J. Exp. Biol.* **222**, jeb194688. doi:10.1242/jeb.194688

Dow, J. (1992). pH gradients in lepidopteran midgut. *J. Exp. Biol.* **172**, 355-375.

Dunn, P. E., Bohnert, T. J. and Russell, V. (1994). Regulation of antibacterial protein synthesis following infection and during metamorphosis of manduca sextaa. *Ann. N. Y. Acad. Sci.* **712**, 117-130. doi:10.1111/j.1749-6632.1994.tb33567.x

Eaton, D. L. and Bammler, T. K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci.* **49**, 156-164. doi:10.1093/toxsci/49.2.156

Eleftherianos, I., Felföldi, G., Ffrench-Constant, R. H. and Reynolds, S. E. (2009). Induced nitric oxide synthesis in the gut of Manduca sexta protects against oral infection by the bacterial pathogen Photorhabdus luminescens. *Insect Mol. Biol.* **18**, 507-516. doi:10.1111/j.1365-2583.2009.00899.x

Enders, C. K. (2003). Performing multivariate group comparisons following a statistically significant MANOVA. *Meas. Eval. Couns. Dev.* **36**, 40-56. doi:10.1080/07481756.2003.12069079

Ferguson, L. V., Dhakal, P., Lebenzon, J. E., Heinrichs, D. E., Bucking, C. and Sinclair, B. J. (2018). Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. *Funct. Ecol.* **32**, 2357-2368. doi:10.1111/1365-2435.13153

Fink, C., Staubach, F., Kuenzel, S., Baines, J. F. and Roeder, T. (2013). Noninvasive analysis of microbiome dynamics in the fruit fly Drosophila melanogaster. *Appl. Environ. Microbiol.* **79**, 6984-6988. doi:10.1128/AEM.01903-13

Fraune, S. and Bosch, T. C. G. (2010). Why bacteria matter in animal development and evolution. *BioEssays* **32**, 571-580. doi:10.1002/bies.200900192

Freitak, D., Wheat, C. W., Heckel, D. G. and Vogel, H. (2007). Immune system responses and fitness costs associated with consumption of bacteria in larvae of Trichoplusia ni. *BMC Biol.* **5**, 56. doi:10.1186/1741-7007-5-56

Freitak, D., Heckel, D. G. and Vogel, H. (2009). Bacterial feeding induces changes in immune-related gene expression and has trans-generational impacts in the cabbage looper (Trichoplusia ni). *Front. Zool.* **6**, 7. doi:10.1186/1742-9994-6-7

Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P. and Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proc. Natl Acad. Sci. USA* **114**, 9641-9646. doi:10.1073/pnas.1707186114

He, Y., Cao, X., Li, K., Hu, Y., Chen, Y.-r., Blissard, G., Kanost, M. R. and Jiang, H. (2015). A genome-wide analysis of antimicrobial effector genes and their transcription patterns in Manduca sexta. *Insect Biochem. Mol. Biol.* **62**, 23-37. doi:10.1016/j.ibmb.2015.01.015

Kim, B. Y., Hui, W. L., Lee, K. S., Wan, H., Yoon, H. J., Gui, Z. Z., Chen, S. and Jin, B. R. (2011). Molecular cloning and oxidative stress response of a sigma-class glutathione S-transferase of the bumblebee Bombus ignitus. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **158**, 83-89. doi:10.1016/j.cbpb.2010.09.012

Koenig, C., Bretschneider, A., Heckel, D. G., Grosse-Wilde, E., Hansson, B. S. and Vogel, H. (2015). The plastic response of Manduca sexta to host and non-host plants. *Insect Biochem. Mol. Biol.* **63**, 72-85. doi:10.1016/j.ibmb.2015.06.001

Krams, I. A., Kecko, S., Jõers, P., Trakimas, G., Elferts, D., Krams, R., Luoto, S., Rantala, M. J., Inashkina, I. and Gudrā, D. (2017). Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. *J. Exp. Biol.* **220**, 4204-4212. doi:10.1242/jeb.169227

Krieg, A. (1987). *Diseases Caused by Bacteria and other Prokaryotes. Epizootiology of Insect Diseases*, pp. 323-355. New York, NY: John Wiley & Sons, Inc.

Kung, V. T. and Wang, J. C. (1977). Purification and characterization of an omega protein from Micrococcus luteus. *J. Biol. Chem.* **252**, 5398-5402.

Lochmiller, R. L. and Deerenberg, C. (2000). Trade–offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98. doi:10.1034/j.1600-0706.2000.880110.x

McMillan, L. (2020). Friend or foe? Effects of host immune activation on the gut microbiome in the caterpillar Manduca sexta. Mendeley Data, V2. doi:10.17632/zkv9mjjm53.2

McMillan, L. E., Miller, D. W. and Adamo, S. A. (2018). Eating when ill is risky: immune defense impairs food detoxification in the caterpillar Manduca sexta. *J. Exp. Biol.* **221**, jeb173336. doi:10.1242/jeb.173336

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D. M., , III and Galindo-Villegas, J. (2015). Selective manipulation of the gut microbiota improves immune status in vertebrates. *Front. Immunol.* **6**, 512. doi:10.3389/fimmu.2015.00512

Neufeld, R. W. J. and Gardner, R. C. (1990). Data aggregation in evaluating psychological constructs: Multivariate and logical deductive considerations. *J. Math. Psychol.* **34**, 276-296. doi:10.1016/0022-2496(90)90033-6

Ojeda-Avila, T., Woods, H. A. and Raguso, R. (2003). Effects of dietary variation on growth, composition, and maturation of Manduca sexta (Sphingidae: Lepidoptera). *J. Insect Physiol.* **49**, 293-306. doi:10.1016/S0022-1910(03)00003-9

Paniagua Voirol, L. R., Frago, E., Kaltenpoth, M., Hilker, M. and Fatouros, N. E. (2018). Bacterial symbionts in lepidoptera: their diversity, transmission, and impact on the host. *Front. Microbiol.* **9**, 556. doi:10.3389/fmicb.2018.00556

Phalnikar, K., Kunte, K. and Agashe, D. (2019). Disrupting butterfly caterpillar microbiomes does not impact their survival and development. *Proc. R. Soc. B* **286**, 20192438. doi:10.1098/rspb.2019.2438

Russell, V. W. and Dunn, P. E. (1991). Lysozyme in the midgut of Manduca sexta during metamorphosis. *Arch. Insect Biochem. Physiol.* **17**, 67-80. doi:10.1002/arch.940170202

Russell, V. W. and Dunn, P. E. (1996). Antibacterial proteins in the midgut of Manduca sexta during metamorphosis. *J. Insect Physiol.* **42**, 65-71. doi:10.1016/0022-1910(95)00083-6

Schwarz, M. T., Kneeshaw, D. and Kembel, S. W. (2018). The gut-associated microbiome of the eastern spruce budworm does not influence larval growth or survival. *bioRxiv* 330928. doi:10.1101/330928

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321. doi:10.1016/0169-5347(96)10039-2

Snyder, M. J., Walding, J. K. and Feyereisen, R. (1995). Glutathione S-transferases from larval Manduca sexta midgut: Sequence of two cdnas and enzyme induction. *Insect Biochem. Mol. Biol.* **25**, 455-465. doi:10.1016/0965-1748(94)00083-B

Soares, M. P. and Weiss, G. (2015). The Iron age of host–microbe interactions. *EMBO Rep.* **16**, 1482-1500. doi:10.15252/embr.201540558

Soares, M. P., Gozzelino, R. and Weis, S. (2014). Tissue damage control in disease tolerance. *Trends Immunol.* **35**, 483-494. doi:10.1016/j.it.2014.08.001

Soares, M. P., Teixeira, L. and Moita, L. F. (2017). Disease tolerance and immunity in host protection against infection. *Nat. Rev. Immunol.* **17**, 83. doi:10.1038/nri.2016.136

Steinhaus, E. (1959). Serratia marcescens Bizio as an insect pathogen. *Hilgardia* **28**, 351-380. doi:10.3733/hilg.v28n14p351

Sullivan, K., Fairn, E. and Adamo, S. A. (2016). Sickness behaviour in the cricket *Gryllus texensis*: Comparison with animals across phyla. *Behav. Process.* **128**, 134-143. doi:10.1016/j.beproc.2016.05.004

Tanisho, S. (1998). Hydrogen production by facultative anaerobe enterobacter aerogenes. In *BioHydrogen* (ed. O. R. Zaborsky, J. R. Benemann, T. Matsunaga, J. Miyake and A. San Pietro), pp. 273-279. Boston, MA: Springer US.

Vallet-Gely, I., Lemaitre, B. and Boccard, F. (2008). Bacterial strategies to overcome insect defences. *Nat. Rev. Microbiol.* **6**, 302. doi:10.1038/nrmicro1870

Weiss, B. and Aksoy, S. (2011). Microbiome influences on insect host vector competence. *Trends Parasitol.* **27**, 514-522. doi:10.1016/j.pt.2011.05.001

Youngblut, N. D., Reischer, G. H., Walters, W., Schuster, N., Walzer, C., Stalder, G., Ley, R. E. and Farnleitner, A. H. (2019). Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nat. Commun.* **10**, 2200. doi:0.1038/s41467-019-10191-3

Zhai, Z., Huang, X. and Yin, Y. (2018). Beyond immunity: The Imd pathway as a coordinator of host defense, organismal physiology and behavior. *Dev. Comp. Immunol.* **83**, 51-59. doi:10.1016/j.dci.2017.11.008

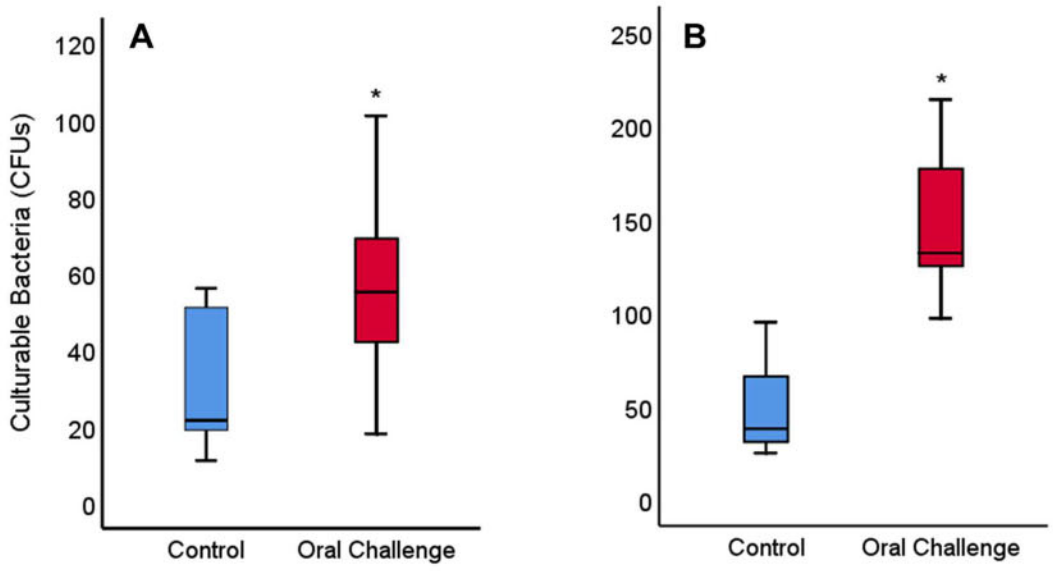

Figure S1. Cultrable bacteria from the midgut contents (A) and frass (B) of *M. sexta,* 4 hours post feeding. Oral challenge group were given a food cube that had been injected with a solution containing ~10^6 bacterial cells (50:50 *M. luteus: E. aerogenes)*. Independent samples T-test from midgut contents indicate a significantly greater amount of bacteria in the oral challenge (N=10, M=57.9, SD=24.7) group relative to the control group (N=10, M=29.1, SD=17.5); t(18)=-3.00, p=0.008. Independent samples T-test from plated frass similarly indicates a significantly greater amount of bacteria in oral challenge (N=10, M=147.5, SD=11.31) compared to control (N=10, M=47.0, SD=23.2); t(18)=-7.5, p<0.0001

Table S1. Forward and reverse primer sequences for target immune-related genes and reference genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Eff.	Reference
Attacin-1	GCAGGCGACGACAAGAAC	ATGCGTGTTGGTAAGAGTAGC	1.0	(An et al., 2010)
Lysozyme	GTGTGCCTCGTGGAGAATG	ATGCCTTGGTGATGTCGTC	0.98	(An et al., 2010)
GST-1	AAGTACCCGTTCCAGCTGAA	TGGGTTGGACAGGACAGTTT	1.0	(Snyder et al., 1995)
Transferrin	CCATTCGTCAGTAGTGTT	GTCAGGGCTATCAAAGTTA	0.98	(Brummett et al., 2017)
NOS	ACGCAAGCGACATTACAGTG	ACCGCTATGTTCACCTCCAC	0.99	(Eleftherianos et al., 2009)
RpL17A	TCCGCATCTCACTGGGTCT	CACGGCAATCACATACAGGTT	0.96	(Rewitz et al., 2006)
Ubiquitin	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	1.0	(Kumar et al., 2012)

3.9 REVISIONS

Methods

Oral inoculation and bacterial colonies

In a pilot study, we found that oral inoculation of bacteria significantly increased the culturable bacteria in both midgut contents (N=10) and frass (N=10) compared with controls N=10 per contrast; Fig S1).
Frass pellets from orally inoculated group as well as control group were collected, suspended in phosphate buffered saline (PBS) and plated on nutrient agar (Sigma-Aldrich cat. No. 70148: by weight: 54% agar, 3% meat extract, 18% peptone, 18% NaCl, 7% yeast extract). The gut transit time of the green coloured food bolus was found to be ~2 h regardless of the presence of inoculated bacteria, which is consistent with previous studies (McMillan et al., 2018). Frass samples from all groups were collected and plated in duplicate at the following time points post-ingestion: 1, 2, 6, 24 and 72 h. Agar plates were kept isolated at 23°C and colonies were counted 48 h post-plating.

Revisions

Group x time interaction

A MANOVA was conducted using challenge type (group) and terminal time point (4 or 24 h) as fixed factors and gut transit time, CFUs, gut pH, and ΔΔCq values of immune genes as variables. This MANOVA showed a significant effect of group as well as group x time point interaction (Wilks' λ=0.03, $F_{52,459}$=12.8, P<0.0001).

CHAPTER 4-PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA* CATERPILLARS PARASITIZED BY WASP *COTESIA CONGREGATA*

4.1 PUBLICATION INFORMATION

In preparation.

4.2 ABSTRACT

The mechanisms mediating parasitic manipulation of behaviour are difficult to elucidate. Previously, this work was usually undertaken by pre-selecting targets such as specific pathways, or proteins. Therefore, research in this area has often been limited by prior knowledge about these pathways. Recently, proteomic analysis of tissue has allowed researchers in the area to examine manipulation mechanisms without this bias. The braconid wasp, *Cotesia congregata* is a parasitoid that injects its eggs along with a polydnavirus (CcBV) into its host, the caterpillar *Manduca sexta*. Upon emergence of the parasitic wasp larvae from the caterpillar, the caterpillar's behaviour changes. It significantly reduces feeding, becomes hypersensitive to stimuli and no longer expresses self-generated behaviour. The caterpillar eventually starves to death. Because feeding is regulated by both the supraesophageal and subesophageal ganglia of the CNS, a proteomic analysis across four different time points was conducted on these two parts of the CNS in both parasitized and non-parasitized (i.e. control) caterpillars. Proteomic profiles in these brain areas varied across time points. Differences in the proteins between parasitized and control brains were observed both before and after the behavioural change of the host. Additionally, we found that parasitized brains had less total protein than controls at all time points. The brains of parasitized caterpillars contained several virally encoded proteins not found in control brains. Although previous works had detected the mRNA for these proteins in the CNS of *M. sexta* caterpillars (Espagne et al., 2005; Hayakawa et al., 1994; Le et al., 2003; Wang et al., 2021), this is the first time that some CcBV-encoded proteins are found in the CNS of M. sexta, as supported by both proteomic and transcriptomic evidence. Many of these changes in protein composition of the CNS coincide with the host's behavioural change, suggesting that they may play a role in changing the host's behaviour.

4.3 INTRODUCTION

When an animal is infected with a pathogen or a parasite their behaviours can be altered as a result (Hart, 1988). Some of these behaviours are an adaptive response by the host in response to the insult; these are called sickness behaviours (Hart, 1988). Occasionally, other behaviours are novel to the animal and are produced by the parasite (Moore, 2002). Any behaviour, whether altered from a pre-existing behaviour or novel in nature, that benefits a parasite's survival or transmission are called a 'behavioural manipulation' and the parasite a parasitic manipulator.

There are numerous examples of parasitoids that manipulate host behaviour. Parasitoids are organisms whose larval development is dependent upon feeding from a host, typically resulting in the host's death (Eggleton and Gaston, 1990; Kuris, 1974). One striking example is found in spiders of the family tetragnathidae. When parasitized by ichneumonid wasps, the spider will spin a protective web, quite distinct in form from its normal web, around the cocoon of their parasitic wasp and protect it from potential predators (Weinersmith, 2019). Having its previous host act as a bodyguard significantly increases the parasite's chances of surviving through pupation (Gonzaga et al., 2017).

Another example of an extreme behavioural manipulation occurs in *Psalis pennatula* caterpillars that have been parasitized by *Microplitis pennatula*, a braconid wasp. After the wasp larva has emerged from within the body of its host, the caterpillar will stand guard over the cocoons of the parasite (Mohan and Sinu, 2017). Using defensive behaviour that the host uses to defend itself against predators, it now uses them to defend the wasps during their cocoon life stage (Mohan and Sinu, 2017).

These two examples of parasitic manipulation have easily discernible benefits to the parasite at the expense of their host, which will never go on to reproduce. However, the exact mechanisms used by the parasites remain rudementary (Gonzaga et al., 2017; Mohan and Sinu, 2017; Weinersmith, 2019).

Cotesia congregata is a parasitic wasp that uses the caterpillar *Manduca sexta* as a host. Similar to many other parasitoids, *C. congregata* is a parasitic manipulator (Adamo et al., 2016b). *M. sexta* is often used as a model for insect endocrinology, therefore provides a convenient organism for exploring how parasites manipulate behaviour (Beckage, 2012; Cole et al., 2002; Gelman et al., 1998). During parasitization of *M. sexta*, the adult female wasp oviposits its eggs into the hemocoel of the caterpillar where they hatch, grow and molt before scraping their way out of the caterpillar through

its body wall (Fulton, 1940). Through the entirety of the endoparasitic lifestage of *C. congregata,* the behaviour of *M. sexta* is indistinguishable from non-parasitized conspecifics (Adamo, 1997). However, 8-12 hours before the parasites emerge, the host exhibits a significant decrease in locomotory behaviour and ceases food consumption (Beckage and Riddiford, 1978; Fulton, 1940; Miles and Booker, 2000). At this point all self-generated behaviours within the caterpillar permanently cease (Adamo, 1997). Although the caterpillar will never resume normal behaviour, this does not appear to be due to damage caused during the wasp's exodus. Evidence for this includes the fact that the caterpillar's defensive reflexes remain intact (Adamo et al., 1997), and its chemosensory abilities to detect food are still active (Miles and Booker, 2000). Moreover, removal of the main brain (i.e. the supraesophageal ganglion) of the caterpillar will result in a return in locomotor activity, demonstrating that the caterpillar is still capable of locomotion (Beckage and Templeton, 1986). The changes in host behaviour are critical for wasp survival qw they prevent the host from eating the wasp cocoons (Adamo et al., 1997) and, as in other parasitoids, the host becomes a bodyguard for the wasp's cocoons, increasing their odds of survival (Adamo, 1998).

Another key aspect of the physiological manipulation that *C. congregata* exerts on *M. sexta* is the ability to halt its development in the larval form such that it never metamorphoses (Beckage and Gelman, 2004; Beckage and Riddiford, 1978). By actively manipulating the endocrine system to ensure that juvenile hormone titers remain elevated, parasitized *M. sexta* caterpillars continue to grow and gain mass far exceeding the critical mass that would normally trigger pupation when harbouring large numbers of wasp larvae (Beckage and Riddiford, 1982; Nijhout and Williams, 1974). There are two main hormone groups of interest when looking at the arrest of larval development in *M. sexta,* juvenile hormone which is released by the corpora allatum and ecdysteroids released by the prothoracic glands. Both of these systems have been shown to be compromised during parasitic manipulation by *Cotesia congregata* (Beckage and Gelman, 2004; Gelman et al., 1998). In addition to manipulating these hormonal systems directly within the hemolymph of the host, *C. congregata* can also potentially impact the neurosecretory cells in the brain (Edwards & Weaver, 2000); these neurosecretory cells have been shown to regulate production and release of the hormones, such as Prothoracicotropic hormone (PTTH) discussed above (Gade et al., 1997). In fact, research by Zitnan et al. (1995) showed a large build up of neuropeptides within the brain of parasitized *M. sexta,* suggesting they were not being released. These findings

indicate that normal functions of the CNS of *M. sexta* are being disrupted by *C. congregata*.

Overall, *Cotesia congregata* exerts a range of behavioural and developmental changes in its host. However, the exact mechanisms behind these changes, and how they might alter host behaviours, remain unclear. The range of possible mechanisms behind behavioural manipulation are as diverse as the parasites themselves. However, multiple parasitic manipulators seem to use some common mechanisms to alter host behaviour (Adamo, 2002; Adamo, 2013; Hughes and Libersat, 2019). There is evidence from fungi (*Ophiocordyceps* in carpenter ants (de Bekker et al., 2014)) , protozoans (*Toxoplasma* in rats (Stibbs, 1985)), and macroparasitoids (*Cotesia* in caterpillars (Žitňan et al., 1995)) that parasites are able to alter the chemical brain composition of their hosts. Alterations to brain chemistry can be achieved whether the parasite is located within the brain specifically, as with the case with *Toxoplasma gondii* (Stibbs, 1985), or without any direct contact with the central nervous system (CNS) as is the case in the gordian worm that resides in the abdomen of its grasshopper host (Biron et al., 2005; Moore, 2002; Thomas et al., 2005). Overall, parasitic manipulators use a variety of mechanisms to alter the chemical composition of the host CNS (Adamo, 2013; Gal and Libersat, 2008; Hughes and Libersat, 2019).

Another potential mechanism of parasitic manipulation is the bidirectional connection between the host's immune system and CNS–such connections are common in both vertebrates and invertebrates (Adamo, 2011; Dantzer et al., 2008). The immune system releases cytokines to which the CNS has receptors, resulting in changes in neural function and behaviour, usually of benefit to the host to aid in recovery (Dantzer, 2004; Hart, 1988). One of the known methods that parasites use to alter host behaviour is by co-opting these pre-existing circuits to their advantage (Adamo, 2013). For instance, the crustacean family of gammarids are subject to parasitic helminths that induce neuroinflammation in their hosts' CNS (Helluy, 2013; Helluy and Thomas, 2010). This neuroinflammation, which is hypothesized to be induced by glial cells releasing cytokines, results in the downstream effect of disruption in the serotonergic systems of the host (Helluy, 2013). These disruptions lead to impacts on the sensorimotor circuits of the gammirid, causing it to ascend the water column at a potentially dangerous time which, in turn, increases instances of predation, thus increasing transmission of the helminth parasite to its next host (Helluy, 2013; Helluy and Thomas, 2003; Helluy and

Thomas, 2010). Previously research has shown that the CNS of *M. sexta* is also capable of responding to immune signals (Adamo and McMillan, 2019).

Within the *Cotesia-Manduca* parasitic relationship there are several instances of *C. congregata* impacting pre-existing circuits to its advantage (Adamo, 1997; Adamo, 2005; Adamo et al., 2016b; Miles and Booker, 2000). *Cotesia congregata* is able to induce immunosuppression in *M. sexta* caterpillars in order to elude and suppress immune functions that would encapsulate or harm the larva growing within the hemocoel of their host (Lavine and Beckage, 1996). If *C. congregata,* as well as other parasites, are able to manipulate host immune systems to evade detection, this ability is hypothesized in Adamo (2013) to have pre-adapted parasitic manipulators for selection to manipulate host immune-neural connections, leading to host behaviour that favours the parasite.

Cessation of feeding is a behaviour that is already within *M. sexta*'s repetoire in the form of illness induced anorexia, a sickness behaviour (Adamo et al., 2007; Adamo et al., 2010; Hart, 1988). In the case of *M. sexta* responding to an immune challenge, illness induced anorexia confers survival benefits by reducing competition with other systems such as the food detoxification system. It also reduces the risk of acquiring a secondary infection (Adamo et al., 2010; McMillan et al., 2018). In the early stages of parasitism by *C. congregata*, the host caterpillar does not exhibit this particular sickness behaviour. *C. congregata* is a koinobiont which means that it needs its host to continue to eat and grow, so that it in turn may feed and grow. It is not until the final stage in the endoparasitic portion of the *C. congregata* parasitic cycle, when the larva emerges from the host, that this behaviour is elicited. This behaviour, as well as decreased locomotion, coincides directly with the time at which cessation of feeding provides an increase in parasite survival (Adamo, 1998).

Previous research into the timing of the induction of reduced locomotion and illness induced anorexia has shown that at the point the larval wasps are emerging from their host, a cytokine storm is taking place within the host (Adamo, 2005; Adamo et al., 2016b). The release of cytokines such as plasmatocyte spreading peptide (PSP), spätzle, and paralytic peptide may be involved in the cessation of feeding and reduced locomotion (Adamo et al., 2016b; Skinner et al., 1991). However, it remains unclear whether this is also one of the mechanisms behind the prolonged feeding suppression witnessed days, and even weeks, after the larval parasites have emerged.

