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RESEARCH ARTICLE

# Reconfiguration of the immune system network during food limitation in the caterpillar *Manduca sexta*

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## ABSTRACT

Dwindling resources might be expected to induce a gradual decline in immune function. However, food limitation has complex and seemingly paradoxical effects on the immune system. Examining these changes from an immune system network perspective may help illuminate the purpose of these fluctuations. We found that food limitation lowered long-term (i.e. lipid) and short-term (i.e. sugars) energy stores in the caterpillar *Manduca sexta*. Food limitation also: altered immune gene expression, changed the activity of key immune enzymes, depressed the concentration of a major antioxidant (glutathione), reduced resistance to oxidative stress, reduced resistance to bacteria (Gram-positive and -negative bacteria) but appeared to have less effect on resistance to a fungus. These results provide evidence that food limitation led to a restructuring of the immune system network. In severely food-limited caterpillars, some immune functions were enhanced. As resources dwindled within the caterpillar, the immune response shifted its emphasis away from inducible immune defenses (i.e. those responses that are activated during an immune challenge) and increased emphasis on constitutive defenses (i.e. immune components that are produced consistently). We also found changes suggesting that the activation threshold for some immune responses (e.g. phenoloxidase) was lowered. Changes in the configuration of the immune system network will lead to different immunological strengths and vulnerabilities for the organism.

**KEY WORDS: Ecological immunology, Nutritional immunology, Lepidopteran, Phenoloxidase, Lysozyme, Attacin, Serpin**

## INTRODUCTION

Animals have evolved against a backdrop of an uncertain food supply. Individuals that can best survive periods of low food availability will have a selective advantage. However, globally suppressing a physiological pathway to save resources may not optimize fitness. Instead, restructuring physiological pathways into different network configurations could minimize resource use while maintaining a high degree of function (Adamo, 2014). One system that seems capable of reconfiguration is the immune system (e.g. during stress, vertebrates, Dhabhar et al., 2012; invertebrates, Adamo, 2014). Although our understanding of immune systems at the network level is limited (Afacan et al., 2012), taking a network perspective can still help us to formulate testable hypotheses about the adaptive purpose of changes in immune function in response to environmental stressors such as food limitation.

The immune system is composed of multiple components that can be separately regulated (e.g. in insects, Beckage, 2008), a necessary precondition for adaptive reconfiguration. The immune system is critical for survival, yet it is energetically expensive (Ardia et al., 2012) and resource intensive (Adamo et al., 2008), suggesting that selection pressure will favor organisms that can reduce resource use while still maintaining function. Recent studies show that reduced food availability induces a mix of positive and negative effects on different immune components (e.g. Ayres and Schneider, 2009; Brunner et al., 2014). For example, brief food deprivation (i.e. 6 h) leads to increased antimicrobial peptide gene transcription in *Drosophila melanogaster*, even in the absence of pathogens (Becker et al., 2010). This response is the opposite of what would be predicted if immune systems gradually decline as resources dwindle. At the molecular level, intracellular immune signaling pathways show intricate interconnections with nutrient signaling pathways (e.g. invertebrates, Becker et al., 2010; vertebrates, Odegaard and Chawla, 2013), providing a mechanism for the complex effects of food deprivation on immunity. Moreover, the existence of these pathways suggests that these complex responses are an evolved response.

We examine the effects of food limitation on the immune system of the last larval instar of the caterpillar *Manduca sexta* (Linnaeus 1763) using a physiological network perspective (Tieri et al., 2010; Cohen et al., 2012). This species is likely to exhibit immune reconfiguration when food is short. *Manduca sexta* caterpillars feed voraciously; their future reproductive success depends on their ability to acquire resources at this developmental stage (Awmack and Leather, 2002). Therefore, when food is limited, *M. sexta* is likely to be under selection pressure to reduce metabolic running costs in order to preserve resources for future reproduction. Moreover, slow larval growth increases predation risk in this species (Kingsolver et al., 2012); therefore, selection is likely to favor animals that maintain growth even when resources are limited. Reducing investment in immune function, then, may be one of the most fitness-sparing options for this species under low food conditions. And, although some insects are able to alter their diet to enhance immune function (Singer et al., 2014), *M. sexta* is a specialist herbivore that typically feeds on a single plant for its entire larval life (Bernays and Woods, 2000). Therefore, this species probably relies on internal reconfigurations to optimize immune function when resources become limited. Furthermore, the immune system of *M. sexta* has the potential for reconfiguration; for example, the expression of some immune genes is sensitive to hormonal concentrations (Zou et al., 2005) and varies depending on the host plant (Koenig et al., 2015).

We assessed the effects of food limitation on what appear to be key points in the immune system network of *M. sexta*, including: a pattern recognition molecule (hemolin), a cytokine that activates antimicrobial gene expression via the toll receptor (spätzle; An et al., 2010; Zhong et al., 2012), antimicrobial molecules (attacin and lysozyme), an antioxidant [glutathione (GSH)], and parts of the phenoloxidase (PO) cascade, including an activator

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**List of symbols and abbreviations**

| | |
|---|---|
| CPC | cetylpridinium chloride |
| $C_q$ | quantitative cycle |
| GSH | glutathione |
| $LD_{50}$ | half-maximal lethal dose |
| PAP-3 | prophenoloxidase activating proteinase-3 |
| PBS | phosphate-buffered saline |
| PO | phenoloxidase |
| qPCR | quantitative real-time PCR |

[prophenoloxidase activating proteinase-3 (PAP-3)] and an inhibitor of PAP-3, Serpin-3 (Fig. 1, see Jiang, 2008; Kanost and Gorman, 2008; Christen et al., 2012; Zhang et al., 2011; Cao et al., 2015; Chevignon et al., 2015). This assessment included different aspects of constitutive immunity, i.e. the branches of the immune system that are maintained regardless of whether there is an ongoing infection (Schmid-Hempel, 2011). These components represent the caterpillar's 'standing army' against pathogens, such as the number of hemocytes (immune cells of the blood; Strand, 2008) and the PO system (Kanost and Gorman, 2008). This assessment also included aspects of the caterpillar's inducible immune system, i.e. those components of the immune system that are synthesized in response to infection (Schmid-Hempel, 2011). These include molecules such as attacin that are capable of killing bacteria (Jiang, 2008). Both vertebrates and invertebrates have examples of constitutive and inducible responses (Schmid-Hempel, 2011). Which type of response requires the most resources depends on pathogen prevalence (Westra et al., 2015), although specific, inducible responses are thought to be less costly overall because of reduced running costs (Lee, 2006, but see Buehler et al., 2009). Finally, our assessment of the *M. sexta* immune system includes host resistance tests using three types of pathogen. Immune assays frequently correlate only weakly with disease resistance (Adamo, 2004a,b), making it difficult to determine the adaptive significance of immune system changes without host resistance tests.

We make three predictions: (1) food limitation will not induce a global immunosuppression, but will cause a shift in the pattern of the immune response (Adamo, 2014); (2) food limitation will lead to a greater reliance on inducible immunity to reduce energetic costs (Lee, 2006); and (3) self-damage from immune-generated molecules (e.g. Sadd and Siva-Jothy, 2006) is also thought to be a major immune system cost (Råberg et al., 1998; Pursall and Rolff, 2012), thus food limitation will reduce immune components that cause collateral damage to an animal's own tissues (e.g. PO activity; Gonzalez-Santoyo and Cordoba-Aguilar, 2012) and upregulate less damaging methods of pathogen control (e.g. antimicrobial peptides; Soares et al., 2014).

