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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

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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

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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).

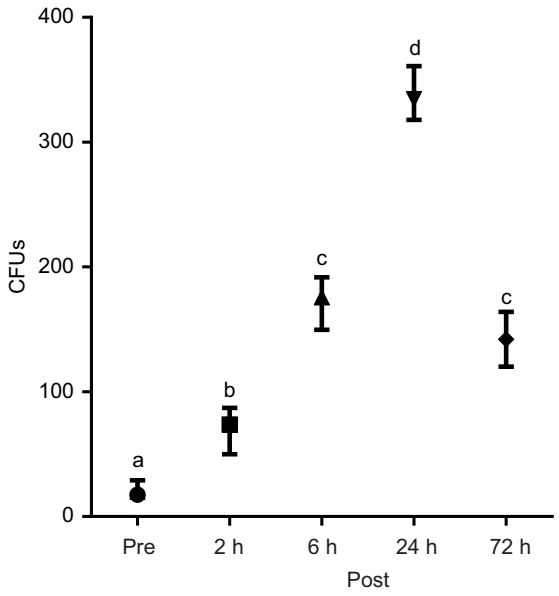

Fig. 1. Bacterial colony forming units cultured from frass collected at different time points. Frass was collected and plated on high nutrient agar from *Manduca sexta* caterpillars prior to oral inoculation of bacteria (Pre) and at several time points after (Post). The number of colony forming units (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.

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).

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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.

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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