Some parasitic manipulators are known to alter transcriptomic and proteomic profiles in the host (Adamo, 2013; Hughes and Libersat, 2019; Thomas et al., 2010). There is evidence that parasitic manipulators are able to both directly and indirectly alter gene expression in their hosts (Adamo, 2013). For example, a baculovirus that infects spongy moth, *Lymantria dispar,* caterpillars and causes tree-top disease uses altered gene expression as a means of behavioural manipulation (Hoover et al., 2011). In this system the baculovirus hijacks the host's transcriptomic machinery to transcribe and translate a virally encoded enzyme that inhibits a host-made hormone in order to alter the host's normal behaviour (Hoover et al., 2011). In doing so, the parasite is able to increase its rates of transmission (Hoover et al., 2011).

One tool that *Cotesia congregata*, along with other members of the monopheletic group microgastroids, possess to potentially help with the manipulation of host behaviour is a domesticated polydnavirus (PDV). PDVs can be thought of as gene delivery vectors from *Cotesia congregata* to *Manduca sexta* (Wang et al., 2021), and therefore could be a powerful mechanism to produce proteomic changes within the CNS. PDVs are double stranded DNA viruses that have fully integrated into the genome of their wasp hosts (Belle et al., 2002; Fleming and Summers, 1991; Savary et al., 1999). In braconid wasps, this PDV is known as a bracovirus. Each species of braconid wasp carries its own genetically unique bracovirus (BV) that is transmitted vertically as an integrated provirus via Mendelian inheritance principles (Stoltz, 1990). Genetic information from the provirus is spread throughout the chromosomes of the wasp, and is present in all tissues (Belle et al., 2002; Gauthier et al., 2021). However, replication of the chromosomally integrated viral sequences only takes place in specialized cells in the calyx of the ovary of female wasps (Bézier et al., 2009; Gauthier et al., 2021; Theilmann and Summers, 1986).

Broadly, BV genes can be sorted into two broad categories: nudiviral-like genes that are responsible for producing the viral particle that will encapsulate proviral dsDNA circles for injection into the host; and proviral segments that are a combination of ancestral viral genes and wasp genes (Belle et al., 2002; Chevignon et al., 2014; Chevignon et al., 2015; Gauthier et al., 2021). In female wasps bracovirus replication is highly targeted to the calyx of the ovary, while within male wasps there has only ever been negligible evidence of any transcription (Drezen et al., 2006; Savary et al., 1999; Theilmann and Summers, 1986). Replication of nudiviral-like particles begins two days into the pupal stage of the wasp lifecycle, with proviral segments being produced after

the first 4 days of pupation (Gauthier et al., 2021). Proviral segments undergo amplification, excision and circularization before being individually packaged into virions that are coded for by the nudiviral-like genes within the wasp calyx (Beck et al., 2007; Burke et al., 2013; Savary et al., 1999). There are a total of 35 distinct dsDNA viral circles containing 222 genes that are packaged in this way (Bézier et al., 2013; Chevignon et al., 2015). The BV will not replicate within the caterpillar host's tissues, so any integration with the lepidopteran host genome that occurs is the direct result of the virions injected initially by the mother.

In *Cotesia congregata* parasitism of *Manduca sexta,* there is estimated to be a total copy number of 5.96×10^{10} BV circles injected per female (Wang et al., 2021). Within the wasp calyx all circles are not produced equally, for instance circle 8 accounts for 22% of the total circles packaged into virions (Wang et al., 2021). Upon injection the BV will integrate itself into the caterpillar host's genome using the host's own integrases. Host integration motifs can be found within the viral circles which direct the hosts' integrases in placement (Chevignon et al., 2018). The main targets for the BV are the caterpillar's immune and endocrine systems (Beckage, 1998; Beckage and Gelman, 2004; Dushay and Beckage, 1993). In as little as 30 minutes post-parasitism, transcripts of BV genes can be found being produced by hemocytes and fat body tissues (Harwood and Beckage, 1994; Harwood et al., 1994; Lavine and Beckage, 1995). By 24 hours post-parasitism BV proteins account for 15% of circulating hemolymph protein (Harwood and Beckage, 1994). Therefore by altering protein composition, BV may offer another feasible mechanism by which behavioural manipulation is induced.

One complication when trying to disentangle the mechanisms behind how parasites are manipulating their host's behaviour is the sheer complexity. Parasites use multiple concurrent methods in order to achieve their end goal (Adamo, 2013). Parasitic manipulators impact neurohormones, cytokines, and gene transcription (Adamo, 2011; Ponton et al., 2006; Poulin, 2010). Recent developments in non-biased methods such as transcriptomics and proteomics are powerful tools in the researcher's kit to elucidate the mechanisms behind the change in host behaviour (Adamo, 2013; Biron and Loxdale, 2013; Hughes and Libersat, 2019).

In recent years, proteomic studies have been used to elucidate aspects of behavioural manipulation in other parasite-host situations. For instance, Ponton et al. (2006) used a proteomic method on the gammarid-helminth system and found that the

parasites were able to alter the production of a protein important for serotonergic synthesis.

Proteomics allows researchers to get a full picture of changes occurring within the system, or a specific tissue of interest. Using this method, many interesting findings have surfaced that would have been difficult to find using traditional methods. For instance, recent studies from differing parasitic systems have found that certain genes/proteins are the target of parasitic manipulation even though they are not currently known to play a direct role in neuronal signaling (Biron et al., 2005; Hayakawa et al., 2000; Ponton et al., 2006). In both the cricket-hairworm system and the gammarid-helminth system proteomic analyses have revealed that proteins involved in neural development are targeted by parasites, even though the hosts are adults, and these proteins should no longer be playing an active role at their current life stage (Biron et al., 2005; Ponton et al., 2006). Using a more directed approach such as blotting or qPCR, these proteins may not have ever been selected as they are off piste.

I propose using proteomic analyses on the two main brain regions of *M. sexta*, the supraesophageal ganglion which governs self-generated behaviours, and the subesophageal ganglion which is where the circuits for feeding are located. Investigating the proteomic changes to these areas over 4 distinct timepoints: pre larval parasite emergence, during parasite emergence, 1 day after parasites have emerged and three days after parasites have emerged; By examining different timepoints, both before and after the visible change in behaviour, I hope to explore whether there is evidence of neuroinflammation, changes to bioamine pathways, or changes in signaling pathways which could lead to changes in behaviour (Biron and Loxdale, 2013; Helluy, 2013; Mangold and Hughes, 2021).

Using a robust method such as proteomics will also allow me to investigate proteins that are present in these brain areas that originated from the domesticated bracovirus (Biron and Loxdale, 2013). We know from previous research that *C. congregata* bracovirus integrates genes into fat body and immune cells (Bézier et al., 2013; Chevignon et al., 2015; Chevignon et al., 2018). Using the host's own cellular machinery, these genes are transcribed and translated into proteins (Bézier et al., 2013; Chevignon et al., 2015; Wang et al., 2021). By integrating the functionally annotated bracovirus genome into our proteomics approach we will be able to determine which viral proteins, if any, are present within the supraesophageal ganglion and

subesophageal ganglion before and after changes in host behaviour are occurring (Chevignon et al., 2014; Louis et al., 2013).

4.4 METHODS

ANIMALS

All studies were performed on larvae of *Manduca sexta* obtained from our colony. The colony was derived from eggs supplied by Great Lakes Hornworms (MI, USA), and was maintained as previously described (Adamo et al., 2016a). *Cotesia congregata* cocoons were donated to us by Dr. Karen Kester from her colony at Virginia Commonwealth University, Virginia, USA. *C. congregata* adults emerged from their cocoons and were given three days to mate. They were then given 3rd instar *M. sexta* larvae in which to lay their eggs. Parasitized *M. sexta* were reared on lab-made high nutrition diet until their 5th larval instar. 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.

TISSUE EXTRACTION

Caterpillars were chilled to induce a cold coma (Robertson et al., 2017) and dissected over ice. The supraesophageal ganglion, the subesophageal ganglion and the were extracted into separate tubes containing 50 µl protease inhibitor (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, Switzerland) and flash frozen in liquid nitrogen. Tissues were kept at -80°C until use.

Parasitized *M. sexta* were sorted into five groups based on the stage of parasitism: Unparasitized (Control), Pre-emergent, Emergent, 1-day post emergence and 3 days post emergence. Pre-emergent *M. sexta* had their tissues extracted two-three days prior to the wasp larva beginning to emerge through the body wall. The Emergent group had their tissues extracted as the wasp larva were in the process of emerging through the body wall. The 1-day post emergence and 3-days post emergence groups had their tissues extracted 1 and 3 days post emergence of the wasp larva emerging from the caterpillar.

PROTEIN 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 were suspended in 30 µl of extraction buffer containing Urea 7M, Thiourea 2M, 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 seconds, followed by 20 seconds on ice. Following homogenization samples were centrifuged at 15 800 g at 4°C for 12 minutes, 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.

RNA EXTRACTION, QUANTIFICATION AND cDNA GENERATION

RNA extraction was performed using an 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. 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 Transcription Supermix for RT-qPCR (Bio-Rad, California, USA) and samples were stored in -80°C until use.

TARGETED QPCR

Primers were designed using sequences available on NCBI and the Primer-BLAST program (Ye et al., 2012), or found in literature (see Table 4.1 below). Reference genes used were Rp17a and ubiquitin which were found to be stable across groups using NormFinder for R (http://moma.dk/normfinder-software). Primers were purchased from integrated DNA technologies (http://www.idtdna.com/site) and stored at -20°C at a working concentration of 10 µmol 1-1.

Table 4.1. Forward and reverse gene primer sequences for target bracovirus genes and reference genes

	Forward Primer (5' to 3')	Reverse Primer5' to 3'	Reference
Ubiquitin	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	(Kumar et al., 2012)
Rp17a	TCCGCATCTCACTGGGTCT	CACGGCAATCACATACAGGTT	(Rewitz et al., 2006)
Cystatin-1	TCGAGCGGCCGCAATGGGCAAG GAATATCGAG	TGGCGCGGCCGCTTAACAATTTT CATATTCCCAAC	(Espagne et al., 2005)
EP-1	GCGCCCGTAGTGTCATTAATG	CCCAGTACTTGATGCGCTTG	(Gad and Kim, 2008)
CcV1	ATTCCTGGGCACCTCCAAG	TGCAACGATCGATCCAGGTC	NA

The reaction mixture used was 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.6 µL forward and reverse primer, 0.8 µL ddH_2O per sample. 6 µL of this reaction mixture was combined with 4 µL cDNA template per well. RT-qPCR was run using a CFX96 real-time system (Bio-rad) with the following parameters: 40 cycles of (95°C for 10 seconds; 55°C for 30 seconds; 60°C for 45 seconds) followed by a final extension of 60°C for 10 minutes.

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

(McMillan, 2022). A MaxQuant database was also used to reduce proteins being identified due to contamination (contaminants_fpp_180320.FASTA) (Cox J and Mann M, 2008). Protein validation was conducted with a 1% false discovery rate filter at both the peptide and the protein level.

PROTEIN RATIO ANALYSIS

In order to perform statistical analyses on the protein data, normalization of protein intensity signals was required. In order to be included in the ratio analysis, 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). 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 (Benjamini and Hochberg, 1995).

PROTEIN GO TERM ANALYSIS

UniProt (The UniProt Consortium, 2021) and InterProScan (Blum et al., 2021) were used to determine gene ontology (GO) terms and sort proteins by homologous families and superfamilies. GO slim analysis was conducted using Flybase ribbon slim.

4.5 RESULTS

PROTEIN AND RNA PROFILES

As previously mentioned, this study was the first to extract proteins from the brains of *Manduca sexta* over different stages of parasitism. As such, before I began looking into differences in protein composition using advanced techniques such as LCMS-MS, I first wanted to look for gross changes to the CNS proteome, such as possible overall differences in protein concentration at different time points.

In order to do this, I extracted the supraesophageal and subesophageal ganglion of both parasitized and control *Manduca sexta*. I then quantified total soluble protein using the Bradford protocol (Table 4.2). The data were found to be normally distributed according to a Shapiro-Wilk test, (Supraesophageal $W(50) = 0.96$, $p = 0.09$; Subesophageal $W(50) = 0.97$, $p = 0.21$). I performed an ANOVA on total protein, with treatment group as a between-group factor and mass of the caterpillars in grams as a co-variate. Post-hoc tests were used to compare all groups to one another, and I applied a Bonferroni correction for multiple comparisons.

Table 4.2. Total protein in the brain regions of *Manduca sexta* in controls and at different timepoints during parasitism

	Supraesophageal Mean +/- SD	Subesophageal Mean +/- SD
Control	7.00 ± 1.1	6.15 ± 1.2
Pre-emergence	4.60 ± 1.5*	4.10 ± 2.1*
Emergence	3.65 ± 1.6*	3.62 ± 1.6*
1-Day Post	4.60± 2.0*	3.50 ± 1.4*
3-Days Post	4.15 ± 1.1*	4.73 ± 1.0*

Protein quantities shown in µg. N=10 for all groups. * indicates a significant difference from control.

As is shown in Table 4.2 unparasitized caterpillars (Control) had significantly higher total protein present in both CNS regions when compared to parasitized caterpillars (Supraesophageal $F(2,4) = 5.7$, $p = 0.001$; Subesophageal $F(2,4) = 5.6$, $p = 0.001$). There were no significant differences between parasitized groups. In addition to these results, the supraesophageal and subesophageal ganglia may contain less fatty substances in parasitized caterpillars than controls, evidenced by the fact that these ganglia appeared more translucent in all parasitized groups (personal observation).

As a follow up to this test, supraesophageal ganglia from all groups were excised and total RNA was extracted. In a subset of these extractions cDNA was generated from total RNA in which mRNA is selectively reverse transcribed by singling out RNA with a poly-adenylated tail. Using a Qubit fluorometer, total RNA/cDNA was measured (Table 4.3). The data were found to be normally distributed using a Shapiro-Wilk test, [RNA: $W(14) = 0.93$, $p = 0.34$; cDNA: $W(16)=0.95$, $p = 0.51$]. I performed independent ANOVAs on total RNA and cDNA respectively. Once again, treatment group was a between-group factor and mass of the caterpillars in grams was a co-variate. For each ANOVA, post-hoc comparisons were performed in the manner described above. Unparasitized caterpillars had significantly higher total RNA and cDNA present in their supraesophageal ganglion than parasitized caterpillars RNA: $F(2, 23) = 8.56$, $p < 0.001$; cDNA: $F(3, 11) = 12.8$, $p < 0.001$. There were no significant differences present between any of the parasitized caterpillars.

Table 4.3. Total RNA and cDNA in the supraesophageal ganglion of *Manduca sexta* in controls and at different timepoints during parasitism

	Total RNA Mean +/- SD	cDNA Mean +/- SD
Control	172.5 ± 24.7	28.7 ± 3.1
Pre-emergence	56.0 ± 25.0*	22.2 ± 2.6*
Emergence	73.4 ± 17.2*	21.5 ± 1.0*
1-Day Post	31.5 ± 12.0*	18.3 ± 1.9*
3-Days Post	39.8 ± 9.16*	-

Amounts shown in ng. RNA N=24 ; cDNA N=16. * indicates a significant difference from control.

PROTEIN DETECTION

Using LCMS-MS tandem mass spectroscopy we were able to identify roughly 2500 proteins in each of the supraesophageal ganglion and the subesophageal ganglion. Of these proteins, using strict statistical criteria for significance (see methods), parasitism at any of our time points resulted in relatively few proteins that had significantly different abundance patterns from non-parasitized controls (Table 4.4).

Table 4.4. Total number of proteins detected in the supraesophageal and subesophageal ganglion in *Manduca sexta*. Percentage of proteins altered during parasitism.

	Supraesophageal		Subesophageal	
	Total Proteins	Percent altered	Total Protein	Percent altered
Control	2771	0.0	2526	0.0
Pre-Emergent	2739	3.2	2506	1.6
Emergent	2671	2.8	2455	3.9
1-Day Post	2639	3.7	2464	3.1
3-Days Post	2654	3.5	2085	2.9

PROTEIN IDENTIFICATION

Supraesophageal ganglion

In total 87 proteins were found to be present in different levels between control and pre-emergent parasitized caterpillars. This number was 76 in emergent caterpillars, 99 in 1-Day post and 93, 3-Days emergence caterpillars. These proteins were identified to be either below detection threshold in parasitized caterpillars (absent), present in lower amounts in parasitized caterpillars (decrease), present in parasitized caterpillars but below detection threshold in control (novel), or in higher amounts in parasitized caterpillars (increase). The differential presence of proteins and the trend compared to control animals was time-point dependent (Figure 4.1).

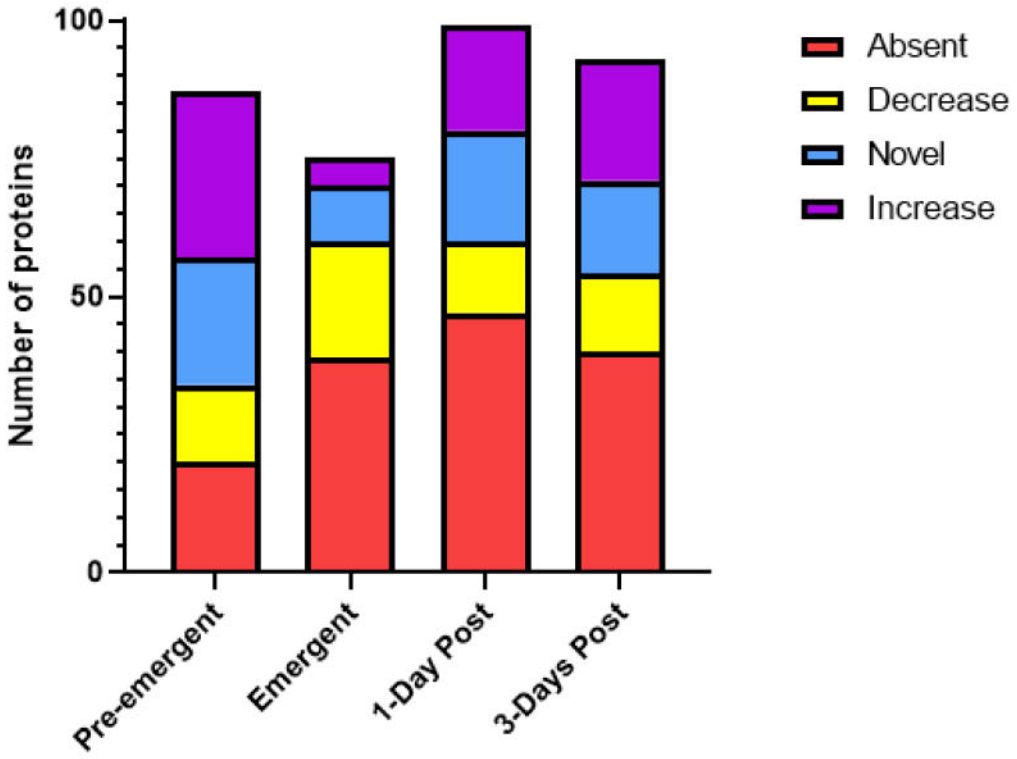

Figure 4.1. Differential presence of proteins within the supraesophageal ganglion of *Manduca sexta* caterpillars that have been parasitized by *Cotesia congregata*. The data show the number of proteins found to be in different concentrations when compared to uninfected control caterpillars.

Some proteins were present in such low quantities that they fell outside of the detection threshold of our method in control caterpillars, but were still present and detectable in parasitized samples. It is possible that these proteins may play a role in the manipulation of the host by the wasp at that time point. In the case of immune proteins, it could also reflect a defensive action on the part of the host. In other words, the caterpillar is mounting an immune defense within the CNS. A third possibility is that the abundance of these proteins is a side-effect, or an unmeant consequence of the parasitic manipulation. Either way, the proteins that we have termed “novel” are part of a diversity of pathways including immunity, cell signaling, cytoskeleton organization and more (Table 4.5).

Table 4.5. Supraesophageal Ganglion: protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and presence at different timepoints during parasitism. During at least one timepoint each protein below was not found in detectable amounts in control caterpillars, but was detectable in all three parasitism samples.

	Supraesophageal Ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Homologous protein family**	**BP GO Terms**
Attacin-1	*	*	*	*	Antimicrobial peptide	Innate immune response
Pro-clotting enzyme	*	*	*	*	Peptidase S1A, chymotrypsin	Proteolysis
Probable chitinase 10	*	*	*	*	Glycoside hydrolase superfamily	Chitin catabolic process
Probable very-long-chain enoyl-CoA reductase art-1	*	*	*	+	3-oxo-5 alpha-steroid 4-dehydrogenase	Very long-chain fatty acid biosynthetic process
Egl nine homologue 1	*	*	+	+	Hypoxia inducible factor	Cellular response to hypoxia
TAR DNA-binding protein	*	+	*	*	RNA binding domain superfamily	Response to oxidative stress
7-methylguanosine phosphate-specific 5'-nucleotidase	*	+	+	+	Pyrimidine 5'nucleotidase	Riconucleoside monophosphate catabolic
DNA-directed RNA polymerase subunit 1	*	+	+	+	DNA-directed RNA pol I, largest subunit	Transcription by RNA polymerase I
GPI transamidase component PIG-S	*	+	+	=	Phophatidylinositol-glycan biosynthesis	GPI anchor biosynthetic process
Protein rogdi	*	=	*	=	RAVE subunit 2	Gamma-aminobutyric acid secretion. neurotransmission
Tyrosine-tRNA ligase, mitochondrial	*	=	+	+	Tyrosine-tRNA ligase	tRNA aminoaculation
Probable alpha-aspartyl dipeptidase	+	*	+	=	Peptidase S51	Proteolysis
Target of rapamycin complex subunit lst8	=	*	=	+	Target of rapamycin complex subunit	TOR signaling
Viral IAP-associated factor homolog	=	*	+	=	Thioredoxin-like superfamily	Apoptotic process
Titin-like	=	+	*	*	Immunoglobin-like domain	Signal transduction
Neurochondrin homolog	=	=	*	*	Neurochondrin-like family	Neuron projection development
Filamin-A	=	=	*	*	Filamin family	Actin cytoskeleton organization

* Present in 3/3 Parasitized samples, Absent in 3/3 Controls
\+ Present in 2/3 Parasitized samples, Absent in 3/3 Controls
= Present in 3/3 Parasitized samples, Present in 3/3 Controls

Table 4.5. continued

	Supraesophageal Ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Homologous protein family**	**BP GO Terms**
Teneurin-a	=	+	+	*	TolB-like superfamily	Synapse organization
ATP synthase subunit s	=	+	+	*	ATP synthase, mitochondrial family	ATP synthesis coupled proton transport
Conserved oligomeric Golgi complex subunit 7	=	=	+	*	Conserved oligomeric Golgi complex	dsRNA transport
Methanethiol oxidase	=	+	*	=	Selenium-binding protein	Protein transport
UDP-glucuronosyltransferase 2	=	=	*	=	Ecdysteroid UDP-glucosyltransferase	NA

* Present in 3/3 Parasitized samples, Absent in 3/3 Controls
\+ Present in 2/3 Parasitized samples, Absent in 3/3 Controls
= Present in 3/3 Parasitized samples, Present in 3/3 Controls

Many proteins were detectable in both control and parasitized caterpillars, but were detected in higher amounts in parasitized caterpillars than in their control counterparts (Table. 4.6). The three proteins with the largest increase seen in parasitized caterpillars versus control caterpillars are listed below. Although the top proteins differ between timepoints of parasitism, a common theme is that the majority play a role in immunity and cellular cytoskeleton.

Table 4.6. Top three most increased proteins present in the supraesophageal ganglion of parasitized caterpillars.

	Protein	Function
Pre-emergence		
	Modular serine protease-like	Involved in innate immunity via Toll pathway (Buchon Nicolas et al., 2009)
	pre-mRNA-splicing factor SPF27	Component of multi-protein spliceosome complex (Chen et al., 2013)
	Gloverin	AMP regulated by Toll-Spatzle pathway (Xu et al., 2012)
Emergence		
	Rho guanine nucleotide exchange factor 28	Immune related protein involved with Rho GTPases (Wang et al., 2019)
	Cysteine and histidine rich domain containing protein	Unknown
	aladin-like	Nuclear pore protein (Carvalhal et al., 2015)
1-Day Post		
	Myosin regulatory light chain 2	Part of the myosin II complex (Park et al., 2011)
	Gloverin	AMP regulated by Toll-Spatzle pathway (Xu et al., 2012)
	calcium-transporting ATPase endoplasmic reticulum type	Ion transport
3-Days Post		
	Hemolin	Cellular immune response (Eleftherianos et al., 2007)
	Gloverin	AMP regulated by Toll-Spatzle pathway (Xu et al., 2012)
	Myosin regulatory light chain 2	Part of the myosin II complex (Park et al., 2011)

Using the GO slim drosophila from the fly base ribbon to group the gene ontology terms, the top three ancestral GO terms for each time point was established (Table 4.7).

Table 4.7. Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon

	1st GO slim	2nd GO slim	3rd GO slim
Pre-emergence	Protein Metabolic process	Response to Stimulus	Cellular component organization
Emergent	Protein Metabolic process	Response to stimulus	NA
1-Day Post	Protein Metabolic process	Response to stimulus	Cellular component organization
3-Days Post	Response to stimulus	Protein metabolic process	Cellular component organization

In addition to proteins being present in higher quantities in parasitized caterpillars, there was also an abundance of proteins that either fall below detection thresholds, or substantially decreased in the supraesophageal ganglion of parasitized caterpillars when compared to control caterpillars (Table 4.8). As with the proteins that were increased in parasitized brains, the proteins found to be absent in parasitized caterpillars were also dependent on the timepoint at which the brains were harvested. There are many more proteins that fall into this “absent” category than into the “novel” category.

Table 4.8. Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and absence at different timepoints during parasitism.