## MATERIALS AND METHODS

### Animals

*Manduca sexta* eggs were obtained from Great Lakes Hornworm (MI, USA) and larvae were cultured as outlined by Bell and Joachim (1976). Caterpillars were fed *ad libitum* on a standard artificial diet designed for *M. sexta* (Recorp, Georgetown, ON, Canada). Caterpillars were reared in individual cups (7 cm diameter×10.5 cm height) after the first instar. Caterpillars were kept at 21±2°C and on a 12 h:12 h light:dark cycle, with a relative humidity of 45–70%.

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

### Food limitation

During the molt to the final larval instar (fifth), all food and fecal pellets were removed. At eclosion (fifth instar-day 0), caterpillars were weighed and head capsule diameter was measured using Vernier calipers. Caterpillars were size-matched and assigned into one of three groups: high nutrition (colony diet), low nutrition (1:3 mixture by volume of colony diet and non-nutritive cellulose) and absent nutrition (140 g of cellulose with 500 ml water to give the same approximate consistency as the other diets). There were no initial size or mass differences across the three groups (Table 1). The low-nutrition diet provided sufficient resources for larval development; however, caterpillars on this diet produce smaller adults (Timmins et al., 1988). The absent-nutrition diet provided water for the caterpillars, but had no nutritional value. Caterpillars were given *ad libitum* access to their allotted diet for 2 days.

Because immune function in *M. sexta* changes with age (Eleftherianos et al., 2008; Beetz et al., 2008; Booth et al., 2015), we included a fourth group of caterpillars that were fed on the high-nutrition diet for a single day. We wanted to exclude the possibility that the results from the food-limited animals could be explained by developmental delay.

### Energy resources

Caterpillars were weighed on fifth instar-day 2 prior to sample collection. We measured the immediate energy resources of the caterpillars by assessing the concentration of two sugars, glucose and trehalose, in their hemolymph (i.e. blood). To collect hemolymph samples, the animals were surface sterilized around the dorsal horn with 70% ethanol. The horn was snipped with clean, chilled dissecting scissors and the blood was collected for a maximum of 30 s into an ice-chilled centrifuge tube. Total hemolymph glucose was determined using a Glucose HK Assay Kit (Sigma-Aldrich, St Louis, MO, USA). Hemolymph was diluted 1:8 in ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich). After centrifugation (10,000 ***g*** for 3 min at 4°C), the samples were added to the kit reagents according to the manufacturer's instructions. The absorbance was measured at 340 nm and values were interpolated from glucose standards. To measure trehalose, trehalase (1 mg $ml^{-1}$) was added to the hemolymph/PBS mixture (10:1 hemolymph-PBS: trehalase). The trehalase breaks down the trehalose to glucose. The mixture was incubated for 3 h. Preliminary tests showed that 3 h was sufficient time to convert the trehalose to glucose. The samples were then tested for glucose concentration using the Glucose HK Assay Kit as previously described. To calculate the total trehalose concentration, the total glucose concentration measured previously for a given sample was subtracted from the final glucose concentration found in this assay, and the result was then divided by two (each trehalose molecule supplies two glucose molecules) (Thompson, 2003). Standards and samples were run in triplicate.

Total hemolymph protein was measured using a Bradford assay (Bradford, 1976). Hemolymph was diluted 1:9 in ice-cold PBS and vortexed. Hemolymph was then centrifuged (10,000 ***g*** for 5 min at 4°C) and 30 µl of the supernatant was added to 180 µl of Bradford reagent (Sigma-Aldrich) in a 96-well plate. After a 10 to 15 min incubation period at room temperature, the samples were read in a plate reader set at 590 nm. Bovine albumin (Sigma-Aldrich) was used for standards. Samples were run in duplicate and standards were run in triplicate.

Total lipid content of *M. sexta* caterpillars was estimated using a chloroform-methanol extraction method (Barnes and Blackstock, 1973). Immediately after hemolymph sampling, larvae were supplied with non-nutritive cellulose for 5 h to clear the contents

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

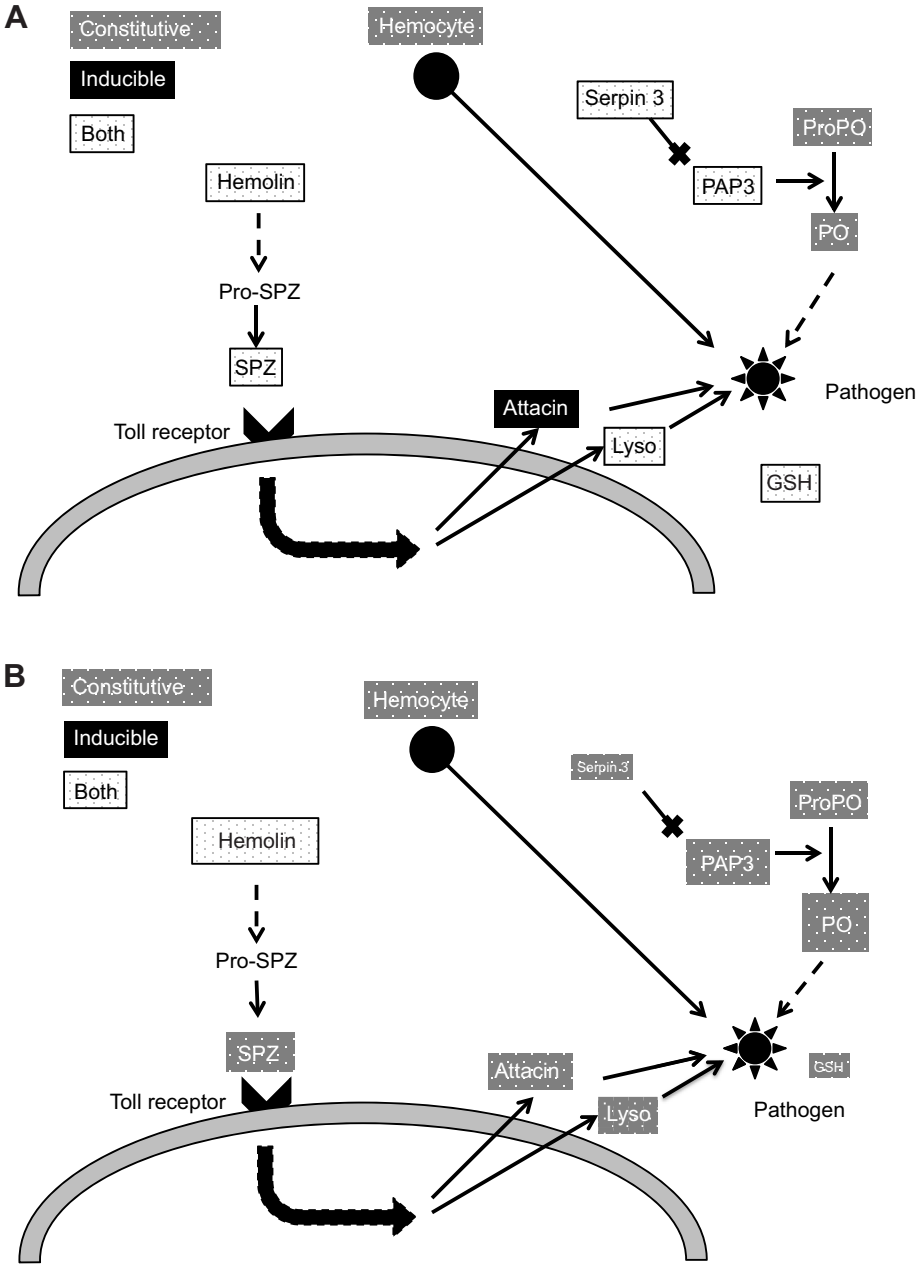


**Fig. 1. Inferred immune system network changes.** A simplified schematic of the *Manduca sexta* immune system (see Jiang, 2008; Jiang et al., 2010; Zhang et al., 2011; Christen et al., 2012; Chevignon et al., 2015) featuring the immune components assessed in this study. The fill pattern for each immune component denotes whether it is important for constitutive responses, capable of induction by an immune challenge, or both. (A) Baseline configuration. (B) Configuration after food limitation (absent-nutrition group). Change in textbox size represents a change in gene expression and/or function in response to food limitation. GSH, glutathione; Lyso, lysozyme; PAP-3, prophenoloxidase activating proteinase-3; PO, phenoloxidase; ProPO, prophenoloxidase; SPZ, spätzle. Dashed lines represent multi-step reactions, while the cross mark represents inhibition.