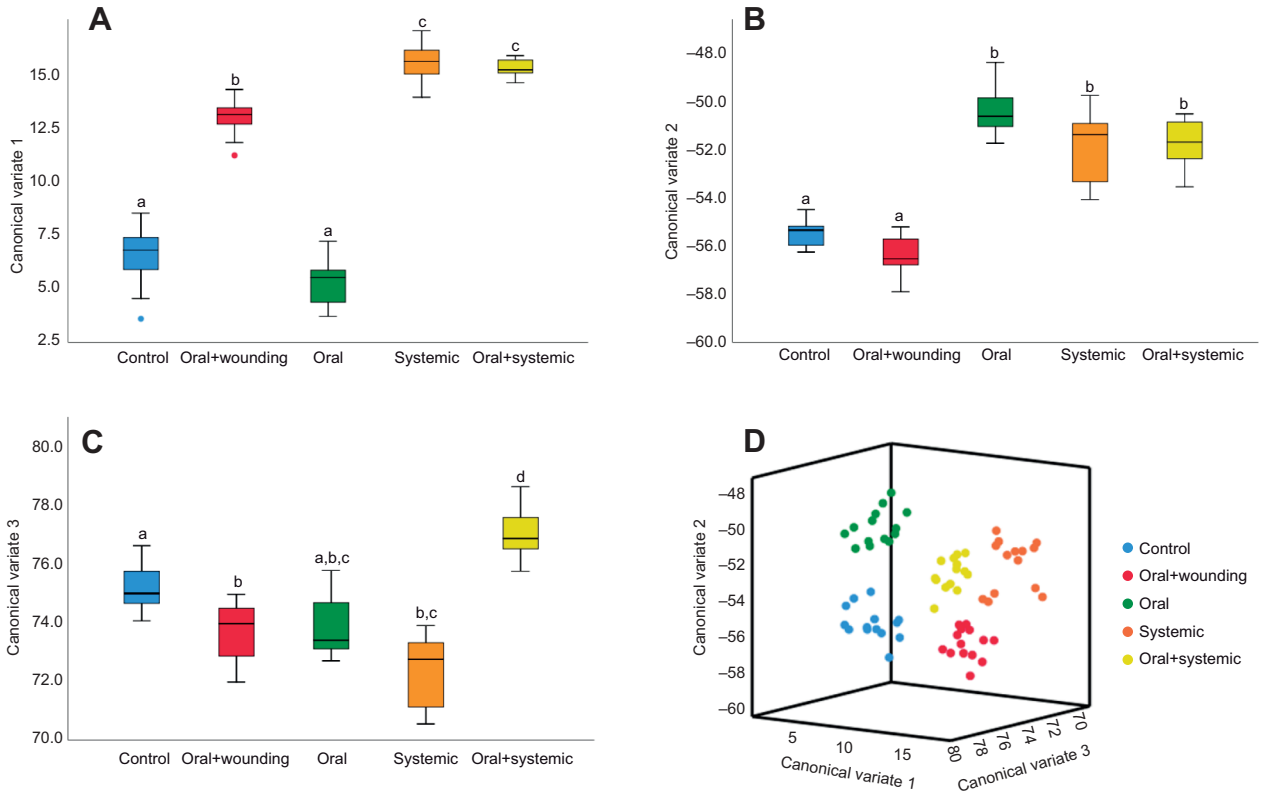

Fig. 2. Canonical variates from the 4 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly 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.

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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

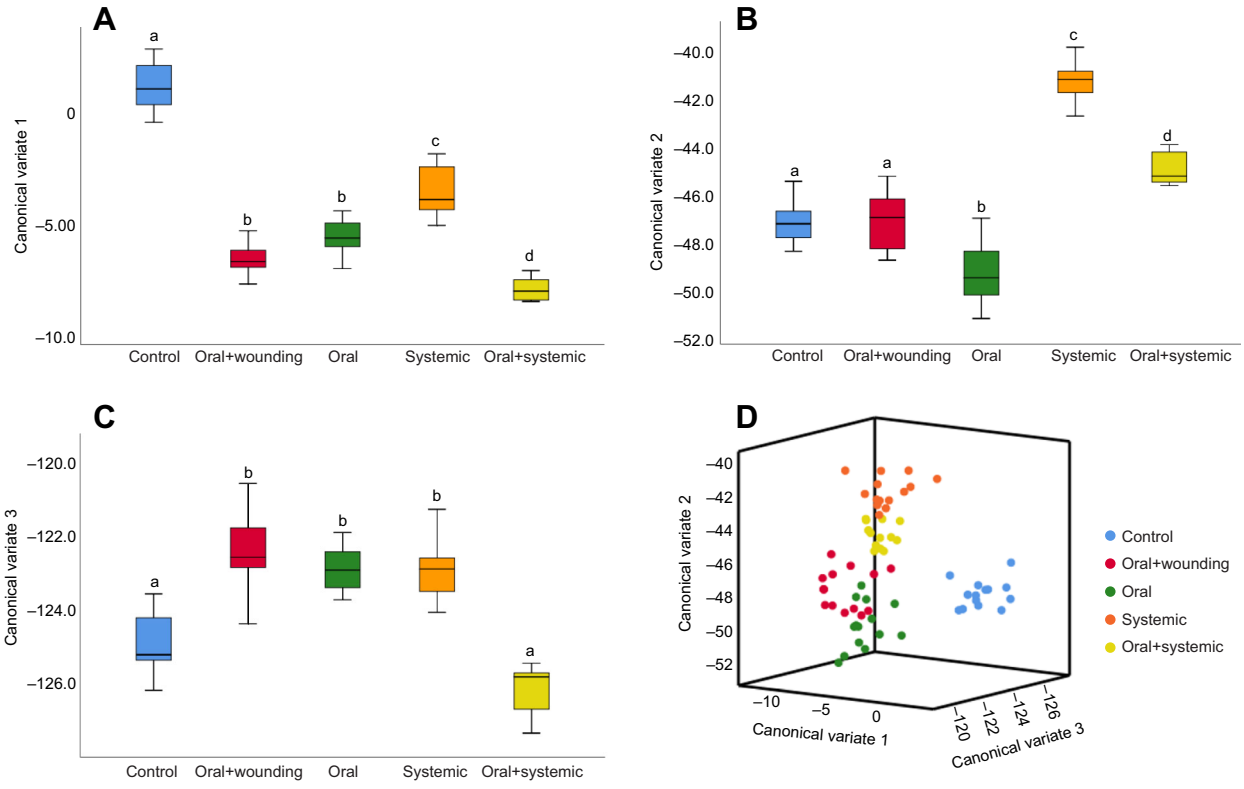

Fig. 3. Canonical variates from the 24 h time point calculated from canonical coefficient factor loadings. (A) Canonical variate 1. Challenge groups with different letters are significantly different from each other (P<0.0002). (B) Canonical variate 2. Challenge groups with different letters are significantly 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.

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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.

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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
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