	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
Hemicentin 1-like	/	=	-	/	Immunoglobin-like domain superfamily	Immune response
G protein-activated inward rectifier potassium channel 3	/	=	=	-	Potassium channel, Kir	Ion transport
Breast carcinoma-amplified sequence 3 homolog	/	=	=	=	BCAS3-like	autophagy
Alpha/beta hydrolase domain-containing protein 17B	/	=	=	=	Alpha/Beta hydrolases	Regulation of post-synpase organization
Serine protease inhibitor 28Dc	/	=	/	-	Serpin family	Immune system process
CBP8-/2--dependent translation initiation factor	/	=	/	=	Armadillo-like helical superfamily	Translational initiation
and polyadenylation specific factor 73	/	=	/	=	Ribonuclease Z superfamily	mRNA cleavage

	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
STE2--related kinase adapter protein alpha	=	/	/	/	Protein kinase-like family	Protein phosphorylation
Modular serine-protease-like	=	/	/	/	Peptidase S1A, chymotrypsin family	Proteolysis
Protein furry	=	/	/	/	Protein furry/Tao3/Mor2 family	Cell morphogenesis
Immulectin-3	=	/	/	/	C-type lectin superfamily	Immune response
Protein obstructor-E	=	/	/	/	Chitin binding superfamily	Chitin-based cuticle development
Chymotrypsin-1	=	/	/	=	Peptidase S1A, chymotrypsin family	Proteolysis
Serine protease nudel-like	=	/	-	-	Peptidase S1A, chymotrypsin family	Proteolysis
Hemicentin-2-like	=	/	-	-	VonWillebrand factor A-like superfamily	Immune response
Guanine nucleotide-binding protein	=	/	=	-	Ras GTPase GNL1-like	Immune response
Serine protease 28	=	/	=	-	Peptidase S1A, chymotrypsin family	Proteolysis

	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
Xaa-Pro aminopeptidase 3-like	=	/	=	=	Creatinase/aminopeptidase-like	Proteolysis
Alkaline phosphatase	=	/	=	=	Alkaline phosphatases	Dephosphorylation
Kazrin-like	=	/	=	=	Kazrin	Histone methylation
Tyrosine-protein kinase transmembrane receptor	=	/	=	=	Protein kinase-like domain superfamily	Protein phosphorylation
Wiskott-Aldrich syndrome protein family member	=	/	=	=	SCAR/WAVE family	Cytoskeleton organization
RNA-binding protein 25	=	/	=	=	PWI domain superfamily	mRNA processing
ralA-binding protein 1	=	/	=	=	RalA-binding protein	Signal transduction
Serine/threonine-protein kinase26	=	/	=	=	Protein kinase-like family	Protein phosphorylation
GATOR complex protein MIOS-B	=	/	=	=	MIOS family	Signal transduction
	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**

	Supraesophageal ganglion					
	Pre	Emergent	1-Day Post	3-Days Post	Protein family	BP GO Terms
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
Peroxisomal biogenesis factor 19	=	/	=	=	Pex19 protein family	Protein import into peroxisome
Protein unc-45 homolog	=	/	=	=	Armadillo-like helical superfamily	Cell differentiation
Peroxidasin	=	/	=	=	Haem peroxidase family	Response to oxidative stress
dnaJ homolog shv	=	=	/	=	HSP4- homologous superfamily	Protein folding
Dynactin	=	=	/	=	Dynactin family	Axonal transport
Plasminogen activator inhibitor 2	=	=	/	=	Serpin family	Immune response
Rap1 GTPase-GDP dissociation stimulator 1	=	=	/	=	Rap1 GTPase dissociation stimulator 1	Positive regulation of GTPase
Exosome complex component RRP46	=	=	/	=	PNPase homologous superfamily	Mushroom body development
ATP-dependent Clp protease	=	=	/	=	P-loop containing nucleoside TP hydrolase superfamily	Proteolysis
Lysosomal Pro-X carboxypeptidase-like	=	=	/	=	Peptidase S28	Proteolysis

	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
U6 snRNA-associated Sm-like protein	=	=	/	=	Like-Sm (LSM) domain containing protein	RNA processing
Bifunctional 3-phosphoadenosine 5	=	=	/	=	Adenylyl-sulfate kinase	Sulfate assimilation
Tyramine beta-hydroxylase	=	=	/	=	Tyramine beta-hydroxylase	Octopamine signaling pathway
Transforming acidic coiled-coil-containing protein	=	=	/	=	TACC family	Mitotic spindle organization
CLIP domain containing serine protease 2	=	=	/	=	Peptidase S1A, chymotrypsin family	Proteolysis
Immunolectin-A precursor	=	=	/	=	C-type lectin superfamily	Immune response
	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Pancreatic triacylglycerol lipase-like	=	=	/	=	Triacylglycerol lipase family	Lipid metabolic process

	Supraesophageal ganglion					
	Pre	Emergent	1-Day Post	3-Days Post	Protein family	BP GO Terms
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
Excitatory amino acid transporter 3	=	=	/	=	Sodium-carboxylate symporter	Chemical synaptic transmission
Serine protease inhibitor 42Dd-like	=	-	/	=	Serpin family	Immune response
Serine/threonine-protein kinase PAK 3	=	=	/	/	Protein kinase like superfamily	Protein phosphorylation
D-2-hydroxyglutarate dehydrogenase	=	=	/	/	FAD-linked oxidase family	Organic acid metabolic process
Sister chromatid cohesion protein PDS5 homolog	=	=	=	/	Sister chromatid cohesion protein PDS5	Chromosome organization
Delta(24)-sterol reductase	=	=	=	/	Delta(24)-sterol reductase	Ras signal transduction
Acid sphingomyelinase-like	=	=	=	/	Metallo-dependent phosphatase-like family	Nucleoside triphosphate catabolic process
protein arginine N-methyltransferase 1	=	=	=	/	Protein arginine N-methyltransferase	Peptidyl-arginine methylation
Digestive cysteine proteinase-2	=	=	=	/	Papain-like cysteine peptidase superfamily	Proteolysis

	Supraesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Alpha amylase 1-like	/	/	/	/	Alpha amylase family	Carbohydrate metabolic process
Lymphocyte antigen 75-like	/	/	/	/	C-type lectin-like superfamily	endocytosis
ATPase family protein 2 homolog	/	-	/	-	P-loop containing nucleoside TP hydrolase superfamily	Negative regulation of protein kinase
High mobility group protein D-like	/	-	=	=	High mobility group box domain superfamily	DNA binding
Esterase GA18864	/	=	-	-	Alpha/Beta hydrolase fold superfamily	
Immulectin-2	/	=	-	-	C-type lectin-like superfamily	Immune response
TWiK family of potassium channels protein 7	/	=	-	/	Two pore domain potassium channel family	Ion transport
DNA replication licensing factor Mcm7	=	=	=	/	Mini-chromosome maintenance protein	DNA replication
N-acetylgalactosaminyltransferase 7	=	=	=	/	Nucleotide-diphospho-sugar transferase	Oligosaccharide biosynthetic process
Vesicle transport protein SEC2-	=	=	=	/	Sec2- glycoprotein and SNARE	Retrograde vesicle-mediated transport
Jupiter microtubule associated homolog-1	=	=	=	/	Microtubule associated protein	
Spermine synthase	=	=	=	/	Spermine synthase	Polyamine biosynthetic process
Clustered mitochondria protein homolog	=	-	-	/	CLU domain containing protein	Organelle organization

Some proteins were again detectable in both control and parasitized caterpillars, but in this case were detected in higher amounts in control caterpillars than in parasitized brains (Table. 4.9). The three proteins with the biggest decrease seen in parasitized caterpillars versus control caterpillars are listed below. Proteins that tended to be present in much lower quantities at a given timepoint in late parasitism do not appear to belong to any one process or function. There are several involved in transcription, some with signaling and others transport.

Table 4.9. Top three most reduced proteins present in the supraesophageal ganglion of parasitized caterpillars.

	Protein	Function
Pre-emergence		
	arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2-like	GTPase-activating protein (Zhang et al., 2003)
	pancreatic lipase-related protein 2-like	Lipid metabolism (Persson et al., 1989)
	7-methylguanosine phosphate-specific 5-nucleotidase	Hydrolyzes m_7GMP, mRNA modification process (Buschmann et al., 2013)
Emergence		
	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E	ATP-dependent annealing helicase that catalyzes the rewinding of the stably unwound DNA (Bétous et al., 2012)
	nucleosome assembly protein 1-like	Histone chaperone (Andrews et al., 2010)
	rogdi	Sleep pathway, metabotropic transmission of GABA (Kim et al., 2017)
1-Day Post		
	arylphorin beta subunit precursor	Larval storage protein, ecdysteroid carrier (Willott et al., 1989)
	arylphorin subunit alpha	Larval storage protein, ecdysteroid carrier (Willott et al., 1989)
	atlastin-like	Endoplasmic reticulum tubular network biogenesis (Orso et al., 2009)
3-Days Post		
	protein canopy homolog 3	Toll-like receptor signaling (Morales and Li, 2017)
	inositol monophosphatase 1	Neuron signal transduction (Qazi and Trimmer, 1999)
	ommochrome-binding protein	Chromophore transport (Martel and Law, 1991)

Using the GO slim drosophila from the fly base ribbon to group the gene ontology terms, the top three ancestral GO terms for each time point was established (Table 4.10).

Table 4.10. Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon

	1st GO slim	2nd GO slim	3rd GO slim
Pre-emergence	Protein metabolic process	Response to Stimulus	Establishment of localization
Emergent	Protein metabolic process	Response to Stimulus	Gene expression
1-Day Post	Protein metabolic process	Response to Stimulus	Gene expression
3-Days Post	Protein metabolic process	Response to Stimulus	Immune system process

Subesophageal Ganglion

In total there were 41 proteins with differential presence in pre-emergent caterpillars, 95 in emergent caterpillars, 76 in 1-Day Post caterpillars and 60 in 3-Days post. These proteins were identified to be either below detection threshold in parasitized caterpillars (absent), decreased amounts in parasitized caterpillars (decrease), present in parasitized caterpillars but below detection threshold in control (novel), or increased amounts in parasitized caterpillars (increase). The differential amounts of proteins and the trend of dysregulation was time-point dependent (Figure 2).

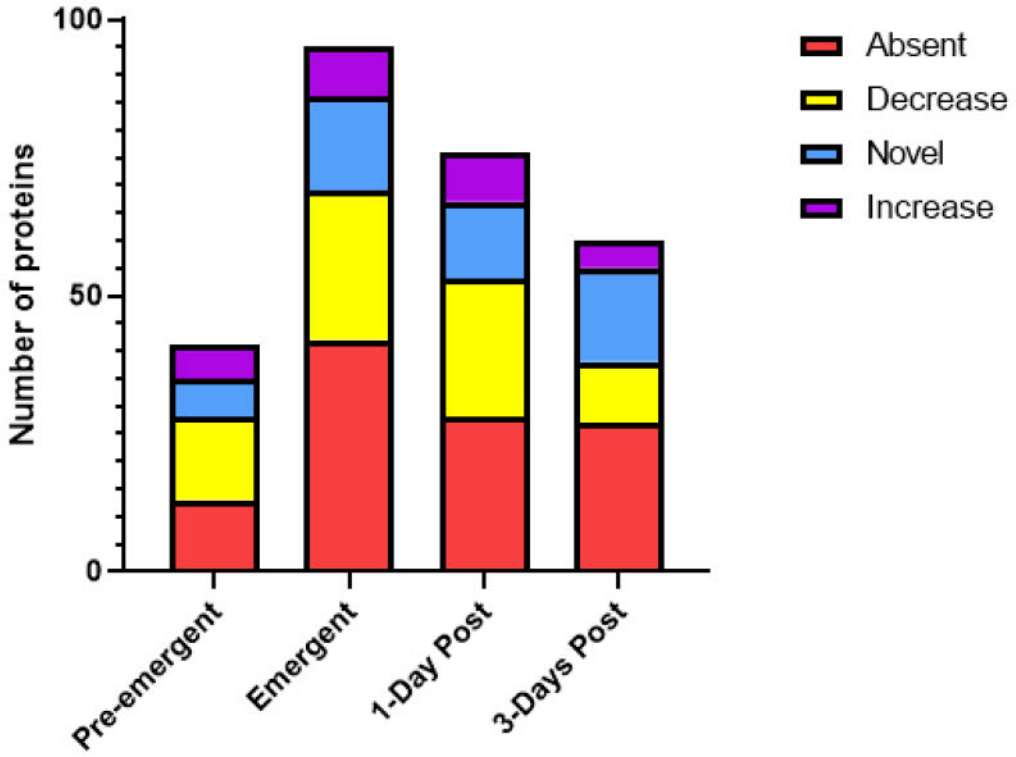

Figure 4.2. Differential presence of proteins within the subesophageal ganglion of *Manduca sexta* caterpillars that have been parsitized by *Cotesia congregata*. The data show the number of proteins found to be in different concentrations when compared to uninfected control caterpillars.

In contrast to the supraesophageal ganglion, in the subesophageal ganglion the most differential protein pattern is seen at the emergent time point. The subesophageal ganglion of pre-emergent caterpillars had overall fewer proteins that were found to be in differing concentrations than those of control caterpillars, indeed less than half of what was seen in the supraesophageal ganglion.

Examining the proteins that are detectable in at least one time point of the subesophageal ganglion of parasitized caterpillars, while appearing to be absent, or in much lower abundance in control caterpillars there was once again a large diversity in function (Table 4.11).

Table 4.11. Subesophageal ganglion: Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and presence at different timepoints during parasitism.

	Subesophageal ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BF GO Terms**
Probable chitinase 10	*	*	*	*	Glycoside hydrolase superfamily	Chitin catabolic process
Signal recognition particle 19kDa	*	*	*	*	Signal recognition particles SRP19	Protein targeting to membrane
Pyruvate dehydrogenase kinase	*	*	+	+	Alpha/Beta hydrolase fold	Proteolysis
Protein numb	*	+	*	*	PH-like domain superfamily	Glial cell migration
Inactive didpeptidyl peptidase	*	*	+	+	Peptidase like	Proteolysis
Methionine aminopeptidase 1	*	+	+	+	Peptidase M24 subfmaily	Proteolysis
brahma-associated protein of 60 kDa	+	*	*	*	SWI/SNF-related matrix associated regulator of chromatin	Positive regulation of gene expression
Coatomer subunit gamma-2 like	+	*	+	=	Coatomer subunit gamma	Intracellular protein transport
MAGUK p55 subfamily member 5	+	*	*	+	MAGUK p55 subfamily	Tight junction assembly
Methionine-R-sulfoxide reductase B1	=	*	+	*	Peptide methionine sulfoxide reductase	Response to oxidative stress
Peroxidase-like	=	*	=	+	Haem peroxidase	Response to oxidative stress
pre-mRNA-splicing factor SPF27	=	*	+	+	Pre-mRNA-splicing factor	mRNA processing
protein N-terminal asparagine amidohydrolase	+	*	+	=	protein N-terminal asparagine amidohydrolase	Protein modification process
putative ferric-chelate reductase 1 homolog	+	*	*	=	Reeler domain	Iron ion homeostasis
putative pre-mRNA-splicing factor ATP-dependent RNA helicase PRP1	+	*	+	+	P-loop containing nucleoside triphosphate hydrolase	mRNA processing
putative serine protease K12H4.7	+	*	+	+	Peptidase S28	Innate immune response
cholinesterase 2-like	+	+	*	+	Alpha/Beta hydrolase homologos superfamily	
DNA topoisomerase 3-beta-1	=	+	*	*	Type IA DNA topoisomerase	DNA topological change
Glutenin	=	=	*	*	Kazal domain	Endopeptidase regulator
rap1 GTPase-GDP dissociation stimulator 1-B	+	+	*	+	Rap1 GTPase dissociation stimulator 1	Positive regulation of GTPase
glycerol kinase	+	+	+	*	Carbohydrate kinase	Carbohydrate metabolic process

	Subesophageal ganglion					
	Pre	Emergent	1-Day Post	3-Days Post	Protein family	BF GO Terms
homeobox protein extradenticle	=	=	=	*	Homeobox-like domain superfamily	Regulation of transcription
cullin-3	+	+	+	*	Cullin	Ubiquitin dependent catabolism
pre-mRNA-processing factor 6	=	=	=	*	Pre-mRNA-splicing factor Syf1-like	mRNA splicing via spliceosome
protein diaphanous	=	=	=	*	Formin, protein diaphanous	Actin cytoskeleton organization
S-phase kinase-associated protein 1	+	+	+	*	S-phase kinase-associated protein	Ubiquitin-dependent protein catabolism

* Present in 3/3 Parasitized samples, Absent in 3/3 Controls
\+ Present in 2/3 Parasitized samples, Absent in 3/3 Controls
= Present in 3/3 Parasitized samples, Present in 3/3 Controls

There were also proteins present in the subesophageal that were present in higher quantities in the subesophageal of parasitized caterpillars when compared to control caterpillars. Timepoint within parasitism still appeared to play a dynamic role in which proteins were being produced in higher amounts (Table 4.12). One particular protein, microtubule-associated protein futsch, is in the top three proteins with the highest increase relative to control in three out of four parasitized groups (emergence, 1-day post and 3-day post).

Table 4.12 Top three biggest increases in proteins present in the subesophageal ganglion of parasitized caterpillars.

	Protein	Function
Pre-emergence		
	methionine-rich storage protein 3	Storage protein that only appears in the final instar (Wang et al., 1993)
	modular serine protease-like	Involved in innate immunity via Toll pathway (Buchon Nicolas et al., 2009)
	hemolin	Cellular immune response (Eleftherianos et al., 2007)
Emergence		
	xenotropic and polytropic retrovirus receptor 1 homolog	Membrane receptor, phosphate transport (Giovannini et al., 2013)
	4-coumarate--CoA ligase 1-like	Phenylpropanoid metabolism (Steinhaus, 1959)
	microtubule-associated protein futsch	Regulates neurotransmitter release and active zone density (Lepicard et al., 2014)
1-Day Post		
	cysteine sulfinic acid decarboxylase	Decarboxylation involved in producing GABA, B-alanine and hypotaurine (Liu et al., 2013)
	unconventional myosin ID	Adherens junction component, cell-cell and signaling platforms (Petzoldt et al., 2012)
	microtubule-associated protein futsch	Regulates neurotransmitter release and active zone density (Lepicard et al., 2014)
3-Days Post		
	microtubule-associated protein futsch	Regulates neurotransmitter release and active zone density (Lepicard et al., 2014)
	cysteine sulfinic acid decarboxylase	Decarboxylation involved in producing GABA, B-alanine and hypotaurine (Liu et al., 2013)
	sodium/calcium exchanger regulatory protein 1-like	Intracellular lipid binding protein

Using the GO slim drosophila from the fly base ribbon to group the gene ontology terms, the top three ancestral GO terms for each time point was established (Table 4.13).

Table 4.13 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon

	1st GO slim	2nd GO slim	3rd GO slim
Pre-emergence	Protein metabolic process	Developmental process	Carbohydrate metabolic process
Emergent	Protein metabolic process	Establishment of localization	Gene expression
1-Day Post	Protein metabolic process	Gene expression	Cellular component organization
3-Days Post	Cellular component organization	Gene expression	Carbohydrate metabolic process

The subesophageal ganglion of caterpillars that have been parasitized also showed patterns of proteins that fall to amounts below the concentration thresholds of our techniques. These proteins are detectable in control caterpillars, and in some cases even different time points of late stage parasitism. These proteins are involved in a variety of pathways including, but not limited to, synaptic transmission, DNA transcription/mRNA translation and cytoskeleton organization (Table 4.14).

Table 4.14. Protein identity, homologus family/superfamily, biological process (BP) Geneontology terms (GO) and absence at different timepoints during parasitism.

	Subesophageal Ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
Ras-related protein Rab-9A	/	/	/	/	Small GTPase	Protein transport
Probable GPI-anchored adhesin-like protein	/	/	/	=	Methylcrotonoyl-CoA carboxylase	Cell adhesion
probable methylcrotonoyl-CoA carboxylase beta chain	/	/	/	=	MCCB/AccD1-like	Leucine catabolic process
Trypsin, alkaline C	/	/	=	/	Peptidase S1A	Proteolysis
X-ray repair cross-complementing protein 6	/	/	=	=	Ku70	Telomere maintenance
Dual specificity protein phosphatase 13	/	=	=	=	Atypical dual specificity phosphatases	Protein dephosphorylation
GILT-like protein 1	/	=	=	=	Gamma interferon inducible lysosomal thiol reductase	Immune response
Golgin subfamily A member 2	/	=	=	=	Golgin subfamily	Golgi organization
15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like	/	=	/	=	Short chain dehydrogenase	Response to LPS
beta-glucuronidase	=	-	/	=	Glycoside hydrolase family	Carbohydrate metabolic process
brachyurin-like	=	/	-	-	Peptidase S1A, chymotrypsin family	Proteolysis
probable NADH dehydrogenase [ubiquinone] 1 alpha subcomplex	=	/	-	-	NADH dehydrogenase [ubiquinone]	Mitochondrial respiratory chain complex I aseembly
probable pterin-4-alpha-carbinolamine dehydratase	=	/	-	-	pterin-4-alpha-carbinolamine dehydratase	Alcohol metabolic process

	Subesophageal Ganglion					
	Pre	Emergent	1-Day Post	3-Days Post	Protein family	BP GO Terms
Alpha amylase 1-like	=	/	/	=	Alpha amylase family	Carbohydrate metabolic process
chymotrypsin-2-like	=	/	/	=	Peptidase S1A, chymotrypsin family	Proteolysis
juvenile hormone epoxide hydrolase-like	=	/	/	/	Epoxide hydrolases	Aromatic catabolic process
putative C1A cysteine protease precursor	=	/	/	/	Papain-like cysteine peptidase	Proteolysis
Hemicentin 1-like	-	/	=	=	Immunoglobin-like domain superfamily	Immune response
acylglycerol kinase	=	/	=	-	NAD kinase/ diaculdlycerol kinase	Glycerolipid metabolic process
calphotin-like	=	/	=	=	Protein MGARP	Anterograde axonal transport
cold shock domain-containing protein E1	=	/	=	=	Cold-shock protein	mRNA stabilization
dystrophin	=	/	=	-	Dystrophin/utrophin	Actin cytoskeleton organization
filamin-A	=	/	=	=	Filamin family	Actin cytoskeleton organization
Immulectin-3	=	/	=	=	C-type lectin superfamily	Immune response
N-acetylgalactosaminyltransferase 7	=	/	=	=	Nucleotide-diphospho-sugar transferase	Oligosaccharide biosynthetic process
nuclear valosin-containing protein-like	=	/	=	=	P-loop containing nucleoside tp hydrolase	Cellular response to LPS
obscurin	=	/	=	-	Immunoglobin-like domain superfamily	Protein phosphorylation
syntaxin-1A	=	/	=	-	Syntaxin	Vesicle-mediated transport
beta-fructofuranosidase 1	=	=	/	=	Glycoside hydrolase family	Carbohydrate metabolic process
excitatory amino acid transporter 3	=	=	/	=	Sodium:dicarboxylate symporter	Chemical synaptic transmission
general transcription factor IIF subunit 1	=	=	/	=	general transcription factor IIF	Transcription by RNA polymeraseII
Immunolectin-A precursor	=	=	/	/	C-type lectin superfamily	Immune response
Protein yellow	=	=	/	/	Major royal jelly protein/protein yellow	Melanin biosynthetic process
splicing factor 3B subunit 6	=	=	/	/	RNA-binding domain superfamily	mRNA splicing via spliceosome

	Subesophageal Ganglion					
	Pre	**Emergent**	**1-Day Post**	**3-Days Post**	**Protein family**	**BP GO Terms**
probable serine/threonine-protein kinase kinX	=	-	-	/	Chitin binding superfamily	Cell adhesion
serine/threonine-protein kinase tricorner	=	=	=	/	Protein kinase-like superfamily	Protein phosphorylation
tyrosine-protein phosphatase Lar	=	-	=	/	Immunoglobulin-like homologous superfamily	Protein dephosphorylation
CD109 antigen	=	=	-	/	Terpenoid cyclase homologous superfamily	Regulation of endopeptidase
patronin-like	=	=	=	/	Calmodulin-regulated spectrin protein	Neuron projection development
vacuolar protein sorting-associated protein 28	=	-	-	/	Vacuolar protein sorting	Endosome transport
CRAL-TRIO domain-containing protein	-	=	-	/	CRAL-TRIO lipid binding superfamily	Axon guidance

= Present in 3/3 Parasitized samples, Present in 3/3 Controls
- Absent in 2/3 Parasitized samples, Present in 3/3 Controls
/ Absent in 3/3 Parasitized samples, Present in 3/3 Controls

In addition to the proteins that were absent in parasitized caterpillars, there were also a number of proteins that were found in lower abundance in the subesophageal ganglion of parasitized caterpillars. Once again the pattern of these proteins was timepoint dependent, with some being drastically reduced at one timepoint in parasitism, while seemingly returning to control levels in others (Table 4.15).

Table 4.15 Top three decreases in proteins present in the subesophageal ganglion of parasitized caterpillars.

	Protein	Function
Pre-emergence		
	carbonic anhydrase 1	Carbon dioxide to water and carbonic acid (Jungreis et al., 1981)
	calphotin-like	Starch and sucrose metabolism
	beta-fructofuranosidase 1	Polysaccharide hydrolases (Gan et al., 2018)
Emergence		
	hemicentin-1	Extracellular immunoglobulin, cell attachments (Vogel and Hedgecock, 2001)
	insulin-like growth factor-binding protein	IGF signaling pathway (Lee et al., 2018)
	chymotrypsin-1-like	Serine endopeptidase (Rawlings and Barrett, 1994)
1-Day Post		
	immulectin-2	Innate immunity, prophenoloxidase pathway (Yu and Kanost, 2003)
	protein yellow-like	Melanin biosynthetic process
	calphotin-like	Starch and sucrose metabolism
3-Days Post		
	DENN domain-containing protein Crag	Basement membrane modeling (Isabella and Horne-Badovinac, 2015)
	aldo-keto reductase AKR2E4-like	Ecdysteroid signaling (Yamamoto et al., 2017)
	probable 4-coumarate--CoA ligase 3	Coumaric acid degradation (Steinhaus, 1959)

Using the GO slim drosophila from the fly base ribbon to group the gene ontology terms, the top three ancestral GO terms for each time point was established (Table 4.16).