of the gut. We used the 5 h time point, because after 5 h on the cellulose diet, the frass was composed of cellulose. Larvae were dehydrated at 60°C for 3 days. Next, dry mass was recorded and remains were ground with mortar and pestle. Grinds were transferred to a vial containing 18 ml of a 2:1 chloroform: methanol mixture (Sigma-Aldrich) and shaken for 4 h. The grinds were filtered through Whatman grade 1 filter paper and the flow-through was collected in a vial containing 2 ml of 0.9% NaCl solution (Sigma-Aldrich). Vials were shaken and refrigerated overnight at 4°C. The bottom phase was collected into pre-weighed vials and heated at 60°C for 8 h to evaporate the solvent. The remaining lipid residue was weighed as an estimate of total lipid content.

### Immune assays

Immune assays were performed on subsamples taken from the same hemolymph sample used for the sugar and protein determinations.

Total PO activity in the hemolymph was quantified using a method modified from Hall et al. (1995). Hemolymph was diluted 1:20.6 in ice-cold PBS and vortexed. For each sample, 30 µl of the hemolymph-PBS mixture was added to 180 µl of 0.2 mol $l^{-1}$ L-3,4-dihydroxyphenylalanine (Sigma-Aldrich) and left to incubate for 5 min at room temperature. To activate the zymogen prophenoloxidase, 2 µl of 10% cetylpridinium chloride (CPC; Sigma-Aldrich) was added to each reaction mixture (Saul and Sugumaran, 1987; Hall et al., 1995). CPC induces a confirmation change in prophenoloxidase, exposing the catalytic site of active PO (Hall et al., 1995). The addition of phenylthiocarbamide (Sigma-Aldrich), a potent inhibitor of PO (Ryazanova et al., 2012), prevented a change in absorbance of the reaction mixture, suggesting that the action of CPC was specific for prophenoloxidase. In addition, no change in absorbance was measured when hemolymph was excluded from the well, indicating that the observed activation was not an artifact of CPC



Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

**Table 1. The effect of diet on long-term and short-term energy availability in *Manduca sexta* caterpillars**

| | High (*N*=22) | Low (*N*=25) | Absent (*N*=22) | Age 5-1 (*N*=21) | $F_{3,86}$ | *P* | Dunnett's test |
|---|---|---|---|---|---|---|---|
| Head capsule width (mm) | 5.6±0.2 | 5.6±0.2 | 5.6±0.2 | 5.6±0.2 | 0.47 | 0.71 | n.s. |
| Initial mass (5-0) (g) | 1.15±0.2 | 1.11±0.2 | 1.12±0.21 | 1.13±0.20 | 0.12 | 0.95 | n.s. |
| Final mass (5-2) (g) | 2.98±0.56 | 3.26±0.64 | 1.44±0.27 | 1.90±0.38 | 70.9 | <0.0001 | H=L<br>*P*=0.47<br>H,L>A<br>*P*<0.0001<br>5-1<H,L<br>5-1>A<br>*P*<0.0001 |
| Dry mass (g) | 0.45±0.09 | 0.43±0.11 | 0.16±0.03 | 0.29±0.09 | 62.2 | <0.0001 | H=L<br>*P*=0.96<br>H,L>A<br>*P*<0.0001<br>5-1<H,L<br>5-1>A<br>*P*<0.0001 |
| Total lipid (g) | 0.039±0.016 | 0.029±0.10 | 0.007±0.014 | 0.018±0.007 | 23.3 | <0.0001 | H>L<br>*P*=0.04<br>H,L>A<br>*P*<0.0001<br>5-1<H,L<br>5-1>A<br>*P*=0.02 |
| Hemolymph protein (mg ml$^{-1}$) | 4.37±1.2 | 3.12±0.84 | 2.89±0.87 | 3.27±0.62 | 12.3 | <0.0001 | H>L<br>*P*=0.001<br>H>A<br>*P*<0.0001<br>L=A<br>*P*=0.73<br>5-1=L,A<br>*P*>0.5<br>5-1<H<br>*P*<0.0001 |
| Trehalose (mg ml$^{-1}$) | 3.16±0.55 | 2.78±0.38 | 1.14±0.56 | 3.31±0.59 | 79.8 | <0.0001 | H>L<br>*P*=0.05<br>H,L>A<br>*P*<0.0001<br>5-1=H<br>*P*=0.99<br>5-1>L,A<br>p<0.008 |
| Glucose (mg ml$^{-1}$) | 0.57±0.48 | 0.21±0.15 | 0.048±0.03 | 0.68±0.45 | 17.4 | <0.0001 | H>L<br>*P*=0.01<br>H,L>A<br>*P*<0.0001<br>5-1=H<br>*P*=0.99<br>5-1>L,A<br>*P*<0.001 |

Multivariate analysis, followed by univariate *post hoc* tests. Values are means±s.d. H, high nutrition; L, low nutrition; A, absent; 5-1, fifth instar-day 1.

alone. Immediately after the addition of CPC, the wells were mixed for 5 s and the change in absorbance at 490 nm was recorded for 10 min at room temperature. A standard curve within the linear range of the assay was run concurrently on each microplate using mushroom tyrosinase (Sigma-Aldrich), which captured the linear range of the reaction. Mushroom tyrosinase catalyzes the formation of the same dopachrome as PO (Xie et al., 2003).

Total lysozyme-like activity in the hemolymph was determined using a turbidity assay modified from Adamo (2004b). Hemolymph samples were diluted 1:4 in ice-cold PBS and vortexed. For each sample, 10 µl of the hemolymph–PBS mixture was transferred to a microplate well containing 180 µl of a *Micrococcus luteus* cell wall suspension in PBS (12.5 mg 25 ml$^{-1}$, Sigma-Aldrich). Microplate wells were mixed for 5 s and the change in absorbance at 450 nm was recorded for 10 min at room temperature. Lysozyme (Sigma) standards in the linear range of the assay were run concurrently.

GSH levels were assessed by measuring both reduced and oxidized GSH species (GSH/GSSG; Cayman Chemicals, Ann Arbor, MI, USA). Although GSH is not typically thought of as part of the immune response, it plays a key role in buffering animals against both pathogen toxins (Aucoin et al., 1995) and the oxidative stress that can be generated by PO activity (Clark et al., 2010). We include it here as a likely component of 'infection tolerance' (Stahlschmidt et al., 2015). Hemolymph was deproteinated immediately after collection by centrifuging at 18,845 ***g*** for 10 min at 4°C and then adding the sample to an equal amount of metaphosphoric acid (0.1 g ml$^{-1}$, Sigma-Aldrich). After incubating

