RESEARCH ARTICLE

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

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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.,

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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$_2$O].

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$_2$O] 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$_2$O. 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

(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, $X_3^2 = 185.2$, 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, $X_1^2 = 71.9$, 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, $X_5^2 = 98.16$, 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, $X_1^2 = 8.68$, 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, $X_1^2 = 3.4$,
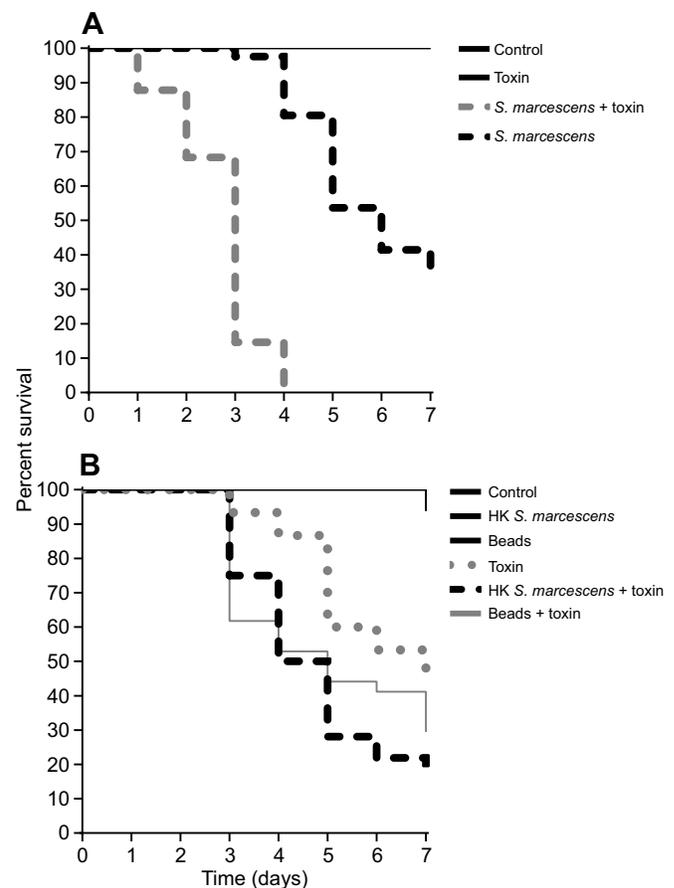

Fig. 1. Effect of immune and toxin challenges on the survival of *Manduca sexta* caterpillars. (A) Effect on survival of a sub-lethal dose of permethrin (toxin; N=41), a live *Serratia marcescens* infection (N=40), and a combined permethrin and live *S. marcescens* challenge (N=42). There was no mortality 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.
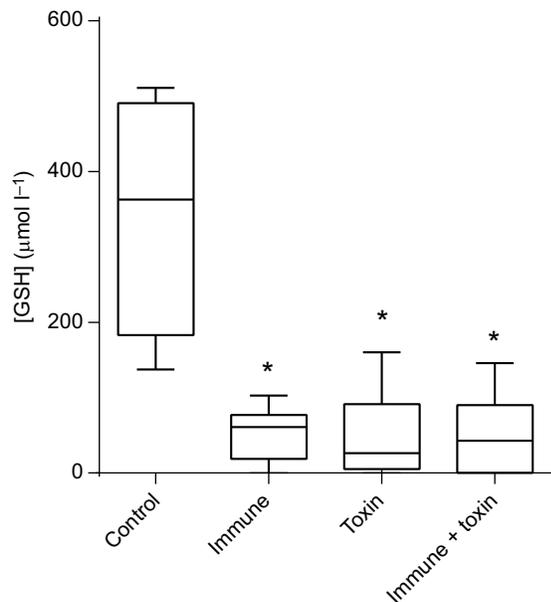
Fig. 2. Effect of immune and toxin challenge on glutathione (GSH) concentration in the hemolymph of *Manduca sexta* caterpillars. Bars represent first and third quartiles, internal bar represents median, and error bars represent the maximum and minimum result. Asterisk represent groups that are significantly different from the control (*P*<0.05). *N*=10 for all groups.
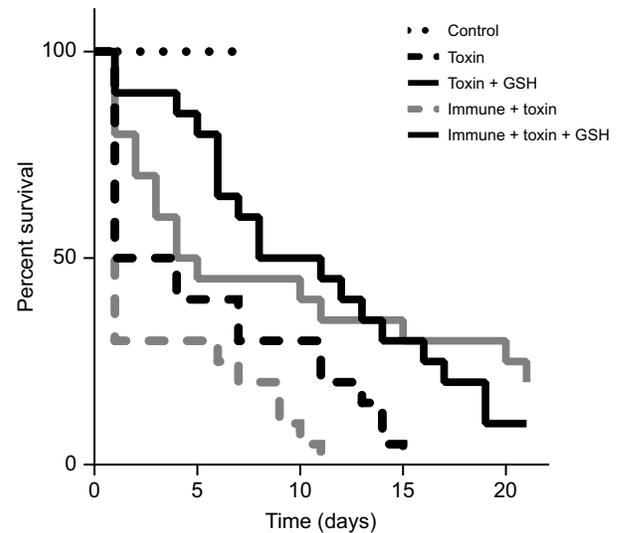

Fig. 3. Effect of supplemental GSH on the survival of *Manduca sexta* caterpillars exposed to immune (i.e. heat-killed *S. marcescens*) and toxin challenges. Effect on survival of a sub-lehtal dose of permethrin, with and without additional GSH (toxin, toxin+GSH), a dual challenge of heat-killed immune 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
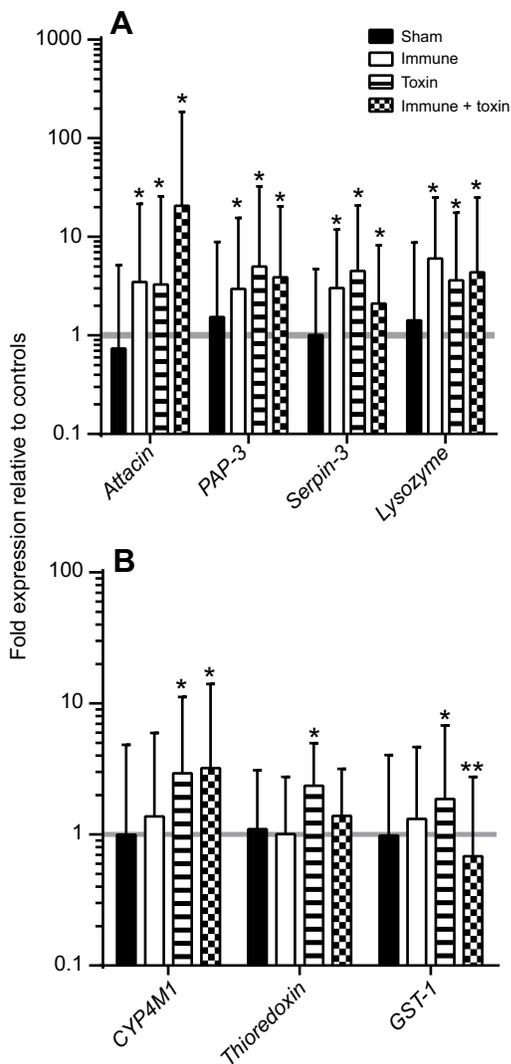
Fig. 4. Gene expression after single and dual immune-toxin challenges in the caterpillar *Manduca sexta*. (A) Expression of immune-related genes in response to immune and toxin challenge. Bars with an asterisk represent those that are upregulated when compared with control animals. Error bars represent 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.

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

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