Table 4.16 Top three ancestral GO terms assigned using the GO slim drosophila from the flybase ribbon

	1st GO slim	2nd GO slim	3rd GO slim
Pre-emergence	Protein metabolic process	Establishment of localization	Response to stimulus
Emergent	Protein metabolic process	Cellular component organization	Establishment of localization
1-Day Post	Protein metabolic process	Response to stimulus	Carbohydrate metabolic process
3-Days Post	Cellular component organization	Response to stimulus	Establishment of localization

UNCHARACTERIZED PROTEINS

Even with the sequencing of the *Manduca sexta* genome, there are still sequences that remain to be annotated. This resulted in a high number of uncharacterized proteins. Similarly to what we have seen with the proteins that can be identified, some of these uncharacterized proteins do seem to differ between parasitized and unparasitized caterpillars (Figure 4.3).

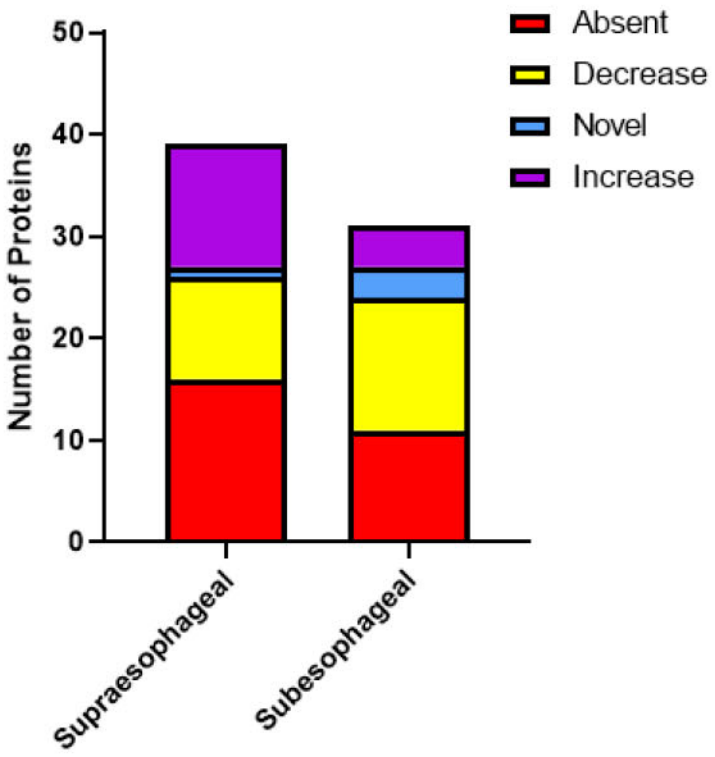

Figure 4.3 Uncharacterized proteins that are present in differing amounts in parasitized caterpillars when compared to control caterpillars.

COTESIA CONGREGATA BRACOVIRUS PROTEINS

By comparing our proteomics output to the annotated genome of *Cotesia congregata* and its bracovirus (CcBV) it was possible to identify viral proteins being produced in the supraesophageal ganglion, as well as the subesophageal ganglion. Interestingly, which proteins were present depended on the stage of parasitism. Some viral proteins appeared in our pre-emergence group and remained detectable through to our 3-Days Post parasite emergence group (Figure 4.4). Others only passed detection thresholds in our post-emergence groups (Figure 4.4).

	Supraesophageal				Subesophageal			
	Pre	Emergent	1-Day Post	3-Days Post	Pre	Emergent	1-Day Post	3-Days Post
CcBV_30.1	+	+	+	+	-	-	-	-
EP2-1	+	+	-	+	-	-	-	-
BV7-6	+	+	+	+	-	-	-	-
CcPL9.004	+	+	+	+	+	+	+	+
EP1-3	+	+	-	+	+	+	+	+
CcV1	+	+	+	+	+	+	+	+
CcBV_31.9	-	+	-	-	-	-	-	-
Cystatin	+	+	+	+	+	+	+	+
BV7-1	-	+	+	+	-	-	-	-
CcPL4.001	-	+	+	+	-	-	-	-

Figure 4.4. Heatmap of bracovirus proteins detected at the different timepoints of parasitism in both the supraesophageal ganglion and the subesophageal ganglion. Green cells (+) indicate presence of protein, pink cells (-) indicate absence of protein.

COTESIA CONGREGATA BRACOVIRUS MRNA

Detecting bracovirus proteins within the central nervous system of *Manduca sexta* was a novel finding. Previous research has found that post-parasitism, virally encoded proteins can make up 15% or more of the proteins found in hemolymph (Amaya et al., 2005; Harwood and Beckage, 1994). This, coupled with the fact that insects possess a barrier called the perineurium (akin to the vertebrate blood-brain barrier) posed the possibility, however small, that the proteins we were seeing were merely blood based proteins adhering to the perineurium and not being expressed within the neural tissue at all. As such, further experiments were undertaken to confirm whether their presence within the tissue was legitimate or the results of possible contamination. As such, supraesophageal ganglion were extracted and the mRNA was extracted from 5 caterpillars from the control (unparasitized), pre-emergence, emergent, and post-emergent groups.

Realtime q-PCR analysis was conducted using primers for the genes for *EP1-3*, *Cystatin,* and *CcV1* (CcV1 is the the *C. congregata* (CcBV) homolog of *C. rubecula* (CrBV) in which this protein was originally annotated). Amplification was found in all of the parasitized groups for our three genes of interest, whereas no amplification was seen in the control groups (Figure 4.4). This result was further replicated in an additional experiment where the supraesophageal ganglion of 4 parasitized post-emergent caterpillars was excised, washed, and then had the inner nervous tissue excised through

a small gauged needle, leaving behind the outer sheath that would have come in contact with the hemolymph. Once again, this was done to minimize possible contaminants from the body of the parasitized caterpillars. Realtime qPCR was conducted using genes for *EP1-3*, *Cystatin,* and *CcV1*. Once again, all four samples had amplification for our genes of interest (Figure 4.4). This evidence supports the indication that viral mRNA is being transcribed and viral proteins are being produced within the CNS of parasitized caterpillars.

	EP1-3	*Cystatin*	*CcV1*
Control	-	-	-
Pre-emergence	+	+	+
Emergent	+	+	+
Post-emergence	+	+	+
Post-emergence -outer sheath	+	+	+

Figure 4.5. Amplification of viral transcripts was observed for all three primer pairs in the supraesophageal ganglion of all parasitized caterpillars, regardless of timepoint. No amplification was seen in the supraesophageal tissues of non-parasitized controls.

4.6 DISCUSSION

Proteomic and RNA profiles

Parasitized caterpillars continue to develop at a normal rate gaining mass and head capsule size at a similar rate to unparasitized caterpillars (Beckage and Templeton, 1986), with the caveat that after the 5th instar, parasitized caterpillars grow more slowly (Alleyne et al., 1997). Conducting a Bradford analysis of total soluble protein revealed that even before behavioural changes occurred in the caterpillar host, there was a significant reduction in total protein in parasitized caterpillar brains compared with brains from controls. This was found to be true of total RNA and cDNA as well, with significant reductions being found between control caterpillars and parasitized caterpillars even prior to wasp larval emergence (i.e. prior to host behavioural change).This indicates that although the parasitized caterpillars continue to grow and proceed through their instars in an apparently normal fashion (with the exception of during the 5th instar; (Alleyne et al., 1997), their brains grow more slowly for the size and age of the caterpillar. It should be noted that this reduction in brain development occurs while the caterpillar is still actively feeding (i.e. in pre-emergence caterpillars, the reduction in brain protein cannot be due to a decline in feeding), meaning that a starved control group was not necessary.

Previous work on the *Cotesia-Manduca* parasitic system has revealed that there are significant changes to overall metabolism within the host immediately after parasitization (Alleyne and Beckage, 1997; Alleyne et al., 1997). The wasp larvae must walk a fine line between enabling host survival and siphoning sufficient energy for its own growth. On the one hand, the wasp larvae must keep their host in robust condition so that it can avoid predation. This results in parasitized caterpillars differing in their intermediate metabolism compared with unparasitized weight-matched control caterpillars (Alleyne and Beckage, 1997). Studies on parasitized *M. sexta* have found that parasitized caterpillars have only ⅙ of storage proteins such as arylphorin circulating in their hemolymph when compared to unparasitized control caterpillars (Beckage and Kanost, 1993). In terms of direct physiological mechanism, the decline in storage proteins in parasitized animals is presumably associated with the maintenance of elevated juvenile hormone titres, which normally decline to undetectable levels in last-instar control animals. Indeed, the expression of lepidopteran storage proteins during the last instar is known to be repressed until JH levels drop. In this respect, it is interesting to note that levels of the Juvenile Hormone Esterase Hypoxide protein were

lower in emergent and post-emergent parasitized subesophageal ganglia than in those of controls (Table 4.14). Parasitism by *Cotesia congregata* also results in atrophy of testes as well as reduction in germline cells of its host (Reed and Beckage, 1997). Redistribution of resources is more than likely the key behind the decline in storage proteins and atrophy of testes. The reduced protein content of the brain suggests that the CNS is not spared from such redistribution of resources.

From personal observation during extraction of the supraesophageal and subesophageal ganglia, ganglia from parasitized caterpillars appeared more translucent, possibly being caused by less lipid within the ganglia. Again, this could be a result of redistribution of resources to accommodate the parasitic larva. There were no significant differences in total RNA or cDNA across different parasitized time points (i.e., pre-emergence caterpillars that were not yet exhibiting behavioural change were not significantly different from any post-emergence group), suggesting that the brain is maintained in terms of genomic and protein content after the wasps emerge, despite the lack of feeding.

Protein Detection and Trends

Overall, in both the supraesophageal ganglion and the subesophageal ganglion we were able to identify approximately 2500 proteins. Of these, approximately 1.5-4% were altered, depending on the stage of parasitism. Proteomic analyses conducted on the CNS of other parasitic systems that result in behavioural change of the host, such as the jewel wasp-cockroach system and the grasshopper-hairworm system had a similar result with approximately 2% of proteins being present in significantly different quantities in their parasitized groups (Biron et al., 2005; Kaiser et al., 2019).

The subesophageal ganglion of pre-emergence caterpillars exhibited the fewest differences in protein concentration changes when compared to the subesophageal of control caterpillars prior to any behavioural changes. Also, at the point of behavioural change, namely cessation of feeding, there was the highest amount of differences in protein profile from control (Table 4.3). This could be of particular interest because the subesophageal ganglion houses the feeding circuits within the CNS (Altman and Kien, 1987).

Looking at the global trends of the changes in proteins at each time point, we can classify the proteins into four categories. Those that increase significantly in comparison to control caterpillars, which we called *increased*; those that decreased significantly in

comparison with control caterpillars, which we called *decreased*; Those that were present above detection threshold in parasitized caterpillars only which we referred to as *novel*; and those that were above the detection threshold in control caterpillars only which we referred to as *absent*. The same protein could fit into multiple different categories depending on time point, but could only have one classification per time point.

Protein identification

Protein identification can give us insight into possible mechanisms involved in permanent change in feeding behaviour observed in parasitized caterpillars. Proteins that were found to be novel/increased in parasitized hosts belong to a wide diversity of pathways. The largest proportion belong to immunity, cell signaling and cytoskeleton organization. Certain AMPs such as Attacin-1 and Gloverin were found in all timepoints, both pre- at- and post- emergence. Other immune molecules (e.g. lysozyme) appeared only after the wasps began to emerge. It is worth highlighting the change in immune-related proteins, given that activation of the immune system has been linked to other changes in feeding behaviour (i.e. illness induced anorexia). The presence of immune-related proteins could be a result of an immune response within the brain, in response to the presence of the bracovirus or due to systemic immune activation. Previous research has confirmed the presence of cytokine receptors within the CNS of *M. sexta* (Adamo and McMillan, 2019). Infection also increases the hemolymph concentration of the neurohormone octopamine (Adamo et al., 1997), suggesting that the CNS receives signals of systemic infection and responses to this information. Either response could lead to neuroinflammation. Recently, evidence was published that, in addition to being antimicrobial, Gloverin may also aid in regulating inflammation in tissues (Lin et al., 2019; Xu et al., 2012). Therefore, Gloverin may be produced in response to neuroinflammation. We also know that cytokines are essential in the bidirectional communication between the immune system and the nervous system (Adamo, 2011; Dantzer, 2004). At the time of emergence, there is a cytokine storm within the hemocoel of the host caterpillar brought on by the wasp larva burrowing through the body wall (Adamo et al., 2016b). Within the fat body of pre-emergence caterpillars, immune genes such as *plasmatocyte-spreading peptide, attacin-1, lysozyme* and *serpin3* were found to be downregulated (Adamo et al., 2016b). At the time of emergence, however, there is a spike in upregulation of these genes within the fat body. The increase in immune activity during wasp emergence is probably a major contributor

to the caterpillar's feeding cessation (Adamo et al., 2016b). However, in this same study, expression of these immune genes was found to return to control levels, or even be downregulated again, 24 hours post emergence of the wasp larva (Adamo et al., 2016b). This makes it difficult to pin permanent feeding change on the cytokine storm. However, in our proteomic analysis we found that the immune factors within the CNS were elevated at least 3 days after the parasitic larva emerged, much longer than what was seen in the fat body. Therefore, it is possible that the cessation of feeding in these caterpillars is, at least in part, due to long term illness-induced anorexia.

The reduction in the amount of soluble protein within the supraesophageal and subesophageal ganglion in all parasitized caterpillars probably helps to explain the large number of proteins in the absent/decreased categories. Once again, these proteins fall within a wide range of categories including immune function and metabolism. Changes in protein concentration have been found in the brains of other parasitized animals. For example, in the jewel wasp-cockroach system, self-generated behaviours of the host cockroach are inhibited 10 minutes after being stung by the wasp, the proteomic profiles of the cockroaches' CNS is already significantly different from control, even though at this time the major change in host behaviour (i.e. hypokinetica) is not yet exhibited (Kaiser et al., 2019).

The proteomic findings of the current work are reminiscent of previous proteomics work on different systems, which has often shown broad changes within the CNS of parasitized hosts that correlate with behavioural alterations. Examples include: the jewel wasp-cockroach system (parasitoid that develops both in and on the cockroach); Trematode-crustacean system (cysts within the CNS); Worm-crustacean system (larva within the body cavity); Hairworm-cricket system (internal parasite) and in the current work: the wasp-caterpillar system (larva within the body cavity). These parasites are from different phyla, exert different behavioural changes within their hosts (ranging from negative geotaxis to hypokinetica) are present in different areas of the host's body, and yet share some overlap in proteomic changes (Biron et al., 2005; Kaiser et al., 2019; Ponton et al., 2006). This overlap between studies is illustrated in Table 4.17. In addition to the specific proteins found to be affected in these different behaviour-altering parasitic systems, there were also broader categories such as immune proteins that saw changes in all five systems, although the specific proteins involved were different. In the *C. congregata-M. sexta* system, some of the most affected proteins (i.e., those with greatest change from control) were immune related, such as

immulectin and attacin. In both the trematode-crustacean and worm-crustacean systems 20-30% of affected proteins were immune related (Ponton et al., 2006). Overall, the fact that these overlapping proteomic changes all lead to some behavioural change strongly suggests that these broad categories of proteins are both susceptible to manipulation and may lead to behavioural changes when disrupted.

Table 4.17. Comparison of altered proteins within the CNS in five different parasite behavioural changing interactions.

	Jewel wasp/cockroach[1]	Trematode/crustacean[2]	Worm/Crustacean[2]	Hairworm/Grasshopper[3]	Wasp/caterpillar[4]
Cytoskeleton					
Tropomyosin					
Troponin					
Actin					
Myosin light/heavy chain					
Signal Transduction					
Tyrosine-kinase					
Arginine-kinase					
Microtubule-associa. protein futsch					
Transcription/Translation					
DNA binding protein					
Translation-initation facto					
Other					
UDP-glucuronosyltransferase					
Hemocyanin					
Rho GTPase singaling					

Red indicates change in protein, blue indicates no change in protein.

1 (Kaiser et al., 2019); 2 (Ponton et al., 2006); 3 (Biron et al., 2005; Biron et al., 2006); 4 (current work)

Changes in protein intensity in the supraesophageal and subesophageal ganglia show a complex pattern. The changes that appeared following wasp emergence may be involved in inducing the behavioural changes seen at that time in the host. Both the supraesophageal and the subesophageal ganglia showed changes that suggest a reduction in proteolysis. These changes may help explain the maintenance of brain protein despite the lack of feeding in the host. Also, there was a marked decrease in trypsins and trypsinogens within the CNS, which are known to play an active role in preventing neurodegeneration (Wang et al., 2008). Other changes were consistent with overall reduction in metabolic activity in the host after wasp emergence (Alleyne and Beckage, 1997). There were few changes in proteins directly involved with electrical signaling in the brain (i.e. ion channels) or synaptic transmission. The few proteins showing major changes post wasp emergence exhibited a mix of up and down regulation (e.g. TolB-like was up and inositol monophosphatase-1 was down in the supraesophageal ganglion, but both of these proteins are involved in signal transduction pathways). These findings indicate that many changes are occurring within the host's CNS during parasitism, although determining how these changes result in changes in neuronal function (i.e.neurophysiology) will require actual electrophysiological measurements–this is a possible direction for future research. Additional issues for interpreting protein changes in terms of neural function is that changes could be occurring in either glia or neurons, with each having different implications for neural function. Similarly, small neuronal changes could be masked by large changes in glia. Changes in glia will also alter neural function, but would do so using indirect means (e.g. perhaps due to changes in ion concentrations or transmitter re-uptake (see Edenfeld et al., 2005)). Overall, it is difficult to draw conclusions about neurophysiology based on the available proteomics data alone.

Proteomic studies of other parasitized brains have faced similar issues (e.g. (Biron et al., 2005; Kaiser et al., 2019; Ponton et al., 2006). In fact, proteomic studies of brains of unparasitized animals also face the wide gap between proteins and produced behaviour. Proteomics cannot stand alone in terms of understanding the brain, but must be combined with transcriptomics, electrophysiology, and other techniques. Neural circuitry is complex and redundant, and this organizational complexity may help to explain the complicated changes observed in proteomics studies of CNS. In this study, some protein changes are in response to the wasp, while some may be produced by the wasp. Moreover, changes in synaptic transmission proteins may be due to upstream

changes in neuronal function (e.g. axonal transport) that are ultimately responsible (i.e. manipulative target).

One consideration is that the current work did not include a starvation control. Therefore, it is possible that some of the changes observed in post-emergence caterpillars were due to lack of feeding. However, it is worth noting that the changes observed in pre-emergence caterpillars occurred before cessation of feeding. This means that, at minimum, changes in those caterpillars must have been precipitated by parasitic infection.

Cotesia Congregata Bracovirus

The domestication of a bracovirus was an evolutionary victory for the ancestor of microgastroid wasps. In domesticating a polydnavirus female wasps now had access to their own tailored gene delivery vector, providing them with a powerful tool with which to manipulate their host (Wang et al., 2021).

The primary tissues that have been studied in respect to impacts of the bracovirus are: hemocytes, which are involved in cell mediated immunity, encapsulating smaller foreign bodies and forming agglutination nodules around larger invaders (Eleftherianos et al., 2009b; Nardi et al., 2006); and the fat body, which is the primary hub of humoural immunity. The fat body produces and releases AMPs and proteins through activation of Toll, Imd and JAK/STAT pathways (Gunaratna and Jiang, 2013). For a more in-depth review of insect immunity see Buchon et al. (2014).

Hemocytes, which are immune cells that circulate within the blood of the caterpillar, are highly impacted by the bracovirus (Amaya et al., 2005; Asgari, 2006; Harwood et al., 1994). Hemocytes are targeted by the injected viral circles and an estimated 3165 genes within the nucleus of hemocytes are impacted by insertion events of viral genes (Chevignon et al., 2018). Genes that code for signal transduction, nutrient transport and metabolic functions are highly impacted and can have up to 10 distinct insertions per gene (Chevignon et al., 2018). Genes that code for transcription and translation machinery are less often the points of insertion for bracoviral genes (Chevignon et al., 2018). Although the information on how and where the bracovirus is inserting itself into the CNS is yet unknown, some of the proteins being produced there are the same as in hemocytes. For instance, Early protein 1 (EP-1) is a virally encoded protein that can be detected within 30 minutes post parasitism (Harwood et al., 1994); by 24 hours post parasitism this particular protein makes up 5% of proteins circulating

within the hemolymph (Chevignon et al., 2018; Harwood and Beckage, 1994). Although to our knowledge, protein function has not yet been assigned, it is hypothesized that it is involved in immunomodulation, specifically interacting with lectins–surface molecules that are involved in regulating self from non-self (Harwood and Beckage, 1994; Le et al., 2003). Unfortunately, like many proteins coded within the bracovirus genome, EP-1 has no conserved motifs that can be compared across taxa, making its exact mechanism hard to predict (Espagne et al., 2004). Its presence within the CNS of *M.sexta* may indicate that immunomodulation is occurring, potentially altering immune-neural communication.

Another, better characterized protein, that was found in both CNS components across all timepoints was CcV1. CcV1 is a virally encoded protein that is expressed in fat body and hemocytes as early as 4 hours post-parasitism (Le et al., 2003). In other braconid wasps this protein ceases to be present within the host 72 hours post parasitism (Le et al., 2003). In the *C. congregata- M. sexta* system however, this protein continues to be present throughout parasitism even after the wasp larvae have emerged from the host in the hemocytes and fat body (Amaya et al., 2005; Le et al., 2003). and CNS (current study). From the literature there are two known functions of CcV1; the first is inhibition of surface-cell exposure of lectin-binding sites and microparticle formation within hemocytes; and the second is actively changing the actin cytoskeleton of cells producing this protein (Amaya et al., 2005). Actin is particularly important for maintaining the shape of neuronal synapses (Dillon and Goda, 2005; Gentile et al., 2022). Therefore, the changes in these proteins in parasitized caterpillar brains could be a sign of altered synaptic cleft structure (Gentile et al., 2022), possibly preventing neurotransmitter release and proper function. CcV1 is heavily present within the hemolymph, fat body and CNS of the host throughout parasitism, but it is also at least partially tissue specific as only scant evidence exists of its presence in the midgut tissues or malpighian tubules (Harwood and Beckage, 1994).

Within the subesophageal ganglion, there do not appear to be any proteins that are confined to a specific time point. The viral proteins that are detected at pre-emergence are also detected at each subsequent time point sampled, and those not detected at pre-emergence are not subsequently detected at any later time point. This may indicate that the role these particular proteins play is necessary throughout parasitism. It may also indicate that the behavioural changes that occur after wasp emergence are not triggered by proteomic changes within the subesophageal ganglion.

The supraesophageal ganglion constitutively expressed viral encoded proteins as well, but, at the point of emergence of the wasp larva from the host, there were also novel viral proteins being expressed. It is likely that the dsDNA for these proteins was inserted into the CNS genome early in parasitism, as previous work by Chevignon (2018) shows that dsDNA circles and host-integration motifs (sequences necessarily for gene insertion into the *M. sexta* genome) are no longer present 48 hours post parasitism. Although characteristics such as the sequence, structure and molecular weight of CcBV-encoded proteins expressed following integration are known, their function and mechanisms remain undefined. Nevertheless, they remain excellent candidates for molecules that play a role in the induction of the adaptive behavioural changes that occur after wasp emergence.

4.7 REFERENCES

Adamo, S. A. (1997). How Parasites Alter the Behavior of their Insect Hosts. In Parasites and Pathogens: Effects On Host Hormones and Behavior (ed. Beckage, N. E.), pp. 231–245. Boston, MA: Springer US.

Adamo, S. A. (1998). Feeding suppression in the tobacco hornworm, Manduca sexta: costs and benefits to the parasitic wasp Cotesia congregata. Can. J. Zool. 76, 1634–1640.

Adamo, S. A. (2002). Modulating the modulators: parasites, neuromodulators and host behavioral change. Brain. Behav. Evol. 60, 370–377.

Adamo, S. A. (2005). Parasitic suppression of feeding in the tobacco hornworm, Manduca sexta: Parallels with feeding depression after an immune challenge. Arch. Insect Biochem. Physiol. 60, 185–197.

Adamo, S. A. (2011). Bidirectional connections between the immune system and the nervous system in insects. In Insect Immunology (ed. Beckage, N. E.), p. Academic Press.

Adamo, S. A. (2013). Parasites: evolution's neurobiologists. J. Exp. Biol. 216, 3–10.

Adamo, S. A. and McMillan, L. E. (2019). Listening to your gut: immune challenge to the gut sensitizes body wall nociception in the caterpillar Manduca sexta. Philos. Trans. R. Soc. B Biol. Sci. 374, 20190278.

Adamo, S., Linn, C. and Beckage, N. (1997). Correlation between changes in host behaviour and octopamine levels in the tobacco hornworm Manduca sexta parasitized by the gregarious braconid parasitoid wasp Cotesia congregata. J. Exp. Biol. 200, 117–127.

Adamo, S. A., Fidler, T. L. and Forestell, C. A. (2007). Illness-induced anorexia and its possible function in the caterpillar, Manduca sexta. Brain. Behav. Immun. 21, 292–300.

Adamo, S. A., Bartlett, A., Le, J., Spencer, N. and Sullivan, K. (2010). Illness-induced anorexia may reduce trade-offs between digestion and immune function. Anim. Behav. 79, 3–10.

Adamo, S. A., Kovalko, I., Turnbull, K. F., Easy, R. H. and Miles, C. I. (2016). The parasitic wasp Cotesia congregata uses multiple mechanisms to control host (Manduca sexta) behaviour. J. Exp. Biol. 219, 3750–3758.