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

**Table 2. Forward and reverse primer sequences for target immune-related genes and reference genes**

| Gene | Primer sequence | Reference |
|---|---|---|
| *Spätzle* | F: 5′-AGTGACCAGTAAGCCAACAAC-3′ R: 5′-CGAAGAGCCAAACGAGTAAATG-3′ | An et al., 2010 |
| *Hemolin* | F: 5′-CAACCAAGCAACAACACAGG-3′ R: 5′-CAGCACAGGCATCTTCTCC-3′ | An et al., 2010 |
| *Attacin-1* | F: 5′-GCAGGCGACGACAAGAAC-3′ R: 5′-ATGCGTGTTGGTAAGAGTAGC-3′ | An et al., 2010 |
| *Lysozyme* | F: 5′-GTGTGCCTCGTGGAGAATG-3′ R: 5′-ATGCCTTGGTGATGTCGTC-3′ | An et al., 2010 |
| *PAP-3* | F: 5′-ATTAAGCTGTTGTGTGGTG-3′ R: 5′-CGGGTGCGGTATTGTCTTC-3′ | Jiang et al., 2003 |
| *Serpin-3* | F: 5′-GATTCCTCGCGATTCGATGC-3′ R: 5′-CATTTACGTCATTAAGTTTCATG-3′ | Zhu et al., 2003 |
| *MsA** | F: 5′-CTCTTCCAGCCTTCCTTCCT-3′ R: 5′-ACAGGTCCTTACGGATGTCG-3′ | Schwartz et al., 1993 |
| *RpL17A** | F: 5′-TCCGCATCTCACTGGGTCT-3′ R: 5′-CACGGCAATCACATACAGGTT-3′ | Rewitz et al., 2006 |
| *MsS3** | F: 5′-CGCGAGTTGACTTCGGT-3′ R: 5′-GCCGTTCTTGCCCTGTT-3′ | Zhu et al., 2003 |
| *Ubiquitin** | F: 5′-AAAGCCAAGATTCAAGATAAG-3′ R: 5′-TTGTAGTCGGATAGCGTGCG-3′ | Kumar et al., 2012 |
| *βFTZ-F1** | F: 5′-CGTGCCTCCTACAATAGTGCTT-3′ R: 5′-AATCCCTAGCGGTTACTGACC-3′ | MacWilliam et al., 2015 |
| *G3PDH** | F: 5′-CGATTAAGGAACTTGAGGACG-3′ R: 5′ATAAGGAAGCGGATGCAAGG -3′ | Mészaros and Morton, 1996 |

All primers were previously reported. Asterisks (*) indicate candidate reference genes.

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. Absorbance was measured at 405 nm. Samples and standards were run in triplicate.

Total hemocyte counts were prepared by diluting hemolymph in ice-cold anti-coagulant (1:10). The anticoagulant consisted of 140 mmol $l^{-1}$ NaCl, 5 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ HEPES, 8 mmol $l^{-1}$ EDTA and 0.16 mmol $l^{-1}$ phenylthiocarbamide dissolved in double-distilled water (Sigma-Aldrich). Diluted hemolymph was placed on a Fuchs-Rosenthal hemocytometer. Cells were counted using phase contrast microscopy.

### RNA extraction and cDNA generation

In *M. sexta*, the fat body makes the majority of immune proteins (Zhang et al., 2014). Fat body was harvested from fifth instar-day 2 caterpillars that had been placed on the three different diets (constitutive expression) at eclosion. To assess inducible expression (i.e. after an immune challenge), a second set of *M.* sexta larvae were randomly assigned to one of the three diets, and injected with 60 µl mixture of heat-killed *Serratia marcescens* [Gram-negative bacterium, Microkwik culture, Carolina Biological, 1/10 half-maximal lethal dose ($LD_{50}$)], *Bacillus cereus* (Gram-positive bacterium, Microkwik culture, Carolina Biological, 1/10 $LD_{50}$) or *Beauveria bassiana* (strain GHA, fungus, 1/10 $LD_{50}$, BotaniGard 22WP; Laverlam, Butte, MT, USA) on fifth instar-day 1. The $LD_{50}$ values had been determined during the disease resistance studies. We also included a control group of unchallenged caterpillars fed the high-nutrition diet.

Fat body was excised from fifth instar-day 2 larvae and tissue was immediately stabilized in 100 µl of RNA*later* (Qiagen, Hilden, Germany) and stored at −80°C. All samples were processed for RNA extraction and cDNA generation in adherence with guidelines to preserve sample quality (Taylor et al., 2010). RNA extraction was performed using the RNeasy Lipid Tissue Mini kit (Qiagen). All steps adhered to the manufacturer's instructions and included a DNase 1 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 was determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only samples with an A260/A280 ratio greater than 1.8 were used. The concentration of extracted total RNA samples ranged from 23 to 290 ng $µl^{-1}$. cDNA was synthesized using iScript (Bio-Rad, Hercules, CA, USA) and samples were stored at −20°C.

### Quantitative real-time PCR

To determine the relative expression of genes encoding immune-related proteins, cDNA levels were measured by quantitative real-time PCR (qPCR). The qPCR experiments used previously reported primer sets for *M. sexta* genes (Table 2). 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 each biological sample and gene, a 25 µl reaction mixture was prepared containing 1 µl of sample cDNA, 12.5 µl of 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 $dH_2O$. Reactions were performed in 96-well plates with a CFX96 real-time system (Bio-Rad). The reaction proceeded as follows: initial denaturation (95°C: 3 min), followed by 45 cycles of denaturation (95°C: 30 s), annealing (52°C: 45 s) and extension (72°C: 30 s). 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 each biological sample, qPCR reactions were performed in duplicate and for each gene target no-template controls were run. 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.

### Reference gene selection

For constitutive expression of immune-related genes, *RpL17A* was selected as a reference gene (Rewitz et al., 2006) after testing the stability of three candidate reference genes suggested from the literature. Studying expression during an immune challenge required additional controls and treatment groups. For this reason, we selected the most stable reference genes from six candidate reference genes used in previous studies in *M. sexta*: *RpL17A*, *actin* (*MsA*), *ribosomal protein S3* (*MsS3*), *ubiquitin*, *beta FTZ-F1* and *glycerol-3-phosphate dehydrogenase* (*G3PDH*) (Table 2). We used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes (Andersen et al., 2004) (i.e. *ubiquitin* and *beta FTZ-F1*), using the $C_q$ values of five biological samples for each candidate reference gene, for each treatment.

### Disease resistance tests

High- and low-nutrition diet caterpillars were injected on fifth instar-day 2 with the $LD_{50}$ of one of three pathogens: *Serratia marcescens* (Gram-negative bacterium, $2×10^5$ cells), *Bacillus cereus* (Gram-positive bacterium, $2×10^4$ cells) or *Beauveria*

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

*bassiana* (strain GHA, fungus, approximately $1\times10^5$ conidia). Caterpillars were maintained on their assigned diet during the live challenge. All three are common pathogens of insects (Fuxa and Tanada, 1987). Caterpillars were checked daily for mortality, or for the exposure of the dorsal vessel (i.e. the start of metamorphosis; Dominick and Truman, 1984). Data were censored at 10 days for the bacterial studies and 12 days for the fungus. By this time, the infected caterpillars had either died or had reached the start of metamorphosis. However, because absent-nutrition caterpillars only live approximately 1 week even when uninfected, the injected absent-nutrition caterpillars were compared with uninfected absent-nutrition controls. Caterpillars fed the absent-nutrition diet were given half the $LD_{50}$, as they weigh approximately half that of high-nutrition caterpillars by fifth instar-day 2 (Table 1). Survival was plotted as Kaplan–Meier survival plots and tested using Mantel–Cox (log-rank) tests.

### Paraquat challenge

Paraquat generates oxidative stress in animals, and drives down GSH levels in *M. sexta* (Guillet et al., 2000). We tested the ability of caterpillars on the different diets to withstand a paraquat challenge. Fifth instar-day 2 caterpillars were injected with 2 µl of 2.5 mg 100 $\mu l^{-1}$ paraquat dichloride hydrate (Fluka, Germany) in double-distilled water. Caterpillars fed the absent-nutrition diet were given half this dose, as they weigh approximately half that of high-nutrition controls by fifth instar-day 2 (Table 1). Caterpillars were observed for mortality for 10 days.