Alleyne, M. and Beckage, N. E. (1997). Parasitism-induced Effects on Host Growth and Metabolic Efficiency in Tobacco Hornworm Larvae Parasitized by Cotesia congregata. J. Insect Physiol. 43, 407–424.

Alleyne, M., Chappell, M. A., Gelman, D. B. and Beckage, N. E. (1997). Effects of Parasitism by the Braconid Wasp Cotesia congregata on Metabolic Rate in Host Larvae of the Tobacco Hornworm, Manduca sexta. J. Insect Physiol. 43, 143–154.

Altman, J. and Kien, J. (1987). Functional organization of the subesophageal ganglion in arthropods. Arthropod Brain Its Evol. Dev. Struct. Funct. Wiley N. Y. 588,.

Amaya, K. E., Asgari, S., Jung, R., Hongskula, M. and Beckage, N. E. (2005). Parasitization of Manduca sexta larvae by the parasitoid wasp Cotesia congregata induces an impaired host immune response. J. Insect Physiol. 51, 505–512.

Andrews, A. J., Chen, X., Zevin, A., Stargell, L. A. and Luger, K. (2010). The Histone Chaperone Nap1 Promotes Nucleosome Assembly by Eliminating Nonnucleosomal Histone DNA Interactions. Mol. Cell 37, 834–842.

Asgari, S. (2006). Venom proteins from polydnavirus-producing endoparasitoids: Their role in host-parasite interactions. Arch. Insect Biochem. Physiol. 61, 146–156.

Beck, M. H., Inman, R. B. and Strand, M. R. (2007). Microplitis demolitor bracovirus genome segments vary in abundance and are individually packaged in virions. Virology 359, 179–189.

Beckage, N. E. (1998). Modulation of immune responses to parasitoids by polydnaviruses. Parasitology 116, S57–S64.

Beckage, N. E. (2012). Chapter 13 - Polydnaviruses as Endocrine Regulators. In Parasitoid Viruses (ed. Beckage, N. E.) and Drezen, J.-M.), pp. 163–168. San Diego: Academic Press.

Beckage, N. E. and Gelman, D. B. (2004). WASP PARASITOID DISRUPTION OF HOST DEVELOPMENT: Implications for New Biologically Based Strategies for Insect Control. Annu. Rev. Entomol. 49, 299–330.

Beckage, N. E. and Kanost, M. R. (1993). Effects of parasitism by the braconid wasp Cotesia congregata on host hemolymph proteins of the tobacco hornworm, Manduca sexta. Insect Biochem. Mol. Biol. 23, 643–653.

Beckage, N. E. and Riddiford, L. M. (1978). Developmental Interactions Between the Tobacco Hornworm Manduca Sexta and Its Braconid Parasite Apan^{TEL}es Congregatus1. Entomol. Exp. Appl. 23, 139–151.

Beckage, N. E. and Riddiford, L. M. (1982). Effects of parasitism by Apanteles congregatus on the endocrine physiology of the tobacco hornworm Manduca sexta. Gen. Comp. Endocrinol. 47, 308–322.

Beckage, N. E. and Templeton, T. J. (1986). Physiological effects of parasitism by Apanteles congregatus in terminalstage tobacco hornworm larvae. J. Insect Physiol. 32, 299–314.

Belle, E., Beckage, N. E., Rousselet, J., Poirié, M., Lemeunier, F. and Drezen, J.-M. (2002). Visualization of Polydnavirus Sequences in a Parasitoid Wasp Chromosome. J. Virol. 76, 5793–5796.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 289–300.

Bétous, R., Mason, A. C., Rambo, R. P., Bansbach, C. E., Badu-Nkansah, A., Sirbu, B. M., Eichman, B. F. and Cortez, D. (2012). SMARCAL1 catalyzes fork regression and Holliday junction migration to maintain genome stability during DNA replication. Genes Dev. 26, 151–162.

Bézier, A., Herbinière, J., Lanzrein, B. and Drezen, J.-M. (2009). Polydnavirus hidden face: The genes producing virus particles of parasitic wasps. J. Invertebr. Pathol. 101, 194–203.

Bézier, A., Louis, F., Jancek, S., Periquet, G., Thézé, J., Gyapay, G., Musset, K., Lesobre, J., Lenoble, P., Dupuy, C., et al. (2013). Functional endogenous viral elements in the genome of the parasitoid wasp Cotesia congregata: insights into the evolutionary dynamics of bracoviruses. Philos. Trans. R. Soc. B Biol. Sci. 368, 20130047.

Biron, D. G. and Loxdale, H. D. (2013). Host–parasite molecular cross-talk during the manipulative process of a host by its parasite. J. Exp. Biol. 216, 148–160.

Biron, D. g, Marché, L., Ponton, F., Loxdale, H. d, Galéotti, N., Renault, L., Joly, C. and Thomas, F. (2005). Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. Proc. R. Soc. B Biol. Sci. 272, 2117–2126.

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G., Paysan-Lafosse, T., Qureshi, M., Raj, S., et al. (2021). The InterPro protein families and domains database: 20 years on. Nucleic Acids Res. 49, D344–D354.

Buchon, N., Silverman, N. and Cherry, S. (2014). Immunity in Drosophila melanogaster—from microbial recognition to whole-organism physiology. Nat. Rev. Immunol. 14, 796–810.

Buchon Nicolas, Poidevin Mickael, Kwon Hyun-Mi, Guillou Aurélien, Sottas Valentin, Lee Bok-Luel, and Lemaitre Bruno (2009). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. Proc. Natl. Acad. Sci. 106, 12442–12447.

Burke, G. R. and Strand, M. R. (2013). Deep Sequencing Identifies Viral and Wasp Genes with Potential Roles in Replication of Microplitis demolitor Bracovirus. J. Virol. 86, 3293–3306.

Buschmann, J., Moritz, B., Jeske, M., Lilie, H., Schierhorn, A. and Wahle, E. (2013). Identification of Drosophila and Human 7-Methyl GMP-specific Nucleotidases *. J. Biol. Chem. 288, 2441–2451.

Chevignon, G., Thézé, J., Cambier, S., Poulain, J., Da Silva, C., Bézier, A., Musset, K., Moreau, S. J. M., Drezen, J.-M. and Huguet, E. (2014). Functional Annotation of Cotesia congregata Bracovirus: Identification of Viral Genes Expressed in Parasitized Host Immune Tissues. J. Virol. 88, 8795–8812.

Chevignon, G., Cambier, S., Da Silva, C., Poulain, J., Drezen, J.-M., Huguet, E. and Moreau, S. J. (2015). Transcriptomic response of Manduca sexta immune tissues to parasitization by the bracovirus associated wasp Cotesia congregata. Insect Biochem. Mol. Biol. 62, 86–99.

Chevignon, G., Periquet, G., Gyapay, G., Vega-Czarny, N., Musset, K., Drezen, J.-M. and Huguet, E. (2018). Cotesia congregata Bracovirus Circles Encoding PTP and Ankyrin Genes Integrate into the DNA of Parasitized Manduca sexta Hemocytes. J. Virol. 92, e00438-18.

Cole, T. J., Beckage, N. E., Tan, F. F., Srinivasan, A. and Ramaswamy, S. B. (2002). Parasitoid–host endocrine relations: self-reliance or co-optation? Insect Biochem. Mol. Biol. 32, 1673–1679.

Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. Eur. J. Pharmacol. 500, 399–411.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9, 46–56.

de Bekker, C., Quevillon, L. E., Smith, P. B., Fleming, K. R., Ghosh, D., Patterson, A. D. and Hughes, D. P. (2014). Species-specific ant brain manipulation by a specialized fungal parasite. BMC Evol. Biol. 14, 166.

Dillon, C. and Goda, Y. (2005). The actin cytoskeleton: Integrating form and function at the synapse. Annu. Rev. Neurosci. 28, 25–55.

Drezen, J.-M., Bézier, A., Lesobre, J., Huguet, E., Cattolico, L., Periquet, G. and Dupuy, C. (2006). The few virus-like genes of Cotesia congregata bracovirus. Arch. Insect Biochem. Physiol. 61, 110–122.

Dushay, M. S. and Beckage, N. E. (1993). Dose-dependent separation of Cotesia congregata-associated polydnavirus effects on Manduca sexta larval development and immunity. J. Insect Physiol. 39, 1029–1040.

Edenfeld, G., Stork, T. and Klämbt, C. (2005). Neuron-glia interaction in the insect nervous system. Curr. Opin. Neurobiol. 15, 34–39.

Edwards, J. P. and Weaver, R. J. (2000). Endocrine changes in lepidopteran larvae - potential challenges to parasitoid development and survival. Comp. Biochem. Physiol. -- Part B Biochem. Mol. Biol. Supplement 1, S31.

Eggleton, P. and Gaston, K. J. (1990). "Parasitoid" Species and Assemblages: Convenient Definitions or Misleading Compromises? Oikos 59, 417–421.

Eleftherianos, I., Gökçen, F., Felföldi, G., Millichap, P. J., Trenczek, T. E., Ffrench-Constant, R. H. and Reynolds, S. E. (2007). The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect Manduca sexta. Cell. Microbiol. 9, 1137–1147.

Eleftherianos, I., Felföldi, G., Ffrench-Constant, R. H. and Reynolds, S. E. (2009a). Induced nitric oxide synthesis in the gut of Manduca sexta protects against oral infection by the bacterial pathogen Photorhabdus luminescens. Insect Mol. Biol. 18, 507–516.

Eleftherianos, I., Xu, M., Yadi, H., ffrench-Constant, R. H. and Reynolds, S. E. (2009b). Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection. J. Exp. Biol. 212, 1840–1848.

Fleming, J. G. and Summers, M. D. (1991). Polydnavirus DNA is integrated in the DNA of its parasitoid wasp host. Proc. Natl. Acad. Sci. 88, 9770–9774.

Fulton, B. B. (1940). The Hornworm Parasite, Apanteles Congregatus Say and the Hyperparasite, Hypopteromalus Tabacum (Fitch)1. Ann. Entomol. Soc. Am. 33, 231–244.

Gade, G., Hoffmann, K. H. and Spring, J. H. (1997). Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963–1032.

Gal, R. and Libersat, F. (2008). A Parasitoid Wasp Manipulates the Drive for Walking of Its Cockroach Prey. Curr. Biol. 18, 877–882.

Gan, Q., Zhang, X., Zhang, D., Shi, L., Zhou, Y., Sun, T., Jiang, S., Gao, J. and Meng, Y. (2018). BmSUC1 is essential for glycometabolism modulation in the silkworm, Bombyx mori. Biochim. Biophys. Acta BBA - Gene Regul. Mech. 1861, 543–553.

Gauthier, J., Boulain, H., van Vugt, J. J. F. A., Baudry, L., Persyn, E., Aury, J.-M., Noel, B., Bretaudeau, A., Legeai, F., Warris, S., et al. (2021). Chromosomal scale assembly of parasitic wasp genome reveals symbiotic virus colonization. Commun. Biol. 4, 1–15.

Gelman, D. B., Reed, D. A. and Beckage, N. E. (1998). Manipulation of fifth-instar host (Manduca sexta) ecdysteroid levels by the parasitoid wasp Cotesia congregata. J. Insect Physiol. 44, 833–843.

Gentile, J. E., Carrizales, M. G. and Koleske, A. J. (2022). Control of Synapse Structure and Function by Actin and Its Regulators. Cells 11, 603.

Giovannini, D., Touhami, J., Charnet, P., Sitbon, M. and Battini, J.-L. (2013). Inorganic Phosphate Export by the Retrovirus Receptor XPR1 in Metazoans. Cell Rep. 3, 1866–1873.

Gonzaga, M. O., Kloss, T. G. and Sobczak, J. F. (2017). Host Behavioural Manipulation of Spiders by Ichneumonid Wasps. In Behaviour and Ecology of Spiders: Contributions from the Neotropical Region (ed. Viera, C.) and Gonzaga, M. O.), pp. 417–437. Cham: Springer International Publishing.

Gunaratna, R. T. and Jiang, H. (2013). A comprehensive analysis of the Manduca sexta immunotranscriptome. Dev. Comp. Immunol. 39, 388–398.

Hart, B. L. (1988). Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12, 123–137.

Harwood, S. H. and Beckage, N. E. (1994). Purification and characterization of an early-expressed polydnavirus-induced protein from the hemolymph of Manduca sexta larvae parasitized by Cotesia congregata. Insect Biochem. Mol. Biol. 24, 685–698.

Harwood, S. H., Grosovsky, A. J., Cowles, E. A., Davis, J. W. and Beckage, N. E. (1994). An Abundantly Expressed Hemolymph Glycoprotein Isolated from Newly Parasitized Manduca sexta Larvae Is a Polydnavirus Gene Product. Virology 205, 381–392.

Hayakawa, Y., Ohnishi, A., Mizoguchi, A. and Yamashika, C. (2000). Distribution of growth-blocking peptide in the insect central nervous tissue. Cell Tissue Res. 300, 459–464.

Helluy, S. (2013). Parasite-induced alterations of sensorimotor pathways in gammarids: collateral damage of neuroinflammation? J. Exp. Biol. 216, 67–77.

Helluy, S. and Thomas, F. (2003). Effects of Microphallus papillorobustus (Platyhelminthes: Trematoda) on serotonergic immunoreactivity and neuronal architecture in the brain of Gammarus insensibilis (Crustacea: Amphipoda). Proc. R. Soc. Lond. B Biol. Sci. 270, 563–568.

Helluy, S. and Thomas, F. (2010). Parasitic manipulation and neuroinflammation: Evidence from the system Microphallus papillorobustus (Trematoda) - Gammarus (Crustacea). Parasit. Vectors 3, 38.

Hoover, K., Grove, M., Gardner, M., Hughes, D. P., McNeil, J. and Slavicek, J. (2011). A Gene for an Extended Phenotype. Science 333, 1401–1401.

Hughes, D. P. and Libersat, F. (2019). Parasite manipulation of host behavior. Curr. Biol. 29, R45–R47.

Isabella, A. J. and Horne-Badovinac, S. (2015). Chapter Nine - Building from the Ground up: Basement Membranes in Drosophila Development. In Current Topics in Membranes (ed. Miner, J. H.), pp. 305–336. Academic Press.

Jungreis, A. M., Barron, N. D. and Johnston, J. W. (1981). Comparative properties of tobacco hornworm, Manduca sexta, carbonic anhydrases. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 241, R92–R99.

Kaiser, M., Arvidson, R., Zarivach, R., Adams, M. E. and Libersat, F. (2019). Molecular cross-talk in a unique parasitoid manipulation strategy. Insect Biochem. Mol. Biol. 106, 64–78.

Kim, M., Jang, D., Yoo, E., Oh, Y., Sonn, J. Y., Lee, J., Ki, Y., Son, H. J., Hwang, O., Lee, C., et al. (2017). Rogdi Defines GABAergic Control of a Wake-promoting Dopaminergic Pathway to Sustain Sleep in Drosophila. Sci. Rep. 7, 11368.

Lavine, M. D. and Beckage, N. E. (1996). Temporal pattern of parasitism-induced immunosuppression in Manduca sexta larvae parasitized by Cotesia congregata. J. Insect Physiol. 42, 41–51.

Le, N. T., Asgari, S., Amaya, K., Tan, F. F. and Beckage, N. E. (2003). Persistence and expression of Cotesia congregata polydnavirus in host larvae of the tobacco hornworm, Manduca sexta. J. Insect Physiol. 49, 533–543.

Lee, G. J., Han, G., Yun, H. M., Lim, J. J., Noh, S., Lee, J. and Hyun, S. (2018). Steroid signaling mediates nutritional regulation of juvenile body growth via IGF-binding protein in Drosophila. Proc. Natl. Acad. Sci. 115, 5992–5997.

Lepicard, S., Franco, B., Bock, F. de and Parmentier, M.-L. (2014). A Presynaptic Role of Microtubule-Associated Protein 1/Futsch in Drosophila: Regulation of Active Zone Number and Neurotransmitter Release. J. Neurosci. 34, 6759–6771.

Lin, Q., Su, G., Wu, A., Chen, D., Yu, B., Huang, Z., Luo, Y., Mao, X., Zheng, P., Yu, J., et al. (2019). Bombyx mori gloverin A2 alleviates enterotoxigenic Escherichia coli-induced inflammation and intestinal mucosa disruption. Antimicrob. Resist. Infect. Control 8, 189.

Liu, P., Torrens-Spence, M. P., Ding, H., Christensen, B. M. and Li, J. (2013). Mechanism of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases. Amino Acids 44, 391–404.

Louis, F., Bézier, A., Periquet, G., Ferras, C., Drezen, J.-M. and Dupuy, C. (2013). The Bracovirus Genome of the Parasitoid Wasp Cotesia congregata Is Amplified within 13 Replication Units, Including Sequences Not Packaged in the Particles. J. Virol. 87, 9649–9660.

Mangold, C. A. and Hughes, D. P. (2021). Insect Behavioral Change and the Potential Contributions of Neuroinflammation—A Call for Future Research. Genes 12, 465.

Martel, R. R. and Law, J. H. (1991). Purification and properties of an ommochrome-binding protein from the hemolymph of the tobacco hornworm, Manduca sexta. J. Biol. Chem. 266, 21392–21398.

McMillan, L. E., Miller, D. W. and Adamo, S. A. (2018). Eating when ill is risky: immune defense impairs food detoxification in the caterpillar Manduca sexta. J. Exp. Biol. 221, jeb173336.

McMillan, L. (2022). Proteomic Databases for Manduca-Cotesia study. *Mendeley Data* **V1** DOI: 10.17632/kvxxm2z.1

Miles, C. I. and Booker, R. (2000). Octopamine mimics the effects of parasitism on the foregut of the tobacco hornworm Manduca sexta. J. Exp. Biol. 203, 1689–1700.

Mohan, P. and Sinu, P. A. (2017). Parasitoid wasp usurps its host to guard its pupa against hyperparasitoids and induces rapid behavioral changes in the parasitized host. PLOS ONE 12, e0178108.

Moore, J. (2002). Parasites and the behavior of animals. Oxford University Press on Demand.

Morales, C. and Li, Z. (2017). Drosophila canopy b is a cochaperone of glycoprotein 93. J. Biol. Chem. 292, 6657–6666.

Nardi, J. B., Pilas, B., Ujhelyi, E., Garsha, K. and Kanost, M. R. (2003). Hematopoietic organs of Manduca sexta and hemocyte lineages. Dev. Genes Evol. 213, 477–491.

Nardi, J. B., Pilas, B., Bee, C. M., Zhuang, S., Garsha, K. and Kanost, M. R. (2006). Neuroglian-positive plasmatocytes of Manduca sexta and the initiation of hemocyte attachment to foreign surfaces. Dev. Comp. Immunol. 30, 447–462.

Nijhout, H. F. and Williams, C. M. (1974). Control of Moulting and Metamorphosis in the Tobacco Hornworm, Manduca Sexta (L.): Cessation of Juvenile Hormone Secretion as a Trigger for Pupation. J. Exp. Biol. 61, 493–501.

Orso, G., Pendin, D., Liu, S., Tosetto, J., Moss, T. J., Faust, J. E., Micaroni, M., Egorova, A., Martinuzzi, A., McNew, J. A., et al. (2009). Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. Nature 460, 978–983.

Persson, B., Bengtsson-Olivecrona, G., Enerbäck, S., Olivecrona, T. and Jörnvall, H. (1989). Structural features of lipoprotein lipase. Eur. J. Biochem. 179, 39–45.

Petzoldt, A. G., Coutelis, J.-B., Géminard, C., Spéder, P., Suzanne, M., Cerezo, D. and Noselli, S. (2012). DE-Cadherin regulates unconventional Myosin ID and Myosin IC in Drosophila left-right asymmetry establishment. Development 139, 1874–1884.

Ponton, F., Lefèvre, T., Lebarbenchon, C., Thomas, F., Loxdale, H., Marché, L., Renault, L., Perrot-Minnot, M.-J. and Biron, D. G. (2006). Do distantly related parasites rely on the same proximate factors to alter the behaviour of their hosts? Proc. R. Soc. B Biol. Sci. 273, 2869–2877.

Poulin, R. (2010). Chapter 5 - Parasite Manipulation of Host Behavior: An Update and Frequently Asked Questions. In Advances in the Study of Behavior (ed. Brockmann, H. J.), Roper, T. J.), Naguib, M.), Wynne-Edwards, K. E.), Mitani, J. C.), and Simmons, L. W.), pp. 151–186. Academic Press.

Qazi, S. and Trimmer, B. A. (1999). The role of inositol 1,4,5-trisphosphate 5-phosphatase in inositol signaling in the CNS of larval Manduca sexta. Insect Biochem. Mol. Biol. 29, 161–175.

Rawlings, N. D. and Barrett, A. J. (1994). Families of serine peptidases. Methods Enzymol. 244, 19–61.

Reed, D. A. and Beckage, N. E. (1997). Inhibition of testicular growth and development in Manduca sexta larvae parasitized by the braconid wasp Cotesia congregata. J. Insect Physiol. 43, 29–38.

Robertson, R. M., Spong, K. E. and Srithiphaphirom, P. (2017). Chill coma in the locust, Locusta migratoria, is initiated by spreading depolarization in the central nervous system. Sci. Rep. 7, 10297.

Savary, S., Drezen, J.-M., Tan, F., Beckage, N. e. and Periquet, G. (1999). The excision of polydnavirus sequences from the genome of the wasp Cotesia congregata (Braconidae, Microgastrinae) is developmentally regulated but not strictly restricted to the ovaries in the adult. Insect Mol. Biol. 8, 319–327.

Skinner, W. S., Dennis, P. A., Li, J. P., Summerfelt, R. M., Carney, R. L. and Quistad, G. B. (1991). Isolation and identification of paralytic peptides from hemolymph of the lepidopteran insects Manduca sexta, Spodoptera exigua, and Heliothis virescens. J. Biol. Chem. 266, 12873–12877.

Steinhaus, E. (1959). Serratia marcescens Bizio as an insect pathogen. Hilgardia 28, 351–380.

Stibbs, H. H. (1985). Changes in brain concentrations of catecholamines and indoleamines in Toxoplasma gondii infected mice. Ann. Trop. Med. Parasitol. 79, 153–157.

Stoltz, D. B. Y. (1990). Evidence for Chromosomal Transmission of Polydnavirus DNA. J. Gen. Virol. 71, 1051–1056.

Theilmann, D. A. and Summers, M. D. Y. (1986). Molecular Analysis of Campoletis sonorensis Virus DNA in the Lepidopteran Host Heliothis virescens. J. Gen. Virol. 67, 1961–1969.

Thomas, F., Adamo, S. and Moore, J. (2005). Parasitic manipulation: where are we and where should we go? Behav. Processes 68, 185–199.

Thomas, F., Poulin, R. and Brodeur, J. (2010). Host manipulation by parasites: a multidimensional phenomenon. Oikos 119, 1217–1223.

Vogel, B. E. and Hedgecock, E. M. (2001). Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented line-shaped junctions. Development 128, 883–894.

Wang, X.-Y., Frohlich, D. R. and Wells, M. A. (1993). Polymorphic cDNAs encode for the methionine-rich storage protein from Manduca sexta. Insect Mol. Biol. 2, 13–20.

Wang, Y., Luo, W. and Reiser, G. (2008). Trypsin and trypsin-like proteases in the brain: Proteolysis and cellular functions. Cell. Mol. Life Sci. 65, 237–252.

Wang, Z., Ye, X., Zhou, Y., Wu, X., Hu, R., Zhu, J., Chen, T., Huguet, E., Shi, M., Drezen, J.-M., et al. (2021). Bracoviruses recruit host integrases for their integration into caterpillar's genome. PLOS Genet. 17, e1009751.

Weinersmith, K. L. (2019). What's gotten into you?: a review of recent research on parasitoid manipulation of host behavior. Curr. Opin. Insect Sci. 33, 37–42.

Willott, E., Wang, X. Y. and Wells, M. A. (1989). cDNA and gene sequence of Manduca sexta arylphorin, an aromatic amino acid-rich larval serum protein. Homology to arthropod hemocyanins. J. Biol. Chem. 264, 19052–19059.

Xu, X.-X., Zhong, X., Yi, H.-Y. and Yu, X.-Q. (2012). Manduca sexta gloverin binds microbial components and is active against bacteria and fungi. Dev. Comp. Immunol. 38, 275–284.

Yamamoto, K., Ozakiya, Y. and Uno, T. (2017). Localization of an Aldo-Keto Reductase (AKR2E4) in the Silkworm Bombyx mori (Lepidoptera: Bombycidae). J. Insect Sci. 17, 94.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Thomas, M. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13,.

Yu, X.-Q. and Kanost, M. R. (2003). Manduca sexta lipopolysaccharide-specific immulectin-2 protects larvae from bacterial infection. Dev. Comp. Immunol. 27, 189–196.

Zhang, C.-J., Bowzard, J. B., Anido, A. and Kahn, R. A. (2003). Four ARF GAPs in Saccharomyces cerevisiae have both overlapping and distinct functions. Yeast 20, 315–330.

CHAPTER 5 -GENERAL DISCUSSION

The immune system, detoxification system, and stress response system are all integral to an animal's survival. These systems are all costly in terms of energy and draw on the same limited pool of resources. While classically these systems have been studied largely in isolation, a new way of framing them has been proposed. Namely, these systems may be considered as components of an integrated defense system (IDS) as put forward by Adamo (2022). In this framework, previous research on how these three systems can borrow, or share resources (Adamo, 2017), as well as take precedence over other systems (Adamo et al., 2017), explains how occurrences that seem counter-intuitive, such as illness-induced anorexia, can be beneficial and even necessary.

5.1 EATING WHEN ILL IS RISKY: IMMUNE DEFENSE IMPAIRS FOOD DETOXIFICATION IN THE CATERPILLAR *MANDUCA SEXTA*

In my first study, *Eating when ill is risky*, I investigated the self-generated sickness behaviour of illness-induced anorexia. Illness-induced anorexia is a widespread phenomenon in which an animal faced with an immune challenge reduces its food consumption (Hart, 1988; Sullivan et al., 2016). On the surface, a reduction in food consumption seems illogical during a time when resources are in high demand (i.e. when mounting an immune response) (Ardia et al., 2012; Freitak et al., 2003). The impacts of illness-induced anorexia can also be long term. For instance, in my study of the insect *Manduca sexta,* illness-induced anorexia decreased mass gain, which can lead to longer periods of time in the caterpillar stage (Kingsolver et al., 2012). Being a caterpillar for longer increases the risks of predation (Bernays, 1997; Kingsolver et al., 2012). Experiencing illness-induced anorexia during the caterpillar stage can also lead to becoming a smaller adult, which is linked to decreased fecundity (Kingsolver et al., 2012). In light of these negative impacts of illness-induced anorexia,it will be selected against, unless it provides some advantage. The fact that it is phylogenetically widespread (Sullivan et al., 2016) further suggests that it provides an advantage.

Manduca sexta caterpillars feed on solanaceous plants, such as tobacco, that must be detoxified thoroughly post-consumption (Snyder and Glendinning, 1996). Much like the immune system, detoxification systems are also resource demanding (Heckel, 2014). In addition to this there are molecules, such as glutathione (GSH), that are

important for both the immune system (Stahlschmidt et al., 2015) and the detoxification system (Jeschke et al., 2016). GSH is a tripeptide that contains the amino acid cysteine, which is in limited supply to insects (Barbehenn et al., 2013). In fact, 20% of total cysteine is invested in having a pool of GSH, which makes it difficult to create more when the available GSH is depleted (Jeschke et al., 2016). Therefore, tradeoffs between these two parts of the IDS might be most evident in redistribution of limited molecules such as GSH.

Hypothesizing that molecules such as GSH could be a pinch point between the immune system and the detoxification system, I examined GSH levels in caterpillars that had their immune systems activated, caterpillars who had their detoxification systems activated, and finally in a group that had both their immune system and detoxification system activated concurrently. In all three groups GSH levels were observed to be significantly lower than in control caterpillars. The dual challenge group that had both immune activation and detoxification activation did not have significantly lower GSH levels compared to the immune challenge only, or the detoxification challenge only. In other words, activating either or both systems left the caterpillars with a similar reduction of circulating GSH within the hemolymph; examination of Figure 2.2 indicates that GSH levels were extremely low (near zero) in all three of these groups. Possibly because one or both systems had less access to this key resource, caterpillars in the dual challenge group had significantly higher mortality than caterpillars in any other group, and, critically, could be rescued with administration of supplemental GSH. These results support GSH as a limited resource that must be shared between the immune system and detoxification system.

I hypothesize that when the immune system releases proinflammatory cytokines, such as plasmatocyte-spreading peptide (PSP), these are detected by the CNS , these cytokines provide a trigger that results in the brain suppressing feeding during an immune response (i.e. illness-induced anorexia). The cessation of feeding in turn reduces the detoxification systems' need for GSH, allowing it to be used optimally by the immune system to recover from the immune challenge.

In this study, I also examined the gene expression levels in the fat body of both immune and detoxification related genes. I found that when exposed to a toxin, *M. sexta* upregulated the genes for immune molecules such as attacin, PAP-3 and lysozyme, even in the absence of a direct immune challenge. In contrast, when facing an immune challenge, there was no increase in detoxification related gene expression. During a

challenge that activated both the immune system and detoxification system the gene expression of glutathione-S-transferase-1 was downregulated, and the gene expression for thioredoxin was not upregulated as it would be under detoxification alone. This suggests that there is a negative relationship between the immune response and detoxification. Possibly the immune response suppresses detoxification mechanisms, although this was not directly tested.

This study supports the view that immune and detoxification systems are interconnected as it demonstrates two things: first, that a shared resource is critical for surviving either type of threat. Moreover, this study is novel in that it is the first to identify GSH as a pinch-point between two arms of the IDS. Second, the findings that activation of the detoxification system also causes upregulation of immune genes, but not vice-versa, and that a dual challenge does not fully overlap with the expression profiles of either challenge alone, suggests that the regulation of detoxification and immune response are probably co-ordinated, supporting the concept of the IDS.

5.2 FRIEND OR FOE? EFFECTS OF HOST IMMUNE ACTIVATION ON THE GUT MICROBIOME IN THE CATERPILLAR *MANDUCA SEXTA*

While the IDS is made up of multiple interconnected systems, it is also important to view each component system as an interconnected network within itself. For instance, within the immunophysiological arm of the IDS there is a network between the different immune tissues within the insect.There can exist trade-offs between specific tissues such as the fatbody and the midgut. Fatbody is an essential immune tissue that also serves as a metabolic center and energy storage facility for insects (Skowronek et al., 2021). The midgut is the main organ for detoxification of food, nutritional absorption and also an integral immune tissue (Caccia et al., 2019). The midgut is an interface between 'outside' and 'inside' and as such must keep any passing microbes under control so that they do not become opportunistic pathogens, which comes at a cost (Krams et al., 2017).

In this study I investigated the strategy undertaken when the caterpillar *Manduca sexta* is exposed to a systemic immune challenge while subsequently being exposed to microbe laced food. Under natural conditions, *Manduca sexta* does not have a robust resident gut microbiome (Hammer et al., 2017), but it is constantly exposed to the leaf microbiome of its food source, the tobacco plant (Zhou et al., 2020). In this study, I found that under the pressure of an immune challenge in the hemolymph, the number of

transient gut bacteria increased. This may be due to less resources being available to the midgut to control the growth of potentially harmful bacteria as it travels the length of the gut. This research suggests that regulation of an individual's microbiome requires immunological resources, and the availability of these resources may be impacted by systemic illness. Indeed, there is evidence for similar immune-microbiome trade-offs in vertebrates. For example, there is evidence from both humans and mice that an immune challenge can lead to widespread disruption of the microbiome leading it to shift into a pathobiome (pathogenic microbiome) (Krezalek et al., 2017;Miller et al., 2021). This pathobiome in turn can lead to exacerbated sepsis and organ failure (Miller et al., 2021).

By examining various immune components in the midgut and fat body, in addition to other physiological parameters (e.g. colony forming units (CFUs) in the frass), I found evidence suggesting that the configuration of the immune response depends on whether the challenge was an oral inoculation of bacteria to the midgut, or an injection of a systematic bacterial challenge to the hemocoel. This finding supports other evidence showing that the immune system alters its configuration (i.e. the details of its response) when faced with multiple challenges (Adamo, 2017; Adamo et al., 2008; Adamo et al., 2016a).

The results from this study broadly support the hypothesis that the immunophysiological response to a challenge depends on the nature and context of the challenge. The immune system will respond in a fluid fashion depending on the number of simultaneous challenges, and how the threat evolves over time. The flexibility of the immune system in terms of recruiting different immune organs and resources, depending on the internal and external context, helps explain the variability in the immune response across individuals.

5.3 PROTEOMIC ANALYSIS OF THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA* CATERPILLARS PARASITIZED BY WASP *COTESIA CONGREGATA*

As in vertebrates (Dantzer et al., 2008), there are bidirectional connections between the immune system and central nervous system in invertebrates (Adamo, 2011). In insects, the central nervous system releases neurohormones such as octopamine that alter immune function (Huang et al., 2012) and the immune system releases cytokines for which there are receptors in the brain (Adamo and McMillan, 2019). While generally beneficial, these lines of communication between the immune system and the CNS can potentially be hijacked by parasitic manipulators, such as the

parasitic wasp *Cotesia congregata* (Adamo, 2013). A hypothesis put forward by Adamo et al. (2016b) postulates that the parasitic wasp *C. congregata* is able to induce changes in host behaviour (e.g. an illness-induced anorexia like state) by hijacking the communication network that exists between the IDS and the CNS.

I investigated this possibility by using a proteomic analysis to look at the proteome of two main areas of the CNS, the supraesophageal ganglion and the subesophageal ganglion, over four different timepoints in late stage parasitism. These areas were specifically chosen due to their role in motivation (supraesophageal ganglion (Emanuel et al., 2020; Schwaerzel et al., 2003), mouthpart control, and CPGs necessary to coordinate feeding (subesophageal ganglion (Blaney and Simmonds, 1987; Rohrbacher, 1994). By comparing the proteomic output to both the genome for *M.sexta* (Gershman et al., 2021; Kanost et al., 2016) and the genome of the domesticated polydnavirus (Espagne et al., 2004). I demonstrated changes in proteomic profiles between control and unparasitized caterpillars at four different stages of parasitism, both before and after behavioural changes took place. Some changes, such as the increase in certain immune factors such as attacin, gloverin and lysozyme were mirrored in previous research that demonstrated an increase in immune factors in hemocytes and fatbody of parasitized caterpillars (Chevignon et al., 2018). Other changes, such as those seen to cytoskeleton proteins are also found in other hosts of parasitic manipulators (Biron et al., 2005; Kaiser et al., 2019; Ponton et al., 2006).

Overall, proteomic analyses of the supraesophageal and subesophageal ganglion pre- and post- behavioural change show a complex pattern of increases and decreases of proteins involved in many different tasks and pathways, such as the immune system, cytoskeleton, and general proteolysis. The wide range of effects is inconsistent with targeting of specific neural circuits. The lack of targeting for particular neuropharmacological pathways has been found in the hosts of other parasitic manipulators. For example, in the jewel wasp-cockroach system the female wasp injects a venom cocktail that attacks over 200 neural targets (Kaiser and Libersat, 2015). Although in this system there was some specificity towards synaptic transmission, there were also immune proteins found within the venom, such as different proteases involved in the Toll pathway, as well as the Toll receptor ligand spätzle (Arvidson et al., 2019).The increase in immune proteins in both manipulated brains could be non-specific effects of foreign agents in the brain, but may also be a reflection of immune/neural circuit exploitation (Mangold and Hughes, 2021).

In addition to changes in proteomic profiles of host produced proteins, I also found complex patterns of bracovirus coded protein production with the tissues of the supraesophageal and subesophageal ganglia. Many of these proteins had not been seen prior in the CNS of *M. sexta* caterpillars. Certain bracovirus encoded proteins were not detected in the CNS until emergence of the wasp larval, which may indicate that they are involved in the behavioural shift away from self-generated behaviours in the host at this point. In future, it will be exciting to test whether and how bracovirus encoded proteins expressed within the CNS affect neuronal activity. One possibility is that bracovirus could influence neuronal activity by manipulating the activity of glial cells, the support and immune cells of the brain (Dheilly et al., 2015). Subsequent research should investigate the specific location of expressed bracovirus coded proteins, both in terms of brain regions (central complex) and cell type (neuron vs glia).

5.4 CONCLUDING REMARKS

In conclusion, this thesis examined three different aspects of the IDS. I demonstrated a trade-off between two systems within the IDS, namely the immune system and the detoxification system. Further, I demonstrated a possible reconfiguration between different immune tissues, the midgut and the fatbody, when faced with multiple different (and biologically relevant) stressors. Finally, I investigated possible mechanisms by which a parasitoid might exploit existing lines of communication between the IDS and the CNS, in order to manipulate behavior in a way that benefits the parasitoid and at the expense of the host. My research demonstrates how molecular studies are critical for understanding the design and function of physiological networks. It also demonstrates that interpreting physiological and biochemical results from an organismal perspective (e.g. physiological networks) can be important for understanding seemingly maladaptive responses.

BIBLIOGRAPHY

Adamo, S. A. (1997). How Parasites Alter the Behavior of their Insect Hosts. In *Parasites and Pathogens: Effects On Host Hormones and Behavior* (ed. Beckage, N. E.), pp. 231–245. Boston, MA: Springer US.

Adamo, S. A. (1998). Feeding suppression in the tobacco hornworm, Manduca sexta: costs and benefits to the parasitic wasp Cotesia congregata. *Can. J. Zool.* **76**, 1634–1640.

Adamo, S. A. (2002). Modulating the modulators: parasites, neuromodulators and host behavioral change. *Brain. Behav. Evol.* **60**, 370–377.

Adamo, S. A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket Gryllus texensis. *J. Insect Physiol.* **50**, 209–216.

Adamo, S. A. (2005). Parasitic suppression of feeding in the tobacco hornworm, Manduca sexta: Parallels with feeding depression after an immune challenge. *Arch. Insect Biochem. Physiol.* **60**, 185–197.

Adamo, S. A. (2008). Norepinephrine and octopamine: linking stress and immune function across phyla. *Invertebr. Surviv. J.* **5**, 12–19.

Adamo, S. A. (2011). Bidirectional connections between the immune system and the nervous system in insects. In *Insect Immunology* (ed. Beckage, N. E.), p. Academic Press.

Adamo, S. A. (2013). Parasites: evolution's neurobiologists. *J. Exp. Biol.* **216**, 3–10.

Adamo, S. A. (2017). The stress response and immune system share, borrow, and reconfigure their physiological network elements: Evidence from the insects. *Horm. Behav.* **88**, 25–30.

Adamo, S. A. (2022). The Integrated Defense System: Optimizing Defense against Predators, Pathogens, and Poisons. *Integr. Comp. Biol.* icac024.

Adamo, S. A. and McMillan, L. E. (2019). Listening to your gut: immune challenge to the gut sensitizes body wall nociception in the caterpillar Manduca sexta. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20190278.

Adamo, S., Linn, C. and Beckage, N. (1997). Correlation between changes in host behaviour and octopamine levels in the tobacco hornworm Manduca sexta parasitized by the gregarious braconid parasitoid wasp Cotesia congregata. *J.*

Exp. Biol. **200**, 117–127.

Adamo, S. A., Fidler, T. L. and Forestell, C. A. (2007). Illness-induced anorexia and its possible function in the caterpillar, Manduca sexta. *Brain. Behav. Immun.* **21**, 292–300.

Adamo, S., Roberts, J., Easy, R. and Ross, N. (2008). Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *J. Exp. Biol.* **211**, 531–538.

Adamo, S. A., Bartlett, A., Le, J., Spencer, N. and Sullivan, K. (2010). Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Anim. Behav.* **79**, 3–10.

Adamo, S. A., Davies, G., Easy, R., Kovalko, I. and Turnbull, K. F. (2016a). Reconfiguration of the immune system network during food limitation in the caterpillar. *J. Exp. Biol.* **219**, 706–718.

Adamo, S. A., Kovalko, I., Turnbull, K. F., Easy, R. H. and Miles, C. I. (2016b). The parasitic wasp Cotesia congregata uses multiple mechanisms to control host (Manduca sexta) behaviour. *J. Exp. Biol.* **219**, 3750–3758.

Adamo, S. A., Easy, R. H., Kovalko, I., MacDonald, J., McKeen, A., Swanburg, T., Turnbull, K. F. and Reeve, C. (2017). Predator exposure-induced immunosuppression: trade-off, immune redistribution or immune reconfiguration? *J. Exp. Biol.* **220**, 868–875.

Ahmad, I. M., Waldbauer, G. P. and Friedman, S. (1989). A defined artificial diet for the larvae of Manduca sexta. *Entomol. Exp. Appl.* **53**, 189–191.

Aitken, A. E., Richardson, T. A. and Morgan, E. T. (2006). Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* **46**, 123–149.

Al Souhail, Q., Hiromasa, Y., Rahnamaeian, M., Giraldo, M. C., Takahashi, D., Valent, B., Vilcinskas, A. and Kanost, M. R. (2016). Characterization and regulation of expression of an antifungal peptide from hemolymph of an insect, Manduca sexta. *Dev. Comp. Immunol.* **61**, 258–268.

Alleyne, M. and Beckage, N. E. (1997). Parasitism-induced Effects on Host Growth and Metabolic Efficiency in Tobacco Hornworm Larvae Parasitized by Cotesia congregata. *J. Insect Physiol.* **43**, 407–424.

Alleyne, M., Chappell, M. A., Gelman, D. B. and Beckage, N. E. (1997). Effects of

Parasitism by the Braconid Wasp Cotesia congregata on Metabolic Rate in Host Larvae of the Tobacco Hornworm, Manduca sexta. *J. Insect Physiol.* **43**, 143–154.

Altincicek, B., Knorr, E. and Vilcinskas, A. (2008). Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. *Dev. Comp. Immunol.* **32**, 585–595.

Altman, J. and Kien, J. (1987). Functional organization of the subesophageal ganglion in arthropods. *Arthropod Brain Its Evol. Dev. Struct. Funct. Wiley N. Y.* **588**,.

Amaya, K. E., Asgari, S., Jung, R., Hongskula, M. and Beckage, N. E. (2005). Parasitization of Manduca sexta larvae by the parasitoid wasp Cotesia congregata induces an impaired host immune response. *J. Insect Physiol.* **51**, 505–512.

Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250.

Andrews, A. J., Chen, X., Zevin, A., Stargell, L. A. and Luger, K. (2010). The Histone Chaperone Nap1 Promotes Nucleosome Assembly by Eliminating Nonnucleosomal Histone DNA Interactions. *Mol. Cell* **37**, 834–842.

Ao, J., Ling, E. and Yu, X.-Q. (2008). A Toll receptor from Manduca sexta is in response to Escherichia coli infection. *Mol. Immunol.* **45**, 543–552.

Aranda, P. S., LaJoie, D. M. and Jorcyk, C. L. (2012). Bleach gel: A simple agarose gel for analyzing RNA quality. *ELECTROPHORESIS* **33**, 366–369.

Ardia, D. R., Gantz, J. E., Brent, C. and Strebel, S. (2012). Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Funct. Ecol.* **26**, 732–739.

Arvidson, R., Kaiser, M., Lee, S. S., Urenda, J.-P., Dail, C., Mohammed, H., Nolan, C., Pan, S., Stajich, J. E., Libersat, F., et al. (2019). Parasitoid Jewel Wasp Mounts Multipronged Neurochemical Attack to Hijack a Host Brain *[S]. *Mol. Cell. Proteomics* **18**, 99–114.

Asgari, S. (2006). Venom proteins from polydnavirus-producing endoparasitoids: Their role in host-parasite interactions. *Arch. Insect Biochem. Physiol.* **61**, 146–156.

Avadhanula, V., Weasner, B. P., Hardy, G. G., Kumar, J. P. and Hardy, R. W. (2009). A

Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. *PLOS Pathog.* **5**, e1000582.

Ayres, J. S. and Schneider, D. S. (2009). The role of anorexia in resistance and tolerance to infections in Drosophila. *PLoS Biol.* **7**, e1000150.

Bajgar, A., Kucerova, K., Jonatova, L., Tomcala, A., Schneedorferova, I., Okrouhlik, J. and Dolezal, T. (2015). Extracellular adenosine mediates a systemic metabolic switch during immune response. *PLoS Biol.* **13**, e1002135.

Barbehenn, R. V., Kochmanski, J., Menachem, B. and Poirier, L. M. (2013). Allocation of cysteine for glutathione production in caterpillars with different antioxidant defense strategies: a comparison of Lymantria dispar and Malacosoma disstria. *Arch. Insect Biochem. Physiol.* **84**, 90–103.

Beck, M. H., Inman, R. B. and Strand, M. R. (2007). Microplitis demolitor bracovirus genome segments vary in abundance and are individually packaged in virions. *Virology* **359**, 179–189.

Beckage, N. E. (1998). Modulation of immune responses to parasitoids by polydnaviruses. *Parasitology* **116**, S57–S64.

Beckage, N. E. (2012). Chapter 13 - Polydnaviruses as Endocrine Regulators. In *Parasitoid Viruses* (ed. Beckage, N. E.) and Drezen, J.-M.), pp. 163–168. San Diego: Academic Press.

Beckage, N. E. and Gelman, D. B. (2004). WASP PARASITOID DISRUPTION OF HOST DEVELOPMENT: Implications for New Biologically Based Strategies for Insect Control. *Annu. Rev. Entomol.* **49**, 299–330.

Beckage, N. E. and Kanost, M. R. (1993). Effects of parasitism by the braconid wasp Cotesia congregata on host hemolymph proteins of the tobacco hornworm, Manduca sexta. *Insect Biochem. Mol. Biol.* **23**, 643–653.

Beckage, N. E. and Riddiford, L. M. (1978). Developmental Interactions Between the Tobacco Hornworm Manduca Sexta and Its Braconid Parasite Apan^{TEL}es Congregatus1. *Entomol. Exp. Appl.* **23**, 139–151.

Beckage, N. E. and Riddiford, L. M. (1982). Effects of parasitism by Apanteles congregatus on the endocrine physiology of the tobacco hornworm Manduca sexta. *Gen. Comp. Endocrinol.* **47**, 308–322.

Beckage, N. E. and Templeton, T. J. (1986). Physiological effects of parasitism by Apanteles congregatus in terminalstage tobacco hornworm larvae. *J. Insect*

Physiol. **32**, 299–314.

Belle, E., Beckage, N. E., Rousselet, J., Poirié, M., Lemeunier, F. and Drezen, J.-M. (2002). Visualization of Polydnavirus Sequences in a Parasitoid Wasp Chromosome. *J. Virol.* **76**, 5793–5796.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 289–300.

Bernays, E. A. (1997). Feeding by lepidopteran larvae is dangerous. *Ecol. Entomol.* **22**, 121–123.

Bernays, E. A. and Woods, H. A. (2000). Foraging in nature by larvae of Manduca sexta—influenced by an endogenous oscillation. *J. Insect Physiol.* **46**, 825–836.

Bétous, R., Mason, A. C., Rambo, R. P., Bansbach, C. E., Badu-Nkansah, A., Sirbu, B. M., Eichman, B. F. and Cortez, D. (2012). SMARCAL1 catalyzes fork regression and Holliday junction migration to maintain genome stability during DNA replication. *Genes Dev.* **26**, 151–162.

Bézier, A., Herbinière, J., Lanzrein, B. and Drezen, J.-M. (2009). Polydnavirus hidden face: The genes producing virus particles of parasitic wasps. *J. Invertebr. Pathol.* **101**, 194–203.

Bézier, A., Louis, F., Jancek, S., Periquet, G., Thézé, J., Gyapay, G., Musset, K., Lesobre, J., Lenoble, P., Dupuy, C., et al. (2013). Functional endogenous viral elements in the genome of the parasitoid wasp Cotesia congregata: insights into the evolutionary dynamics of bracoviruses. *Philos. Trans. R. Soc. B Biol. Sci.* **368**, 20130047.

Biron, D. G. and Loxdale, H. D. (2013). Host–parasite molecular cross-talk during the manipulative process of a host by its parasite. *J. Exp. Biol.* **216**, 148–160.

Biron, D. g, Marché, L., Ponton, F., Loxdale, H. d, Galéotti, N., Renault, L., Joly, C. and Thomas, F. (2005). Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. *Proc. R. Soc. B Biol. Sci.* **272**, 2117–2126.

Biron, D., Ponton, F., Marché, L., Galeotti, N., Renault, L., Demey-Thomas, E., Poncet, J., Brown, S., Jouin, P. and Thomas, F. (2006). 'Suicide'of crickets harbouring hairworms: a proteomics investigation. *Insect Mol. Biol.* **15**, 731–742.

Blaney, W. M. and Simmonds, M. S. J. (1987). Control of mouthparts by the subesophageal ganglion. *Arthropod Brain* 303–322.

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G., Paysan-Lafosse, T., Qureshi, M., Raj, S., et al. (2021). The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res.* **49**, D344–D354.

Boucias, D. G. and Pendland, J. C. (1998). Insect-pathogen relationships. In *Principles of Insect Pathology*, pp. 1–30. Springer.

Brandt, C. R., Adang, M. J. and Spence, K. D. (1978). The peritrophic membrane: Ultrastructural analysis and function as a mechanical barrier to microbial infection in Orgyia pseudotsugata. *J. Invertebr. Pathol.* **32**, 12–24.

Brummett, L. M., Kanost, M. R. and Gorman, M. J. (2017). The immune properties of Manduca sexta transferrin. *Insect Biochem. Mol. Biol.* **81**, 1–9.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev. Microbiol.* **12**, 168.

Buchon, N., Silverman, N. and Cherry, S. (2014). Immunity in Drosophila melanogaster—from microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* **14**, 796–810.

Buchon Nicolas, Poidevin Mickael, Kwon Hyun-Mi, Guillou Aurélien, Sottas Valentin, Lee Bok-Luel, and Lemaitre Bruno (2009). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. *Proc. Natl. Acad. Sci.* **106**, 12442–12447.

Burke, G. R., Thomas, S. A., Eum, J. H. and Strand, M. R. (2013). Mutualistic Polydnaviruses Share Essential Replication Gene Functions with Pathogenic Ancestors. *PLOS Pathog.* **9**, e1003348.

Buschmann, J., Moritz, B., Jeske, M., Lilie, H., Schierhorn, A. and Wahle, E. (2013). Identification of Drosophila and Human 7-Methyl GMP-specific Nucleotidases *. *J. Biol. Chem.* **288**, 2441–2451.

Caccia, S., Casartelli, M. and Tettamanti, G. (2019). The amazing complexity of insect midgut cells: types, peculiarities, and functions. *Cell Tissue Res.* **377**, 505–525.

Cao, X., He, Y., Hu, Y., Wang, Y., Chen, Y.-R., Bryant, B., Clem, R. J., Schwartz, L. M., Blissard, G. and Jiang, H. (2015). The immune signaling pathways of Manduca sexta. *Insect Biochem. Mol. Biol.* **62**, 64–74.

Carlsson, A., Nyström, T., de Cock, H. and Bennich, H. 1998 (1998). Attacin - an insect immune protein - binds LPS and triggers the specific inhibition of bacterial

outer-membrane protein synthesis. *Microbiology* **144**, 2179–2188.

Carvalhal, S., Ribeiro, S. A., Arocena, M., Kasciukovic, T., Temme, A., Koehler, K., Huebner, A. and Griffis, E. R. (2015). The nucleoporin ALADIN regulates Aurora A localization to ensure robust mitotic spindle formation. *Mol. Biol. Cell* **26**, 3424–3438.

Cerenius, L. and Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* **198**, 116–126.