### Test of gut integrity

Food limitation could lead to leakage from the gut into the hemocoel. To test for this possibility, caterpillars were fed on the three diets for the first 2 days of the fifth instar. On fifth instar-day 2, food and fecal pellets were removed. After 1 h, 5 $mm^3$ cubes of each diet were injected with 2 µl of food coloring (Club House no. 900806843R, containing red FD and C no. 40 and FD and C red no. 3) and these were presented to the caterpillars. A similar method was used to test the integrity of the gut in *D. melanogaster* (Rera et al., 2012). Five hours later, caterpillars began to produce pinkish fecal pellets. Blood was collected from each caterpillar by snipping the dorsal horn. One hundred microliters of hemolymph was added to 800 µl of a supersaturated solution of phenylthiocarbamide in water. After vortexing, the absorbance was measured at 500 nmol $l^{-1}$. Preliminary tests found that this wavelength was the most sensitive to the presence of dye in the blood. After blood collection, caterpillars were chilled and dissected carefully to avoid damaging the gut.

### Statistical analysis

Data were analyzed using SPSS (v. 21.0) and GraphPad Prizm (v. 5.0); the qPCR data were analyzed using the CFX Manager v. 3.1 (Bio-Rad). The normalized expression (ΔΔCq) was calculated as the relative quantity of the target gene normalized to the quantities of the reference genes. The *P*-values comparing the expression values relative to controls were calculated using a modified *t*-statistic using the normalized expression values (http://www.bio-rad.com/

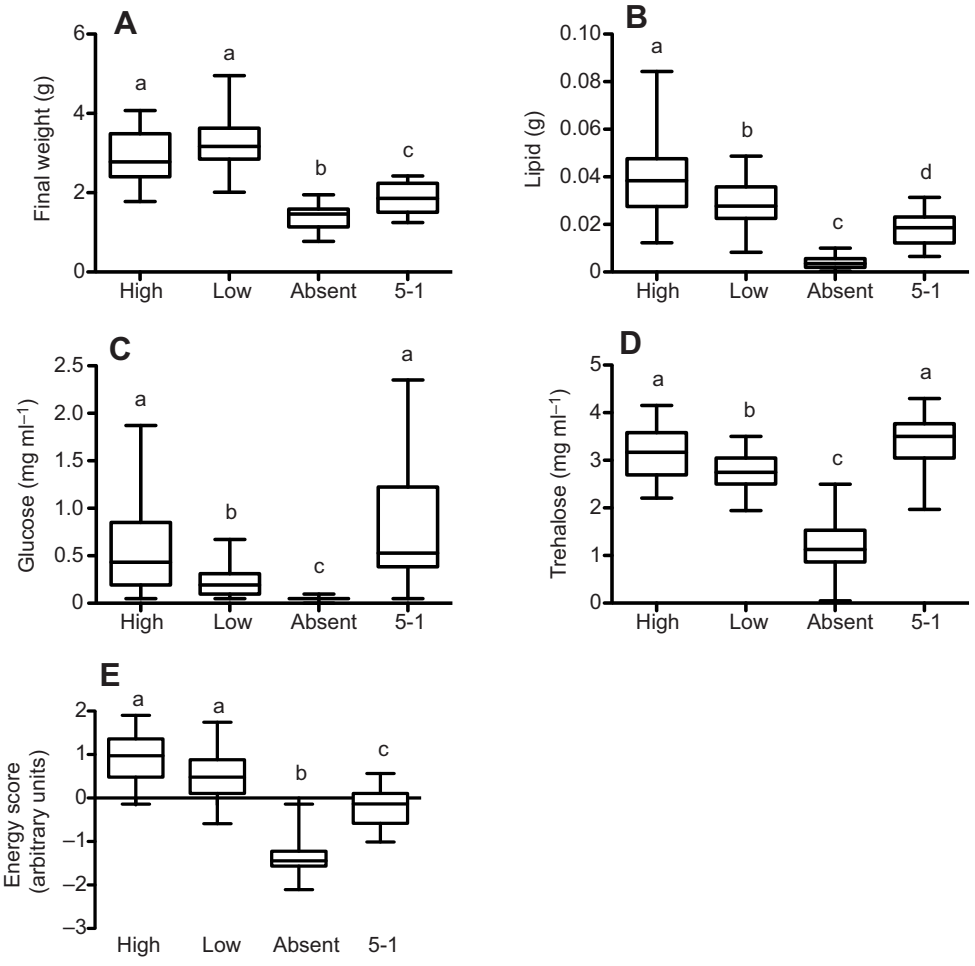


**Fig. 2. Effect of diet and age on energy-related variables in *Manduca sexta* caterpillars.** (A) Final mass; (B) total lipid content; (C) hemolymph glucose concentration; (D) hemolymph trehalose concentration; and (E) energy factor score. 5-1, fifth instar-day 1. Bars represent first and third quartiles, with the internal line representing the median. Error bars denote maximum and minimum values. Values that are significantly different have dissimilar letters above the bars. See Results for statistics; sample sizes are given in Table 1.

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

**Table 3. Standardized canonical discriminant function coefficients**

| | Function 1 | Function 2 |
|---|---|---|
| Glutathione | 0.89 | 0.16 |
| Lysozyme-like activity | –0.25 | –0.38 |
| Hemocyte number | –0.41 | 0.83 |
| Phenoloxidase activity | 0.77 | 0.24 |

webroot/web/pdf/lsr/literature/10021337.pdf, p. 131). Other data were tested for normality using Shapiro–Wilk tests. Outliers were removed using the outlier labeling rule (Hoaglin and Iglewicz, 1987). Two or fewer data points were removed from the total data set/variable. *Post hoc* treatment comparisons used Dunnett's test. Although there were no significant differences in initial mass (see Table 1), changes in mass and lipid content were examined using initial mass as a covariate. Other variables, which were measured per microliter hemolymph, were not corrected for initial mass. Energy measures were found to be correlated; therefore, we used a principal components analysis (PCA) with a direct Oblimin rotation. Final mass, dry mass, total lipid, trehalose and total protein were combined in a PCA. Glucose concentration was omitted as some caterpillars in the absent-nutrition group had undetectable levels. The first factor explained 62.3% of the variance and had an eigenvalue of 3.115. All other eigenvalues had scores less than 1. Therefore, the first factor was used as the 'energy availability score'. To test for changes in immune configuration, hemolymph samples that had values for all four immune measures (i.e. hemocyte number, lysozyme activity, PO activity and GHS concentration) were normalized and log transformed, resulting in a normal distribution and homoscedasticity across groups. A linear discriminant analysis was performed.

## RESULTS

### Energy resources

Diet had a significant effect on all energy-related variables [multivariate ANOVA (MANOVA), Pillai's trace=1.9, $F_{24,243}$=17.8, $P$<0.0001, followed by *post hoc* univariate tests; Fig. 2, Table 1]. Caterpillars fed an absent-nutrition diet were lighter (Fig. 2A) and had less lipid (Fig. 2B) than the other groups (Table 1). They had less than one-tenth of the hemolymph glucose concentration, and approximately one-third the hemolymph trehalose concentration, of caterpillars fed a high nutrient diet (Fig. 2C,D). Total hemolymph protein was also reduced in absent-nutrition caterpillars and low-nutrition caterpillars compared with caterpillars fed a high-nutrition diet (Table 1). Diet affected the PCA-derived energy score ($F_{3,90}$=87.2, $P$<0.0001; Fig. 1E). The high- and low-nutrition groups had the highest scores (Dunnett's $P$=0.18), followed by the nutrient absent group (high and low nutrition>nutrient absent, $P$<0.0001).