Chapman, R. F. (2013). *The insects : structure and function*. Fifth edition / edited by Stephen J. Simpson, the University of Sydney, Australia, Angela E. Douglas, Cornell University, New York, USA.. New York: New York : Cambridge University Press.

Chase, M. R., Raina, K., Bruno, J. and Sugumaran, M. (2000). Purification, characterization and molecular cloning of prophenoloxidases from Sarcophaga bullata. *Insect Biochem. Mol. Biol.* **30**, 953–967.

Chen, P.-H., Lee, C.-I., Weng, Y.-T., Tarn, W.-Y., Tsao, Y.-P., Kuo, P.-C., Hsu, P.-H., Huang, C.-W., Huang, C.-S., Lee, H.-H., et al. (2013). BCAS2 is essential for Drosophila viability and functions in pre-mRNA splicing. *RNA* **19**, 208–218.

Chevignon, G., Thézé, J., Cambier, S., Poulain, J., Da Silva, C., Bézier, A., Musset, K., Moreau, S. J. M., Drezen, J.-M. and Huguet, E. (2014). Functional Annotation of Cotesia congregata Bracovirus: Identification of Viral Genes Expressed in Parasitized Host Immune Tissues. *J. Virol.* **88**, 8795–8812.

Chevignon, G., Cambier, S., Da Silva, C., Poulain, J., Drezen, J.-M., Huguet, E. and Moreau, S. J. (2015). Transcriptomic response of Manduca sexta immune tissues to parasitization by the bracovirus associated wasp Cotesia congregata. *Insect Biochem. Mol. Biol.* **62**, 86–99.

Chevignon, G., Periquet, G., Gyapay, G., Vega-Czarny, N., Musset, K., Drezen, J.-M. and Huguet, E. (2018). Cotesia congregata Bracovirus Circles Encoding PTP and Ankyrin Genes Integrate into the DNA of Parasitized Manduca sexta Hemocytes. *J. Virol.* **92**, e00438-18.

Clark, K. D., Lu, Z. and Strand, M. R. (2010). Regulation of melanization by glutathione in the moth Pseudoplusia includens. *Insect Biochem. Mol. Biol.* **40**, 460–467.

Cole, T. J., Beckage, N. E., Tan, F. F., Srinivasan, A. and Ramaswamy, S. B. (2002). Parasitoid–host endocrine relations: self-reliance or co-optation? *Insect Biochem. Mol. Biol.* **32**, 1673–1679.

Collison, E., Hird, H., Cresswell, J. and Tyler, C. (2016). Interactive effects of pesticide exposure and pathogen infection on bee health–a critical analysis. *Biol. Rev.* **91**, 1006–1019.

Contreras-Garduno, J., Lanz-Mendoza, H., Franco, B., Nava, A., Pedraza-Reyes, M. and Canales-Lazcano, J. (2016). Insect immune priming: ecology and experimental evidences. *Ecol. Entomol.* **41**, 351–366.

Costa, A., Jan, E., Sarnow, P. and Schneider, D. (2009). The Imd Pathway Is Involved in Antiviral Immune Responses in Drosophila. *PLOS ONE* **4**, e7436.

Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. *J. Exp. Biol.* **222**, jeb194688.

Cox J and Mann M (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*.

Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *Eur. J. Pharmacol.* **500**, 399–411.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* **9**, 46–56.

de Bekker, C., Quevillon, L. E., Smith, P. B., Fleming, K. R., Ghosh, D., Patterson, A. D. and Hughes, D. P. (2014). Species-specific ant brain manipulation by a specialized fungal parasite. *BMC Evol. Biol.* **14**, 166.

Demas, G. E., Chefer, V., Talan, M. I. and Nelson, R. J. (1997). Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **273**, R1631–R1637.

Derksen, A. C. G. and Granados, R. R. (1988). Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology* **167**, 242–250.

Dheilly, N. M., Maure, F., Ravallec, M., Galinier, R., Doyon, J., Duval, D., Leger, L., Volkoff, A.-N., Missé, D., Nidelet, S., et al. (2015). Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proc. R. Soc. B Biol. Sci.* **282**, 20142773.

Diamond, S. E. and Kingsolver, J. G. (2010). Fitness consequences of host plant choice: a field experiment. *Oikos* **119**, 542–550.

Dillon, C. and Goda, Y. (2005). The actin cytoskeleton: Integrating form and function at the synapse. *Annu. Rev. Neurosci.* **28**, 25–55.

Dolezal, T., Krejcova, G., Bajgar, A., Nedbalova, P. and Strasser, P. (2019). Molecular regulations of metabolism during immune response in insects. *Insect Biochem. Mol. Biol.* **109**, 31–42.

Dow, J. (1992). pH gradients in lepidopteran midgut. *J. Exp. Biol.* **172**, 355–375.

Drezen, J.-M., Bézier, A., Lesobre, J., Huguet, E., Cattolico, L., Periquet, G. and Dupuy, C. (2006). The few virus-like genes of Cotesia congregata bracovirus. *Arch. Insect Biochem. Physiol.* **61**, 110–122.

Dunn, P. E., Bohnert, T. J. and Russell, V. (1994). Regulation of Antibacterial Protein Synthesis Following Infection and During Metamorphosis of Manduca sextaa. *Ann. N. Y. Acad. Sci.* **712**, 117–130.

Dunphy, G. B. and Downer, R. G. (1994). Octopamine, a modulator of the haemocytic nodulation response of non-immune Galleria mellonella larvae. *J. Insect Physiol.* **40**, 267–272.

Dushay, M. S. and Beckage, N. E. (1993). Dose-dependent separation of Cotesia congregata-associated polydnavirus effects on Manduca sexta larval development and immunity. *J. Insect Physiol.* **39**, 1029–1040.

Eaton, D. L. and Bammler, T. K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci. Off. J. Soc. Toxicol.* **49**, 156–164.

Edenfeld, G., Stork, T. and Klämbt, C. (2005). Neuron-glia interaction in the insect nervous system. *Curr. Opin. Neurobiol.* **15**, 34–39.

Edwards, J. P. and Weaver, R. J. (2000). Endocrine changes in lepidopteran larvae - potential challenges to parasitoid development and survival. *Comp. Biochem. Physiol. -- Part B Biochem. Mol. Biol.* **Supplement 1**, S31.

Eggleton, P. and Gaston, K. J. (1990). “Parasitoid” Species and Assemblages: Convenient Definitions or Misleading Compromises? *Oikos* **59**, 417–421.

Eleftherianos, I., Gökçen, F., Felföldi, G., Millichap, P. J., Trenczek, T. E., Ffrench-Constant, R. H. and Reynolds, S. E. (2007). The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect Manduca sexta. *Cell. Microbiol.* **9**, 1137–1147.

Eleftherianos, I., Felföldi, G., Ffrench-Constant, R. H. and Reynolds, S. E. (2009a). Induced nitric oxide synthesis in the gut of Manduca sexta protects against oral infection by the bacterial pathogen Photorhabdus luminescens. *Insect Mol. Biol.* **18**, 507–516.

Eleftherianos, I., Xu, M., Yadi, H., ffrench-Constant, R. H. and Reynolds, S. E. (2009b). Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection. *J. Exp. Biol.* **212**, 1840–1848.

Elrod-Erickson, M., Mishra, S. and Schneider, D. (2000). Interactions between the cellular and humoral immune responses in Drosophila. *Curr. Biol.* **10**, 781–784.

Emanuel, S., Kaiser, M., Pflueger, H.-J. and Libersat, F. (2020). On the Role of the Head Ganglia in Posture and Walking in Insects. *Front. Physiol.* **11**,.

Enayati, A. A., Ranson, H. and Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol.* **14**, 3–8.

Enders, C. K. (2003). Performing multivariate group comparisons following a statistically significant MANOVA. *Meas. Eval. Couns. Dev.* **36**, 40–56.

Erdem, M., Küçük, C., Büyükgüzel, E. and Büyükgüzel, K. (2016). Ingestion of the anti-bacterial agent, gemifloxacin mesylate, leads to increased gst activity and peroxidation products in hemolymph of Galleria mellonella (lepidoptera: pyralidae). *Arch. Insect Biochem. Physiol.* **93**, 202–209.

Espagne, E., Dupuy, C., Huguet, E., Cattolico, L., Provost, B., Martins, N., Poirié, M., Periquet, G. and Drezen, J. M. (2004). Genome sequence of a polydnavirus: insights into symbiotic virus evolution. *Science* **306**, 286–289.

Espagne, E., Douris, V., Lalmanach, G., Provost, B., Cattolico, L., Lesobre, J., Kurata, S., Iatrou, K., Drezen, J.-M. and Huguet, E. (2005). A Virus Essential for Insect Host-Parasite Interactions Encodes Cystatins. *J. Virol.* **79**, 9765–9776.

Ferguson, L. V., Dhakal, P., Lebenzon, J. E., Heinrichs, D. E., Bucking, C. and Sinclair, B. J. (2018). Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. *Funct. Ecol.* **32**, 2357–2368.

Fink, C., Staubach, F., Kuenzel, S., Baines, J. F. and Roeder, T. (2013). Noninvasive analysis of microbiome dynamics in the fruit fly Drosophila melanogaster. *Appl Env. Microbiol* **79**, 6984–6988.

Fleming, J. G. and Summers, M. D. (1991). Polydnavirus DNA is integrated in the DNA of its parasitoid wasp host. *Proc. Natl. Acad. Sci.* **88**, 9770–9774.

Fraune, S. and Bosch, T. C. (2010). Why bacteria matter in animal development and evolution. *Bioessays* **32**, 571–580.

Freeman, J. C., Smith, L. B., Silva, J. J., Fan, Y., Sun, H. and Scott, J. G. (2021). Fitness studies of insecticide resistant strains: lessons learned and future directions. *Pest Manag. Sci.* **77**, 3847–3856.

Freitak, D., Ots, I., Vanatoa, A. and Hörak, P. (2003). Immune response is energetically costly in white cabbage butterfly pupae. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, S220–S222.

Freitak, D., Heckel, D. G. and Vogel, H. (2009). Bacterial feeding induces changes in immune-related gene expression and has trans-generational impacts in the cabbage looper (Trichoplusia ni). *Front. Zool.* **6**, 7.

Fulton, B. B. (1940). The Hornworm Parasite, Apanteles Congregatus Say and the Hyperparasite, Hypopteromalus Tabacum (Fitch)1. *Ann. Entomol. Soc. Am.* **33**, 231–244.

Gad, W. and Kim, Y. 2008 (2008). A viral histone H4 encoded by Cotesia plutellae bracovirus inhibits haemocyte-spreading behaviour of the diamondback moth, Plutella xylostella. *J. Gen. Virol.* **89**, 931–938.

Gade, G., Hoffmann, K. H. and Spring, J. H. (1997). Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.* **77**, 963–1032.

Gal, R. and Libersat, F. (2008). A Parasitoid Wasp Manipulates the Drive for Walking of Its Cockroach Prey. *Curr. Biol.* **18**, 877–882.

Gan, Q., Zhang, X., Zhang, D., Shi, L., Zhou, Y., Sun, T., Jiang, S., Gao, J. and Meng, Y. (2018). BmSUC1 is essential for glycometabolism modulation in the silkworm, Bombyx mori. *Biochim. Biophys. Acta BBA - Gene Regul. Mech.* **1861**, 543–553.

Gao, Y., Kim, J. H., Jeong, I. H., Clark, J. M. and Lee, S. H. (2021). Transcriptomic identification and characterization of genes commonly responding to sublethal concentrations of six different insecticides in the common fruit fly, Drosophila melanogaster. *Pestic. Biochem. Physiol.* **175**, 104852.

Gauthier, J., Boulain, H., van Vugt, J. J. F. A., Baudry, L., Persyn, E., Aury, J.-M., Noel, B., Bretaudeau, A., Legeai, F., Warris, S., et al. (2021). Chromosomal scale assembly of parasitic wasp genome reveals symbiotic virus colonization.

Commun. Biol. **4**, 1–15.

Gelman, D. B., Reed, D. A. and Beckage, N. E. (1998). Manipulation of fifth-instar host (Manduca sexta) ecdysteroid levels by the parasitoid wasp Cotesia congregata. *J. Insect Physiol.* **44**, 833–843.

Gentile, J. E., Carrizales, M. G. and Koleske, A. J. (2022). Control of Synapse Structure and Function by Actin and Its Regulators. *Cells* **11**, 603.

Gershman, A., Romer, T. G., Fan, Y., Razaghi, R., Smith, W. A. and Timp, W. (2021). De novo genome assembly of the tobacco hornworm moth (Manduca sexta). *G3 GenesGenomesGenetics* **11**, jkaa047.

Giovannini, D., Touhami, J., Charnet, P., Sitbon, M. and Battini, J.-L. (2013). Inorganic Phosphate Export by the Retrovirus Receptor XPR1 in Metazoans. *Cell Rep.* **3**, 1866–1873.

Gonzaga, M. O., Kloss, T. G. and Sobczak, J. F. (2017). Host Behavioural Manipulation of Spiders by Ichneumonid Wasps. In *Behaviour and Ecology of Spiders: Contributions from the Neotropical Region* (ed. Viera, C.) and Gonzaga, M. O.), pp. 417–437. Cham: Springer International Publishing.

Gorman, M. J. and Paskewitz, S. M. (2001). Serine proteases as mediators of mosquito immune responses. *Insect Biochem. Mol. Biol.* **31**, 257–262.

Gui, Z., Hou, C., Liu, T., Qin, G., Li, M. and Jin, B. (2009). Effects of insect viruses and pesticides on glutathione S-transferase activity and gene expression in Bombyx mori. *J. Econ. Entomol.* **102**, 1591–1598.

Guillet, G., Harmatha, J., Waddell, T. G., Philogène, B. J. and Arnason, J. T. (2000). Symposium-in-Print Synergistic Insecticidal Mode of Action between Sesquiterpene Lactones and a Phototoxin, α-Terthienyl. *Photochem. Photobiol.* **71**, 111–115.

Gunaratna, R. T. and Jiang, H. (2013). A comprehensive analysis of the Manduca sexta immunotranscriptome. *Dev. Comp. Immunol.* **39**, 388–398.

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130–7139.

Haine, E. R., Moret, Y., Siva-Jothy, M. T. and Rolff, J. (2008). Antimicrobial Defense and Persistent Infection in Insects. *Science* **322**, 1257–1259.

Hall, M., Scott, T., Sugumaran, M., Söderhäll, K. and Law, J. H. (1995). Proenzyme of Manduca sexta phenol oxidase: purification, activation, substrate specificity of the active enzyme, and molecular cloning. *Proc. Natl. Acad. Sci.* **92**, 7764–7768.

Hamilton, R., Siva-Jothy, M. and Boots, M. (2008). Two arms are better than one: parasite variation leads to combined inducible and constitutive innate immune responses. *Proc. R. Soc. B Biol. Sci.* **275**, 937–945.

Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P. and Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proc. Natl. Acad. Sci.* **114**, 9641–9646.

Han, Y. S., Thompson, J., Kafatos, F. C. and Barillas-Mury, C. (2000). Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J.* **19**, 6030–6040.

Harrison, D. A. (2012). The JAK/STAT Pathway. *Cold Spring Harb. Perspect. Biol.* **4**, a011205.

Hart, B. L. (1988). Biological basis of the behavior of sick animals. *Neurosci. Biobehav. Rev.* **12**, 123–137.

Harwood, S. H. and Beckage, N. E. (1994). Purification and characterization of an early-expressed polydnavirus-induced protein from the hemolymph of Manduca sexta larvae parasitized by Cotesia congregata. *Insect Biochem. Mol. Biol.* **24**, 685–698.

Harwood, S. H., Grosovsky, A. J., Cowles, E. A., Davis, J. W. and Beckage, N. E. (1994). An Abundantly Expressed Hemolymph Glycoprotein Isolated from Newly Parasitized Manduca sexta Larvae Is a Polydnavirus Gene Product. *Virology* **205**, 381–392.

Hayakawa, Y., Yazaki, K., Yamanaka, A. and Tanaka, T. (1994). Expression of polydnavirus genes from the parasitoid wasp Cotesia kariyai m two noctuid hosts. *Insect Mol. Biol.* **3**, 97–103.

Hayakawa, Y., Ohnishi, A., Mizoguchi, A. and Yamashika, C. (2000). Distribution of growth-blocking peptide in the insect central nervous tissue. *Cell Tissue Res.* **300**, 459–464.

He, Y., Cao, X., Li, K., Hu, Y., Chen, Y., Blissard, G., Kanost, M. R. and Jiang, H. (2015). A genome-wide analysis of antimicrobial effector genes and their transcription patterns in Manduca sexta. *Insect Biochem. Mol. Biol.* **62**, 23–37.

Heckel, D. G. (2014). Insect Detoxification and Sequestration Strategies. In *Annual Plant Reviews*, pp. 77–114. John Wiley & Sons, Ltd.

Helluy, S. (2013). Parasite-induced alterations of sensorimotor pathways in gammarids: collateral damage of neuroinflammation? *J. Exp. Biol.* **216**, 67–77.

Helluy, S. and Thomas, F. (2003). Effects of Microphallus papillorobustus (Platyhelminthes: Trematoda) on serotonergic immunoreactivity and neuronal architecture in the brain of Gammarus insensibilis (Crustacea: Amphipoda). *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 563–568.

Helluy, S. and Thomas, F. (2010). Parasitic manipulation and neuroinflammation: Evidence from the system Microphallus papillorobustus (Trematoda) - Gammarus (Crustacea). *Parasit. Vectors* **3**, 38.

Hermann, S. L. and Landis, D. A. (2017). Scaling up our understanding of non-consumptive effects in insect systems. *Curr. Opin. Insect Sci.* **20**, 54–60.

Hill, R. W., Wyse, G. A., Anderson, M. and Anderson, M. (2016). *Animal physiology*. 4th ed. Sinauer Associates Massachusetts.

Hillyer, J. F. (2016). Insect immunology and hematopoiesis. *Dev. Comp. Immunol.* **58**, 102–118.

Hoffmann, J. A. (2003). The immune response of Drosophila. *Nature* **426**, 33–38.

Hoffmann, J. A. and Reichhart, J.-M. (2002). Drosophila innate immunity: an evolutionary perspective. *Nat. Immunol.* **3**, 121–126.

Hoover, K., Grove, M., Gardner, M., Hughes, D. P., McNeil, J. and Slavicek, J. (2011). A Gene for an Extended Phenotype. *Science* **333**, 1401–1401.

Horohov, D. W. and Dunn, P. E. (1983). Phagocytosis and nodule formation by hemocytes of Manduca sexta larvae following injection of Pseudomonas aeruginosa. *J. Invertebr. Pathol.* **41**, 203–213.

Huang, J., Wu, S.-F., Li, X.-H., Adamo, S. A. and Ye, G.-Y. (2012). The characterization of a concentration-sensitive α-adrenergic-like octopamine receptor found on insect immune cells and its possible role in mediating stress hormone effects on immune function. *Brain. Behav. Immun.* **26**, 942–950.

Hughes, D. P. and Libersat, F. (2019). Parasite manipulation of host behavior. *Curr. Biol.* **29**, R45–R47.

Hultmark, D. (2003). Drosophila immunity: paths and patterns. *Curr. Opin. Immunol.* **15**, 12–19.

Imler, J.-L. and Zheng, L. (2004). Biology of Toll receptors: lessons from insects and mammals. *J. Leukoc. Biol.* **75**, 18–26.

Isabella, A. J. and Horne-Badovinac, S. (2015). Chapter Nine - Building from the Ground up: Basement Membranes in Drosophila Development. In *Current Topics in Membranes* (ed. Miner, J. H.), pp. 305–336. Academic Press.

Janeway, C. A. (1989). Approaching the Asymptote? Evolution and Revolution in Immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13.

Janssens, L. and Stoks, R. (2014). Reinforcing effects of non-pathogenic bacteria and predation risk: from physiology to life history. *Oecologia* **176**, 323–332.

Jeschke, V., Gershenzon, J. and Vassão, D. G. (2016). A mode of action of glucosinolate-derived isothiocyanates: Detoxification depletes glutathione and cysteine levels with ramifications on protein metabolism in Spodoptera littoralis. *Insect Biochem. Mol. Biol.* **71**, 37–48.

Jiang, H., Vilcinskas, A. and Kanost, M. R. (2010). Immunity in lepidopteran insects. *Adv. Exp. Med. Biol.* **708**, 181–204.

Jiravanichpaisal, P., Lee, B. L. and Söderhäll, K. (2006). Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**, 213–236.

Jungreis, A. M., Barron, N. D. and Johnston, J. W. (1981). Comparative properties of tobacco hornworm, Manduca sexta, carbonic anhydrases. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **241**, R92–R99.

Jutras, I. and Desjardins, M. (2005). Phagocytosis: At the Crossroads of Innate and Adaptive Immunity. *Annu. Rev. Cell Dev. Biol.* **21**, 511–27.

Kaiser, M. and Libersat, F. (2015). The role of the cerebral ganglia in the venom-induced behavioral manipulation of cockroaches stung by the parasitoid jewel wasp. *J. Exp. Biol.* **218**, 1022–1027.

Kaiser, M., Arvidson, R., Zarivach, R., Adams, M. E. and Libersat, F. (2019). Molecular cross-talk in a unique parasitoid manipulation strategy. *Insect Biochem. Mol. Biol.* **106**, 64–78.

Kaneko, Y., Tanaka, H., Ishibashi, J., Iwasaki, T. and Yamakawa, M. (2008). Gene

Expression of a Novel Defensin Antimicrobial Peptide in the Silkworm, <I>Bombyx mori</I>. *Biosci. Biotechnol. Biochem.* **72**, 2353–2361.

Kanost, M. R., Jiang, H. and Yu, X.-Q. (2004). Innate immune responses of a lepidopteran insect, Manduca sexta. *Immunol. Rev.* **198**, 97–105.

Kanost, M. R., Arrese, E. L., Cao, X., Chen, Y.-R., Chellapilla, S., Goldsmith, M. R., Grosse-Wilde, E., Heckel, D. G., Herndon, N. and Jiang, H. (2016). Multifaceted biological insights from a draft genome sequence of the tobacco hornworm moth, Manduca sexta. *Insect Biochem. Mol. Biol.* **76**, 118–147.

Keene, A. C., Krashes, M. J., Leung, B., Bernard, J. A. and Waddell, S. (2006). Drosophila Dorsal Paired Medial Neurons Provide a General Mechanism for Memory Consolidation. *Curr. Biol.* **16**, 1524–1530.

Khan, I., Agashe, D. and Rolff, J. (2017). Early-life inflammation, immune response and ageing.p. 20170125. The Royal Society.

Kim, B. Y., Hui, W. L., Lee, K. S., Wan, H., Yoon, H. J., Gui, Z. Z., Chen, S. and Jin, B. R. (2011). Molecular cloning and oxidative stress response of a sigma-class glutathione S-transferase of the bumblebee Bombus ignitus. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **158**, 83–89.

Kim, M., Jang, D., Yoo, E., Oh, Y., Sonn, J. Y., Lee, J., Ki, Y., Son, H. J., Hwang, O., Lee, C., et al. (2017). Rogdi Defines GABAergic Control of a Wake-promoting Dopaminergic Pathway to Sustain Sleep in Drosophila. *Sci. Rep.* **7**, 11368.

Kingsolver, J. G., Diamond, S. E., Seiter, S. A. and Higgins, J. K. (2012). Direct and indirect phenotypic selection on developmental trajectories in Manduca sexta. *Funct. Ecol.* **26**, 598–607.

Kingsolver, M. B., Huang, Z. and Hardy, R. W. (2013). Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections. *J. Mol. Biol.* **425**, 4921–4936.

Kodrík, D., Bednářová, A., Zemanová, M. and Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects—an update. *Int. J. Mol. Sci.* **16**, 25788–25816.

Koenig, C., Bretschneider, A., Heckel, D. G., Grosse-Wilde, E., Hansson, B. S. and Vogel, H. (8AD). The plastic response of Manduca sexta to host and non-host plants. *Insect Biochem. Mol. Biol.* **63**, 72–85.

Köhler, A., Pirk, C. W. and Nicolson, S. W. (2012). Simultaneous stressors: Interactive effects of an immune challenge and dietary toxin can be detrimental to

honeybees. *J. Insect Physiol.* **58**, 918–923.

Krams, I. A., Krama, T., Moore, F. R., Rantala, M. J., Mänd, R., Mierauskas, P. and Mänd, M. (2015). Resource availability as a proxy for terminal investment in a beetle. *Oecologia* **178**, 339–345.

Krams, I. A., Kecko, S., Jõers, P., Trakimas, G., Elferts, D., Krams, R., Luoto, S., Rantala, M. J., Inashkina, I. and Gudrā, D. (2017). Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. *J. Exp. Biol.* **220**, 4204–4212.

Krashes, M. J. and Waddell, S. (2008). Rapid Consolidation to a radish and Protein Synthesis-Dependent Long-Term Memory after Single-Session Appetitive Olfactory Conditioning in Drosophila. *J. Neurosci.* **28**, 3103–3113.

Krieg, A. (1987). Diseases caused by bacteria and other prokaryotes. *Epizoot. Insect Dis. John Wiley Sons Inc N. Y. NY* 323–355.

Kumar, P., Pandit, S. S. and Baldwin, I. T. (2012). Tobacco rattle virus vector: a rapid and transient means of silencing Manduca sexta genes by plant mediated RNA interference. *PloS One* **7**, e31347.

Kung, V. T. and Wang, J. C. (1977). Purification and characterization of an omega protein from Micrococcus luteus. *J. Biol. Chem.* **252**, 5398–5402.

Kuris, A. M. (1974). Trophic Interactions: Similarity of Parasitic Castrators to Parasitoids. *Q. Rev. Biol.* **49**, 129–148.

Kurtz, J. (2004). Memory in the innate and adaptive immune systems. *Microbes Infect.* **6**, 1410–1417.