The high-nutrition group ($n$=21) developed more quickly than the low-nutrition group, reaching the wandering stage 7.1±0.9 days into the fifth instar. The low-nutrition group required 9.0±1.1 days ($n$=19; $F_{1,39}$=32.2, $P$<0.0001). The absent-nutrition group ($n$=10) did not reach the wandering stage, but died 6.8±1.0 days into the fifth instar.

### Immune assays

Diet had a significant effect on immune function (MANOVA, Pillai's trace=0.77, $F_{12,231}$=6.65, $P$<0.0001, followed by *post hoc* univariate tests). High-nutrition caterpillars ($n$=22) had significantly more hemocytes per microliter hemolymph than low-nutrition caterpillars ($n$=24; $F_{3,78}$=8.7, $P$<0.0001; Dunnett's, $P$=0.016; Fig. 3A). Absent-nutrition caterpillars ($n$=15) had the same number of hemocytes as high-nutrition caterpillars ($P$=0.99), and had significantly more than low-nutrition caterpillars ($P$=0.045). Absent-nutrition caterpillars had significantly higher levels of total PO activity compared with high- ($F_{3,78}$=4.84, $P$=0.004, Dunnett's $P$=0.026; Fig. 3B) or low-nutrition caterpillars ($P$=0.027). There was no significant difference between high- and low-nutrition caterpillars ($P$=1.0). Diet did not have a significant effect on lysozyme-like activity ($F_{3,78}$=1.05, $P$=0.37; Fig. 3C). However, absent-nutrition caterpillars had significantly lower concentrations of GSH in their hemolymph compared with low- and high-nutrition caterpillars ($F_{3,78}$=17.76, both $P$<0.0001; Fig. 3D). Low- and high-nutrition caterpillars did not differ in GSH hemolymph concentration ($P$=0.41). Absent-nutrition caterpillars were also less resistant to paraquat ($n$=56) compared

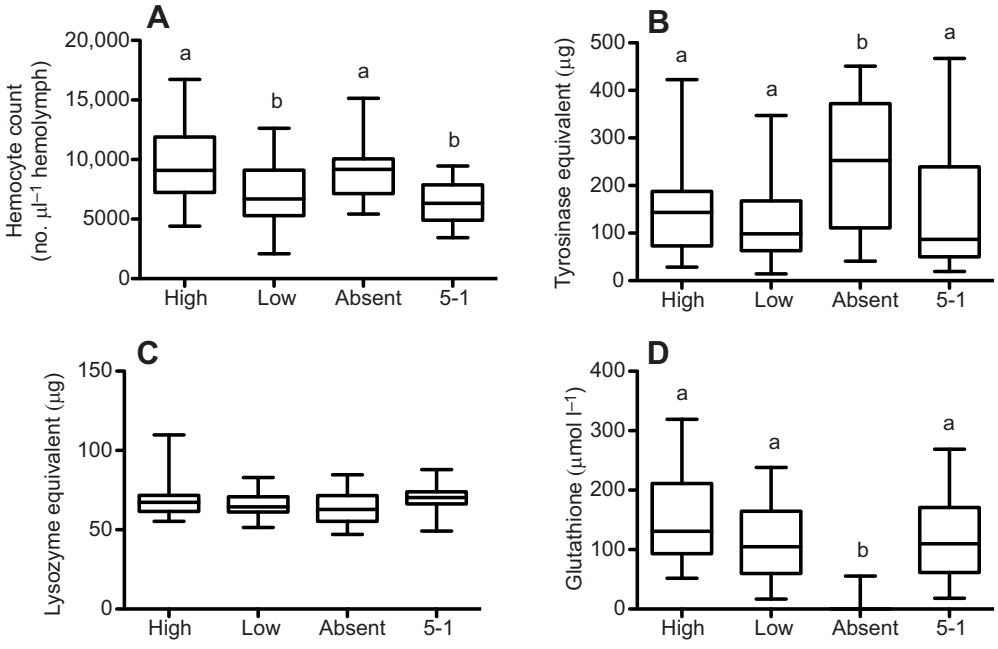


**Fig. 3. Effect of diet and age on immune function in *Manduca sexta* caterpillars.** (A) Hemocyte count; (B) phenoloxidase activity; (C) lysozyme-like activity; and (D) hemolymph glutathione concentration. 5-1, fifth instar-day 1. Bars represent first and third quartiles, with the internal line representing the median. Error bars denote maximum and minimum values. Values that are significantly different have dissimilar letters above the bars. See 'Immune assays' section in the Results for statistics and sample sizes and 'Changes due to age' for additional *post hoc* analyses.



Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

with high- ($n$=62) and low-nutrition groups ($n$=62; Mantel-Cox, $\chi^2_2$=102.5, $P$<0.0001). The high-nutrition group was significantly more resistant to paraquat than the low-nutrition group (Mantel–Cox, $\chi^2_1$=14.06, $P$=0.0002).

A linear discriminant analysis using the four immune responses found two significant canonical discriminant factors (factor 1, Wilks' lambda, $P$<0.0001; factor 2, Wilks' lambda, $P$=0.052; Table 3). The first factor (eigenvalue=1.32) accounted for 89.6% of the variance. The two functions derived from these factors were able to correctly classify 67.2% of the caterpillars ($n$=61) into one of the three food treatments.

We found no correlation between lysozyme-like activity and PO activity under high-nutrition conditions (Spearman's rho, $r$=+0.06, n.s., $N$=24). However, there was a positive correlation between the two assays in both the low-nutrition group (Spearman's rho, $r$=+0.724, $P$<0.0001, $n$=26) and the absent-nutrition group (Spearman's rho, $r$=+0.438, $P$=0.038, $n$=20). Moreover, there was a negative correlation between GSH concentrations and PO activity under high-nutrition conditions (Spearman's rho, $r$=−0.41, $P$=0.05, $n$=24), and no correlation in either the low-nutrition (Spearman's rho $r$=−0.24, $P$=0.24) or absent-nutrition (Spearman's rho, $r$=−0.09, $P$=0.72, $n$=20) groups, even though GSH is important for reducing PO-generated damage (Clark et al., 2010).

## Immune gene expression

### Constitutive

The relative expression of six immune-related genes did not differ between caterpillars fed the low- ($n$=6) and high-nutrition diets ($n$=6; Fig. 4). However, caterpillars fed the absent-nutrition diet showed a significant upregulation of *spätzle* (5.4-fold, $P$=0.005), *hemolin* (13.6-fold, $P$=0.006), *attacin-1* (6.2-fold, $P$=0.04) and *PAP-3* (5.4-fold, $P$<0.0001) compared with caterpillars reared on the high-nutrition diet (Fig. 4).

### Inducible

An immune challenge caused an upregulation of *hemolin* (54.9-fold, $P$=0.02), *attacin-1* (23.1-fold, $P$=0.007), *PAP-3* (19.2-fold, $P$=0.003), *lysozyme* (5.1-fold, $P$=0.01) and *spätzle* (5.3-fold, $P$=0.006) in high-nutrition caterpillars ($n$=5) relative to unchallenged high-nutrition controls ($n$=5). Only *serpin-3* was unchanged ($P$=0.83). Low-nutrition caterpillars ($n$=5) showed the same pattern of upregulation, but the upregulation was smaller than that of controls, and for some genes (e.g. *lysozyme*, $P$=0.25 and *spätzle*, $P$=0.25) the upregulation was not statistically significant (Fig. 5). Absent-nutrition caterpillars ($n$=5) given an immune challenge had higher levels of expression of *hemolin* (11.4-fold, $P$=0.005) than did unchallenged high-nutrition controls. However, this increase is similar to the increase in *hemolin* gene expression found in unchallenged absent-nutrition caterpillars relative to high-nutrition controls (13.6-fold, $P$=0.006; Fig. 4). Absent-nutrition caterpillars did not upregulate the other assessed immune genes (Fig. 5). Instead, *serpin-3* (0.06-fold, $P$=0.048) was significantly downregulated relative to high-nutrition controls.

## Host resistance tests

Caterpillars fed the high-nutrition diet ($n$=30) had greater resistance to the Gram-negative bacterium *S. marcescens* than caterpillars fed the low-nutrition diet ($n$=21; log-rank Mantel–Cox, $\chi^2_1$=30.64, $P$<0.0001; Fig. 6). High-nutrition caterpillars ($n$=52) also had greater resistance to the Gram-positive bacterium *B. cereus* than low-nutrition caterpillars ($n$=46; log-rank Mantel–Cox, $\chi^2_1$=30.48, $P$<0.0001; Fig. 6). High- ($n$=50) and low-nutrition ($n$=52) animals did not differ in susceptibility to fungal challenge (log-rank Mantel-Cox, $\chi^2_1$=0.06, $P$=0.80). Injection of caterpillars fed the absent-nutrition diet exhibited reduced lifespan compared with uninjected controls ($n$=20) for all three pathogens (*S. marcescens*, $n$=15, log-rank Mantel–Cox, $\chi^2_1$=34.9, $P$<0001; *B. cereus*, $n$=45, log-rank Mantel–Cox, $\chi^2_1$=52.6, $P$<0.0001; *B. bassiana*, $n$=41, log-rank Mantel–Cox, $\chi^2_1$=28.0, $P$<0.0001).

## Changes due to age

Younger caterpillars (fifth instar-day 1, $n$=21) were lighter and had less lipid than fifth instar-day 2 caterpillars fed the high-nutrition (i.e. normal) diet (Fig. 2, Table 1). However, the concentrations of trehalose and glucose in the hemolymph were the same as on day 2 (Table 1). Fifth instar-day 1 caterpillars had significantly higher concentrations of trehalose and glucose in their hemolymph than caterpillars fed a low- or absent-nutrition diet (Table 1). Younger caterpillars, like older fifth instar-day 2 caterpillars, had higher GSH

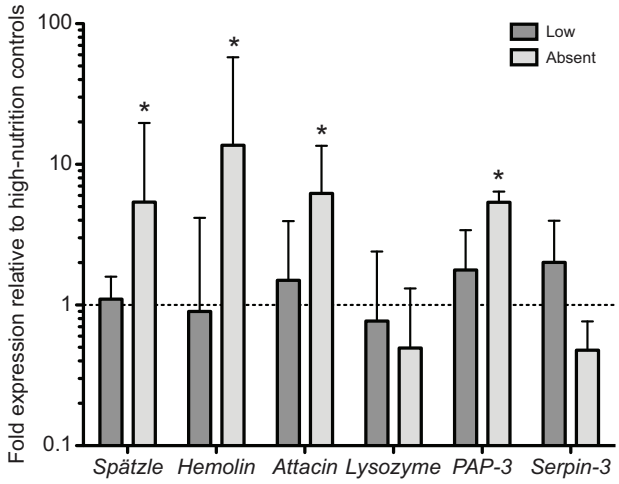


**Fig. 4. Constitutive immune gene expression varies according to diet in *Manduca sexta* caterpillars.** Bars represent means and error bars represent the s.e.m. ($n$=6 for all groups). Asterisks represent values statistically different ($P$<0.05) from high-nutrition controls, whose expression values have been normalized to 1.

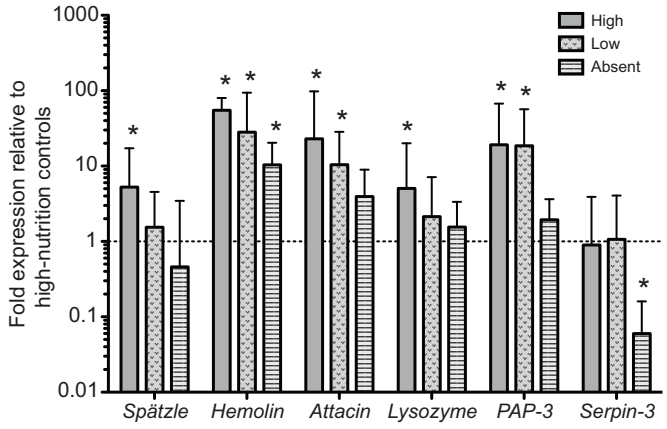


**Fig. 5. Inducible immune gene expression varies according to diet in *Manduca sexta* caterpillars.** Bars represent means and error bars represent the s.e.m. ($n$=5 for all groups). Asterisks represent values statistically different ($P$<0.05) from unchallenged high-nutrition controls, whose expression values have been normalized to 1.

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

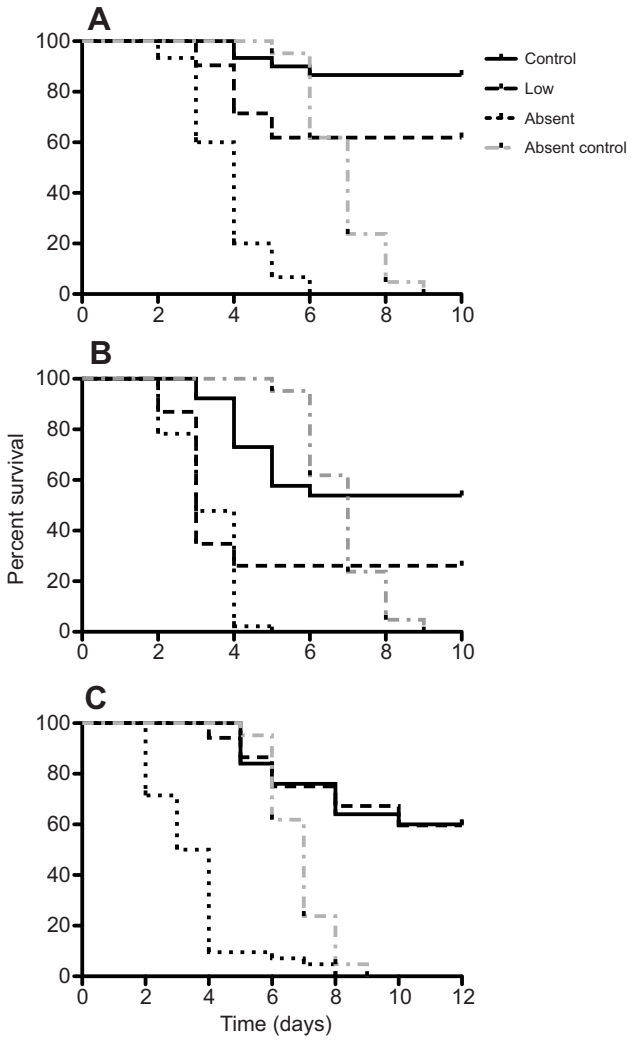


**Fig. 6. Effect of diet on disease resistance in *Manduca sexta* caterpillars.** (A) Gram-negative bacterium *Serratia marcescens*. (B) Gram-positive bacterium *Bacillus cereus*. (C) Fungus *Beauveria bassiana*. Solid line represents high-nutrition controls and the dashed line represents the low-nutrition group. Dotted line represents the absent-nutrition group; Dash and line represents the absent-nutrition control group. See Results, 'Host resistance tests', for statistics and sample sizes.

levels in their hemolymph than absent-nutrition caterpillars (Dunnett's $P<0.001$; Fig. 3), but had fewer hemocytes than older caterpillars (Dunnett's $P=0.001$; Fig. 3). Their PO activity (Dunnett's $P=0.99$) and lysozyme activity (Dunnett's $P=1.0$) were the same as those of fifth instar-day 2 caterpillars (Fig. 3).