Kyriazakis, I., Tolkamp, B. J. and Hutchings, M. R. (1998). Towards a functional explanation for the occurrence of anorexia during parasitic infections. *Anim. Behav.* **56**, 265–274.

Lavine, M. D. and Beckage, N. E. (1995). Polydnaviruses: potent mediators of host insect immune dysfunction. *Parasitol. Today* **11**, 368–378.

Lavine, M. D. and Beckage, N. E. (1996). Temporal pattern of parasitism-induced immunosuppression in Manduca sexta larvae parasitized by Cotesia congregata. *J. Insect Physiol.* **42**, 41–51.

Lavine, M. D. and Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol.* **32**, 1295–1309.

Le, N. T., Asgari, S., Amaya, K., Tan, F. F. and Beckage, N. E. (2003). Persistence and expression of Cotesia congregata polydnavirus in host larvae of the tobacco hornworm, Manduca sexta. *J. Insect Physiol.* **49**, 533–543.

Lee, G. J., Han, G., Yun, H. M., Lim, J. J., Noh, S., Lee, J. and Hyun, S. (2018). Steroid signaling mediates nutritional regulation of juvenile body growth via IGF-binding protein in Drosophila. *Proc. Natl. Acad. Sci.* **115**, 5992–5997.

LeGrand, E. K. and Alcock, J. (2012). Turning up the heat: immune brinksmanship in the acute-phase response. *Q. Rev. Biol.* **87**, 3–18.

Lemaitre, B., Reichhart, J.-M. and Hoffmann, J. A. (1997). Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl. Acad. Sci.* **94**, 14614–14619.

Lepicard, S., Franco, B., Bock, F. de and Parmentier, M.-L. (2014). A Presynaptic Role of Microtubule-Associated Protein 1/Futsch in Drosophila: Regulation of Active Zone Number and Neurotransmitter Release. *J. Neurosci.* **34**, 6759–6771.

Lin, Q., Su, G., Wu, A., Chen, D., Yu, B., Huang, Z., Luo, Y., Mao, X., Zheng, P., Yu, J., et al. (2019). Bombyx mori gloverin A2 alleviates enterotoxigenic Escherichia coli-induced inflammation and intestinal mucosa disruption. *Antimicrob. Resist. Infect. Control* **8**, 189.

Liu, S., Niu, H., Xiao, T., Xue, C., Liu, Z. and Luo, W. (2009). Does Phenoloxidase Contributed to the Resistance? Selection with Butane-Fipronil Enhanced its Activities from Diamondback Moths. *Open Biochem. J.* **3**, 9–13.

Liu, P., Torrens-Spence, M. P., Ding, H., Christensen, B. M. and Li, J. (2013). Mechanism of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases. *Amino Acids* **44**, 391–404.

Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87–98.

Lord, J. C., Anderson, S. and Stanley, D. W. (2002). Eicosanoids mediate Manduca sexta cellular response to the fungal pathogen Beauveria bassiana: A role for the lipoxygenase pathway. *Arch. Insect Biochem. Physiol.* **51**, 46–54.

Louis, F., Bézier, A., Periquet, G., Ferras, C., Drezen, J.-M. and Dupuy, C. (2013). The Bracovirus Genome of the Parasitoid Wasp Cotesia congregata Is Amplified within 13 Replication Units, Including Sequences Not Packaged in the Particles. *J. Virol.* **87**, 9649–9660.

Mangold, C. A. and Hughes, D. P. (2021). Insect Behavioral Change and the Potential Contributions of Neuroinflammation—A Call for Future Research. *Genes* **12**, 465.

Manniello, M. D., Moretta, A., Salvia, R., Scieuzo, C., Lucchetti, D., Vogel, H., Sgambato, A. and Falabella, P. (2021). Insect antimicrobial peptides: potential weapons to counteract the antibiotic resistance. *Cell. Mol. Life Sci.* **78**, 4259–4282.

Marsh, K. J., Wallis, I. R., Andrew, R. L. and Foley, W. J. (2006). The detoxification limitation hypothesis: where did it come from and where is it going? *J. Chem. Ecol.* **32**, 1247–1266.

Martel, R. R. and Law, J. H. (1991). Purification and properties of an ommochrome-binding protein from the hemolymph of the tobacco hornworm, Manduca sexta. *J. Biol. Chem.* **266**, 21392–21398.

McMillan, L. (2022). Proteomic Databases for Manduca-Cotesia study. *Mendeley Data* **V1** DOI: 10.17632/kvxxm2z.1

McMillan, L. E., Miller, D. W. and Adamo, S. A. (2018). Eating when ill is risky: immune defense impairs food detoxification in the caterpillar Manduca sexta. *J. Exp. Biol.* **221**, jeb173336.

Medzhitov, R. (2009). Approaching the Asymptote: 20 Years Later. *Immunity* **30**, 766–775.

Meister, S., Kanzok, S. M., Zheng, X., Luna, C., Li, T.-R., Hoa, N. T., Clayton, J. R., White, K. P., Kafatos, F. C., Christophides, G. K., et al. (2005). Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito Anopheles gambiae. *Proc. Natl. Acad. Sci.* **102**, 11420–11425.

Michel, T., Reichhart, J.-M., Hoffmann, J. A. and Royet, J. (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756–759.

Miles, C. I. and Booker, R. (2000). Octopamine mimics the effects of parasitism on the foregut of the tobacco hornworm Manduca sexta. *J. Exp. Biol.* **203**, 1689–1700.

Miyashita, A. and Adamo, S. A. (2020). Stayin' Alive: Endocrinological Stress Responses in Insects. In *Advances in Invertebrate (NEURO)Endocrinology*, p. Apple Academic Press.

Mohan, P. and Sinu, P. A. (2017). Parasitoid wasp usurps its host to guard its pupa against hyperparasitoids and induces rapid behavioral changes in the parasitized

host. *PLOS ONE* **12**, e0178108.

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin III, D. M. and Galindo-Villegas, J. (2015). Selective manipulation of the gut microbiota improves immune status in vertebrates. *Front. Immunol.* **6**, 512.

Moore, J. (2002). *Parasites and the behavior of animals*. Oxford University Press on Demand.

Morales, C. and Li, Z. (2017). Drosophila canopy b is a cochaperone of glycoprotein 93. *J. Biol. Chem.* **292**, 6657–6666.

Mowlds, P. and Kavanagh, K. (2008). Effect of pre-incubation temperature on susceptibility of Galleria mellonella larvae to infection by Candida albicans. *Mycopathologia* **165**, 5–12.

Mowlds, P., Coates, C., Renwick, J. and Kavanagh, K. (2010). Dose-dependent cellular and humoral responses in Galleria mellonella larvae following β-glucan inoculation. *Microbes Infect.* **12**, 146–153.

Mullen, L. M., Lightfoot, M. E. and Goldsworthy, G. J. (2004). Induced hyperlipaemia and immune challenge in locusts. *J. Insect Physiol.* **50**, 409–417.

Murray, M. and Murray, A. (1979). Anorexia of infection as a mechanism of host defense. *Am. J. Clin. Nutr.* **32**, 593–596.

Nardi, J. B., Pilas, B., Ujhelyi, E., Garsha, K. and Kanost, M. R. (2003). Hematopoietic organs of Manduca sexta and hemocyte lineages. *Dev. Genes Evol.* **213**, 477–491.

Nardi, J. B., Pilas, B., Bee, C. M., Zhuang, S., Garsha, K. and Kanost, M. R. (2006). Neuroglian-positive plasmatocytes of Manduca sexta and the initiation of hemocyte attachment to foreign surfaces. *Dev. Comp. Immunol.* **30**, 447–462.

Neufeld, R. W. and Gardner, R. C. (1990). Data aggregation in evaluating psychological constructs: Multivariate and logical deductive considerations. *J. Math. Psychol.* **34**, 276–296.

Nijhout, H. F. and Williams, C. M. (1974). Control of Moulting and Metamorphosis in the Tobacco Hornworm, Manduca Sexta (L.): Cessation of Juvenile Hormone Secretion as a Trigger for Pupation. *J. Exp. Biol.* **61**, 493–501.

Ojeda-Avila, T., Woods, H. A. and Raguso, R. (2003). Effects of dietary variation on growth, composition, and maturation of Manduca sexta (Sphingidae:

Lepidoptera). *J. Insect Physiol.* **49**, 293–306.

Orso, G., Pendin, D., Liu, S., Tosetto, J., Moss, T. J., Faust, J. E., Micaroni, M., Egorova, A., Martinuzzi, A., McNew, J. A., et al. (2009). Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. *Nature* **460**, 978–983.

Otti, O., Gantenbein-Ritter, I., Jacot, A. and Brinkhof, M. W. (2012). Immune response increases predation risk. *Evolution* **66**, 732–739.

Paniagua Voirol, L. R., Frago, E., Kaltenpoth, M., Hilker, M. and Fatouros, N. E. (2018). Bacterial symbionts in lepidoptera: Their diversity, transmission, and impact on the host. *Front. Microbiol.* **9**, 556.

Park, I., Han, C., Jin, S., Lee, B., Choi, H., Kwon, J. T., Kim, D., Kim, J., Lifirsu, E., Park, W. J., et al. (2011). Myosin regulatory light chains are required to maintain the stability of myosin II and cellular integrity. *Biochem. J.* **434**, 171–180.

Pauchet, Y., Wilkinson, P., Vogel, H., Nelson, D. R., Reynolds, S. E., Heckel, D. G. and Ffrench-Constant, R. H. (2010). Pyrosequencing the Manduca sexta larval midgut transcriptome: messages for digestion, detoxification and defence. *Insect Mol. Biol.* **19**, 61–75.

Persson, B., BENGTSSON-OLIVECRONA, G., ENERBÄCK, S., OLIVECRONA, T. and JÖRNVALL, H. (1989). Structural features of lipoprotein lipase. *Eur. J. Biochem.* **179**, 39–45.

Peters, W. (2012). *Peritrophic Membranes*. Springer Science & Business Media.

Petzoldt, A. G., Coutelis, J.-B., Géminard, C., Spéder, P., Suzanne, M., Cerezo, D. and Noselli, S. (2012). DE-Cadherin regulates unconventional Myosin ID and Myosin IC in Drosophila left-right asymmetry establishment. *Development* **139**, 1874–1884.

Phalnikar, K., Kunte, K. and Agashe, D. (2019). Disrupting butterfly caterpillar microbiomes does not impact their survival and development. *Proc. R. Soc. B Biol. Sci.* **286**, 20192438.

Ponton, F., Lefèvre, T., Lebarbenchon, C., Thomas, F., Loxdale, H., Marché, L., Renault, L., Perrot-Minnot, M.-J. and Biron, D. G. (2006). Do distantly related parasites rely on the same proximate factors to alter the behaviour of their hosts? *Proc. R. Soc. B Biol. Sci.* **273**, 2869–2877.

Poulin, R. (2010). Chapter 5 - Parasite Manipulation of Host Behavior: An Update and

Frequently Asked Questions. In *Advances in the Study of Behavior* (ed. Brockmann, H. J.), Roper, T. J.), Naguib, M.), Wynne-Edwards, K. E.), Mitani, J. C.), and Simmons, L. W.), pp. 151–186. Academic Press.

Qazi, S. and Trimmer, B. A. (1999). The role of inositol 1,4,5-trisphosphate 5-phosphatase in inositol signaling in the CNS of larval Manduca sexta. *Insect Biochem. Mol. Biol.* **29**, 161–175.

Qiu, P., Pan, P. C. and Govind, S. (1998). A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. *Development* **125**, 1909–1920.

Rao, X.-J. and Yu, X.-Q. (2010). Lipoteichoic acid and lipopolysaccharide can activate antimicrobial peptide expression in the tobacco hornworm Manduca sexta. *Dev. Comp. Immunol.* **34**, 1119–1128.

Rao, S., Schieber, A. M. P., O'Connor, C. P., Leblanc, M., Michel, D. and Ayres, J. S. (2017). Pathogen-Mediated Inhibition of Anorexia Promotes Host Survival and Transmission. *Cell* **168**, 503-516.e12.

Rawlings, N. D. and Barrett, A. J. (1994). Families of serine peptidases. *Methods Enzymol.* **244**, 19–61.

Reed, D. A. and Beckage, N. E. (1997). Inhibition of testicular growth and development in Manduca sexta larvae parasitized by the braconid wasp Cotesia congregata. *J. Insect Physiol.* **43**, 29–38.

Renton, K. W. (2001). Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacol. Ther.* **92**, 147–163.

Rewitz, K. F., Rybczynski, R., Warren, J. T. and Gilbert, L. I. (2006). Developmental expression of Manduca shade, the P450 mediating the final step in molting hormone synthesis. *Mol. Cell. Endocrinol.* **247**, 166–174.

Robertson, R. M., Spong, K. E. and Srithiphaphirom, P. (2017). Chill coma in the locust, Locusta migratoria, is initiated by spreading depolarization in the central nervous system. *Sci. Rep.* **7**, 10297.

Rohrbacher, J. (1994). Fictive chewing activity in motor neurons and interneurons of the suboesophageal ganglion of Manduca sexta larvae. *J. Comp. Physiol. A* **175**, 629–637.

Russell, V. W. and Dunn, P. E. (1991). Lysozyme in the midgut of Manduca sexta during metamorphosis. *Arch. Insect Biochem. Physiol.* **17**, 67–80.

Sadd, B. M. and Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proc. R. Soc. Lond. B Biol. Sci.* **273**, 2571–2574.

Savary, S., Drezen, J.-M., Tan, F., Beckage, N. e. and Periquet, G. (1999). The excision of polydnavirus sequences from the genome of the wasp Cotesia congregata (Braconidae, Microgastrinae) is developmentally regulated but not strictly restricted to the ovaries in the adult. *Insect Mol. Biol.* **8**, 319–327.

Schmid-Hempel, P. (2011). Evolutionary Parasitology: The Integrated Study of Infections, Immunology. *Ecol. Genet. Oxf. Univ. Press*.

Schmid-Hempel, P. and Ebert, D. (2003). On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* **18**, 27–32.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (2003). Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in Drosophila. *J. Neurosci.* **23**, 10495–10502.

Schwarz, M. T., Kneeshaw, D. and Kembel, S. W. (2018). The Gut-Associated Microbiome of the Eastern Spruce Budworm Does Not Influence Larval Growth or Survival. *bioRxiv* 330928.

Schwenke, R. A., Lazzaro, B. P. and Wolfner, M. F. (2016). Reproduction–Immunity Trade-Offs in Insects. *Annu. Rev. Entomol.* **61**, 239–256.

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317–321.

Singh, G. J. P. (1986). Hemolymph carbohydrate and lipid mobilization in Locusta migratoria in relation to the progress of poisoning following bioresmethrin treatment. *Pestic. Biochem. Physiol.* **25**, 264–269.

Siva-Jothy, M. T., Moret, Y. and Rolff, J. (2005). Insect Immunity: An Evolutionary Ecology Perspective. In *Advances in Insect Physiology* (ed. Simpson, S. J.), pp. 1–48. Academic Press.

Skinner, W. S., Dennis, P. A., Li, J. P., Summerfelt, R. M., Carney, R. L. and Quistad, G. B. (1991). Isolation and identification of paralytic peptides from hemolymph of the lepidopteran insects Manduca sexta, Spodoptera exigua, and Heliothis virescens. *J. Biol. Chem.* **266**, 12873–12877.

Skowronek, P., Wójcik, Ł. and Strachecka, A. (2021). Fat Body—Multifunctional Insect Tissue. *Insects* **12**, 547.

Snyder, M. J. and Glendinning, J. I. (1996). Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A* **179**, 255–261.

Snyder, M. J., Stevens, J. L., Andersen, J. F. and Feyereisen, R. (1995). Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, Manduca sexta. *Arch. Biochem. Biophys.* **321**, 13–20.

Soares, M. P. and Weiss, G. (2015). The Iron age of host–microbe interactions. *EMBO Rep.* **16**, 1482–1500.

Soares, M. P., Gozzelino, R. and Weis, S. (2014). Tissue damage control in disease tolerance. *Trends Immunol.* **35**, 483–494.

Soares, M. P., Teixeira, L. and Moita, L. F. (2017). Disease tolerance and immunity in host protection against infection. *Nat. Rev. Immunol.* **17**, 83.

Stahlschmidt, Z. R., Acker, M., Kovalko, I. and Adamo, S. A. (2015). The double-edged sword of immune defence and damage control: do food availability and immune challenge alter the balance? *Funct. Ecol.* **29**, 1445–1452.

Steinhaus, E. (1959). Serratia marcescens Bizio as an insect pathogen. *Hilgardia* **28**, 351–380.

Stibbs, H. H. (1985). Changes in brain concentrations of catecholamines and indoleamines in Toxoplasma gondii infected mice. *Ann. Trop. Med. Parasitol.* **79**, 153–157.

Stoltz, D. B. Y. 1990 (1990). Evidence for Chromosomal Transmission of Polydnavirus DNA. *J. Gen. Virol.* **71**, 1051–1056.

Sugumaran, M., Nellaiappan, K. and Valivittan, K. (2000). A New Mechanism for the Control of Phenoloxidase Activity: Inhibition and Complex Formation with Quinone Isomerase. *Arch. Biochem. Biophys.* **379**, 252–260.

Sullivan, K., Fairn, E. and Adamo, S. A. (2016). Sickness behaviour in the cricket Gryllus texensis: Comparison with animals across phyla. *Behav. Processes* **128**, 134–143.

Tanisho, S. (1998). Hydrogen Production by Facultative Anaerobe Enterobacter aerogenes. In *BioHydrogen* (ed. Zaborsky, O. R.), Benemann, J. R.), Matsunaga, T.), Miyake, J.), and San Pietro, A.), pp. 273–279. Boston, MA: Springer US.

Tanji, T., Hu, X., Weber, A. N. R. and Ip, Y. T. (2007). Toll and IMD Pathways

Synergistically Activate an Innate Immune Response in Drosophila melanogaster. *Mol. Cell. Biol.* **27**, 4578–4588.

The UniProt Consortium (2021). UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res.* **49**, D480–D489.

Theilmann, D. A. and Summers, M. D. Y. 1986 (1986). Molecular Analysis of Campoletis sonorensis Virus DNA in the Lepidopteran Host Heliothis virescens. *J. Gen. Virol.* **67**, 1961–1969.

Theopold, U., Rissler, M., Fabbri, M., Schmidt, O. and Natori, S. (1999). Insect Glycobiology: A Lectin Multigene Family in Drosophila melanogaster. *Biochem. Biophys. Res. Commun.* **261**, 923–927.

Thomas, F., Adamo, S. and Moore, J. (2005). Parasitic manipulation: where are we and where should we go? *Behav. Processes* **68**, 185–199.

Thomas, F., Poulin, R. and Brodeur, J. (2010). Host manipulation by parasites: a multidimensional phenomenon. *Oikos* **119**, 1217–1223.

Tokusumi, Y., Tokusumi, T. and Schulz, R. A. (2018). Mechanical stress to Drosophila larvae stimulates a cellular immune response through the JAK/STAT signaling pathway. *Biochem. Biophys. Res. Commun.* **502**, 415–421.

Vallet-Gely, I., Lemaitre, B. and Boccard, F. (2008). Bacterial strategies to overcome insect defences. *Nat. Rev. Microbiol.* **6**, 302.

Viljakainen, L. (2015). Evolutionary genetics of insect innate immunity. *Brief. Funct. Genomics* **14**, 407–412.

Vincent, J. F. V. and Wegst, U. G. K. (2004). Design and mechanical properties of insect cuticle. *Arthropod Struct. Dev.* **33**, 187–199.

Vogel, B. E. and Hedgecock, E. M. (2001). Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented line-shaped junctions. *Development* **128**, 883–894.

Wang, X.-Y., Frohlich, D. R. and Wells, M. A. (1993). Polymorphic cDNAs encode for the methionine-rich storage protein from Manduca sexta. *Insect Mol. Biol.* **2**, 13–20.

Wang, Y., Luo, W. and Reiser, G. (2008). Trypsin and trypsin-like proteases in the brain: Proteolysis and cellular functions. *Cell. Mol. Life Sci.* **65**, 237–252.

Wang, A., Huen, S. C., Luan, H. H., Yu, S., Zhang, C., Gallezot, J.-D., Booth, C. J. and Medzhitov, R. (2016). Opposing effects of fasting metabolism on tissue tolerance in bacterial and viral inflammation. *Cell* **166**, 1512-1525. e12.

Wang, H.-Z., Zhong, X., Gu, L., Li, S.-S., Zhang, G.-R. and Liu, X. (2019). Analysis of the Gynaephora qinghaiensis pupae immune transcriptome in response to parasitization by Thektogaster sp. *Arch. Insect Biochem. Physiol.* **100**, e21533.

Wang, Z., Ye, X., Zhou, Y., Wu, X., Hu, R., Zhu, J., Chen, T., Huguet, E., Shi, M., Drezen, J.-M., et al. (2021). Bracoviruses recruit host integrases for their integration into caterpillar's genome. *PLOS Genet.* **17**, e1009751.

Weinersmith, K. L. (2019). What's gotten into you?: a review of recent research on parasitoid manipulation of host behavior. *Curr. Opin. Insect Sci.* **33**, 37–42.

Weiss, B. and Aksoy, S. (2011). Microbiome influences on insect host vector competence. *Trends Parasitol.* **27**, 514–522.

Willott, E., Wang, X. Y. and Wells, M. A. (1989). cDNA and gene sequence of Manduca sexta arylphorin, an aromatic amino acid-rich larval serum protein. Homology to arthropod hemocyanins. *J. Biol. Chem.* **264**, 19052–19059.

Xu, X.-X., Zhong, X., Yi, H.-Y. and Yu, X.-Q. (2012). Manduca sexta gloverin binds microbial components and is active against bacteria and fungi. *Dev. Comp. Immunol.* **38**, 275–284.

Yamamoto, K., Ozakiya, Y. and Uno, T. (2017). Localization of an Aldo-Keto Reductase (AKR2E4) in the Silkworm Bombyx mori (Lepidoptera: Bombycidae). *J. Insect Sci.* **17**, 94.

Yan, H., Meng, F., Jia, H., Guo, X. and Xu, B. (2012). The identification and oxidative stress response of a zeta class glutathione S-transferase (GSTZ1) gene from Apis cerana cerana. *J. Insect Physiol.* **58**, 782–791.

Yokoi, K., Koyama, H., Minakuchi, C., Tanaka, T. and Miura, K. (2012). Antimicrobial peptide gene induction, involvement of Toll and IMD pathways and defense against bacteria in the red flour beetle, Tribolium castaneum. *Results Immunol.* **2**, 72–82.

Youngblut, N. D., Reischer, G. H., Walters, W., Schuster, N., Walzer, C., Stalder, G., Ley, R. E. and Farnleitner, A. H. (2019). Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nat. Commun.* **10**,.

Yu, X.-Q. and Kanost, M. R. (2003). Manduca sexta lipopolysaccharide-specific immulectin-2 protects larvae from bacterial infection. *Dev. Comp. Immunol.* **27**, 189–196.

Yu, X.-Q., Zhu, Y.-F., Ma, C., Fabrick, J. A. and Kanost, M. R. (2002). Pattern recognition proteins in Manduca sexta plasma. *Insect Biochem. Mol. Biol.* **32**, 1287–1293.

Zambon, R. A., Nandakumar, M., Vakharia, V. N. and Wu, L. P. (2005). The Toll pathway is important for an antiviral response in Drosophila. *Proc. Natl. Acad. Sci.* **102**, 7257–7262.

Zhai, Z., Huang, X. and Yin, Y. (2018). Beyond immunity: The Imd pathway as a coordinator of host defense, organismal physiology and behavior. *Dev. Comp. Immunol.* **83**, 51–59.

Zhang, Z.-T. and Zhu, S.-Y. (2009). Drosomycin, an essential component of antifungal defence in Drosophila. *Insect Mol. Biol.* **18**, 549–556.

Zhang, C.-J., Bowzard, J. B., Anido, A. and Kahn, R. A. (2003). Four ARF GAPs in Saccharomyces cerevisiae have both overlapping and distinct functions. *Yeast* **20**, 315–330.

Zhang, S., Gunaratna, R. T., Zhang, X., Najar, F., Wang, Y., Roe, B. and Jiang, H. (2011). Pyrosequencing-based expression profiling and identification of differentially regulated genes from Manduca sexta, a lepidopteran model insect. *Insect Biochem. Mol. Biol.* **41**, 733–746.

Zhang, S., Cao, X., He, Y., Hartson, S. and Jiang, H. (2014). Semi-quantitative analysis of changes in the plasma peptidome of Manduca sexta larvae and their correlation with the transcriptome variations upon immune challenge. *Insect Biochem. Mol. Biol.* **47**, 46–54.

Zhong, X., Xu, X.-X., Yi, H.-Y., Lin, C. and Yu, X.-Q. (2012). A Toll-Spätzle pathway in the tobacco hornworm, Manduca sexta. *Insect Biochem. Mol. Biol.* **42**, 514–524.

Zhou, J., Yu, L., Zhang, J., Zhang, X., Xue, Y., Liu, J. and Zou, X. (2020). Characterization of the core microbiome in tobacco leaves during aging. *MicrobiologyOpen* **9**, e984.

Zhu, Y., Johnson, T. J., Myers, A. A. and Kanost, M. R. (2003). Identification by subtractive suppression hybridization of bacteria-induced genes expressed in Manduca sexta fat body. *Insect Biochem. Mol. Biol.* **33**, 541–559.

Žitňan, D., Kingan, T. G., Kramer, S. J. and Beckage, N. E. (1995). Accumulation of neuropeptides in the cerebral neurosecretory system of Manduca sexta Larvae Parasitized by the Braconid Wasp Cotesia congregata. *J. Comp. Neurol.* **356**, 83–100.

Zou, Z., Najar, F., Wang, Y., Roe, B. and Jiang, H. (2008). Pyrosequence analysis of expressed sequence tags for Manduca sexta hemolymph proteins involved in immune responses. *Insect Biochem. Mol. Biol.* **38**, 677–682.