### Test of gut integrity

Caterpillars from all three diets had bright red digestive tracts ($n=10$ per group). However, the fat body, Malpighian tubules and other tissues showed no traces of dye. There was no evidence of dye in any of the blood samples ($F_{2,29}=0.66$, $P=0.53$). Therefore, there is no evidence for a lack of integrity of the gut in the food-limited caterpillars.

## DISCUSSION

As predicted, declining resource availability did not result in a global suppression of immune function, but instead resulted in a shift in the pattern of the immune response (Fig. 1). Caterpillars faced with the most severe reduction in resources showed increased, not decreased, constitutive expression of some immune genes (Fig. 5) and greater constitutive immune function (Fig. 4) than did better-fed controls.

Contrary to our prediction, severe food limitation induced a shift towards constitutive immune function and away from inducible responses (Fig. 1). For example, constitutive expression of *lysozyme* was maintained on the absent-nutrition diet, and this result was corroborated by the maintenance of lysozyme-like activity in this group. However, lysozyme, which is both a constitutive and an inducible immune component (He et al., 2015), was not upregulated during an immune challenge in the absent-nutrition group, suggesting a decrease in lysozyme as an inducible response (Fig. 5). Furthermore, some immune components that were inducible in well-fed controls were constitutively upregulated in the absent-nutrition group (e.g. *attacin-1* and *spätzle*), but were not induced upon challenge (Figs 4 and 5). In fact, absent-nutrition caterpillars showed little, if any, inducible responses, especially when the fact that their immune genes are constitutively upregulated was taken into account (Figs 4 and 5). Food-deprived *Drosophila* (Becker et al., 2010) and mosquitoes (*Aedes aegypti*; Price et al., 2015) also show a similar shift towards a more constitutive expression of inducible antimicrobial genes (Becker et al., 2010), suggesting that this may be a common response in insects.

Prioritizing the early constitutive response may give resource-strapped caterpillars the best disease resistance when investment in immunity is reduced. Insects, including *M. sexta* (Dunn and Drake, 1983), respond to the presence of pathogens using a two-stage process (Haine et al., 2008; Johnston et al., 2014). The initial response, using constitutively available components (e.g. PO activity), removes most of the invading pathogens and the slower, inducible responses mop up any pathogens that escape the original attack (Haine et al., 2008). The initial constitutive response may be the immune response that is the most important for survival (e.g. Lochmiller and Deerenberg, 2000; Dubovskiy et al., 2013).

We had predicted that PO activity would be reduced with food limitation because it can be damaging (Gonzalez-Santoyo and Cordoba-Aguilar, 2012); however, this important constitutive response was enhanced (Figs 1, 3). Food limitation also induced changes that we speculate would lead to a lowered threshold for PO activation. The gene for PAP-3, an activator of the PO cascade, was upregulated constitutively with severe food limitation, whereas the gene for serpin-3, an inhibitor of PAP-3 (Kanost and Gorman, 2008), and probably of spätzle as well (Christen et al., 2012), was one of the few genes not upregulated by food limitation. Furthermore, during an immune challenge, *serpin-3* was downregulated in absent-nutrition caterpillars, possibly decreasing inhibition of PO activation. In other words, absent-nutrition caterpillars were in a pro-inflammatory state (Paddibhatla et al., 2010), potentially allowing them to have an augmented early response to pathogens; however, this remains to be empirically tested. Once an immune response was initiated, further amplification was probably prevented, in part, by the lack of upregulation of the cytokine *spätzle*.

The importance of buttressing the first line of defense appears to outweigh the potential costs of this new configuration. Flies (*Scathophaga stercoraria*) selected for high PO levels show decreased longevity when starved (Schwarzenbach and Ward, 2006), suggesting that the shifts observed in the absent-nutrition caterpillars carry costs. The reduction in GSH, which buffers insects against reactive molecules (Guillet et al., 2000), such as those generated by PO (Gonzalez-Santoyo and Cordoba-Aguilar, 2012), likely contributes to these costs. However, by reducing GSH levels,



Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 706-718 doi:10.1242/jeb.132936

food-limited caterpillars may maximize the ability of immune-generated reactive molecules to destroy pathogens because GSH can also inhibit PO activation if levels are locally high (Clark et al., 2010). Unfortunately, this strategy will also increase the risk of damage to the host, and probably helps explain why absent-nutrition caterpillars were more sensitive to paraquat, a chemical that generates oxidative stress (Halliwell and Gutteridge, 2007). GSH requires the sulphur-containing amino acid cysteine, and this amino acid is not abundant in the diet of leaf-eating caterpillars (Barbehenn et al., 2013); therefore, a reduction in GSH with food limitation may be unavoidable.

Network reconfiguration also led to changes that may mitigate costs. For example, hemolin, an immune recognition molecule (Jiang, 2008), was the only immune gene tested that showed both constitutive and inducible upregulation in nutrient absent caterpillars (Fig. 5). Hemolin can bind to bacterial lipopolysaccharides and by facilitating interactions with lipophorins, lead to the detoxification of these compounds (Schmidt et al., 2010). This may be an important alternative method of reducing pathogen-generated damage if molecules such as GSH are unavailable. Hemolin is a much larger molecule than GSH (it contains 411 amino acids, including several that contain sulphur; Ladendorff and Kanost, 1991), but it may be less likely to dampen early immune responses than GSH. The changes in hemolin and GSH may reflect network changes designed to both enhance PO activity and to mitigate some of the costs due to damage. Food limitation also activates the stress response in insects (Davenport and Evans, 1984), and leads to the upregulation of molecules that buffer homeostasis (e.g. heat shock proteins; King and MacRae, 2015). This stress response may also mitigate some of the costs of reducing GSH and increasing PO.

Hemocyte number exhibited a complex response to food limitation. Hemocyte numbers declined in low-nutrition caterpillars, but were maintained in absent-nutrition caterpillars. The decline in hemocyte number in low-nutrition caterpillars may be immunologically significant; *M. sexta* showed reduced encapsulation when fed a low-nutrition diet (Diamond and Kingsolver, 2011). However, changes in hemocyte number are difficult to interpret. Hemocytes can reside in the hematopoietic organ (Nardi et al., 2003), as well as in the hemolymph and along the surface of organs, and can be released upon challenge (Strand, 2008). Therefore, absent-nutrition animals may have maintained their hemocyte number by mobilizing hemocyte stores, not because they maintained hemocyte production. If this second possibility were correct, this would also suggest a shift from an inducible to constitutive response type.

We found no evidence of a negative trade-off between PO activity and lysozyme-like activity under control or food-limited conditions, although this has been reported for some insects (e.g. Cotter et al., 2004, 2011; Ardia et al., 2012). This difference may be due, in part, to species differences, but may also be due to methodological issues, especially regarding how PO activity is measured (e.g. Laughton and Siva-Jothy, 2011; Moreno-Garcia et al., 2013; Kohlmeier et al., 2015). Lysozyme appears to regulate PO activity during an immune response (e.g. Rao et al., 2010; Zdybicka-Barabas et al., 2014), but this does not mean that there is a negative trade-off between the two at the systemic level.

Both experimental diet treatments lowered the energy resources of the caterpillars (Table 1). The values found in this study are within the range of those found in earlier studies on food-limited *M. sexta* (Kramer et al., 1978; Siegert, 1986, 1987; Siegert et al., 1993; Bedoyan et al., 1992; Ismail and Matsumura, 1992; Meyer-Fernandes et al., 2001; Beetz et al., 2008). Surprisingly, the caterpillars on the low-nutrition diet managed to maintain their mass, including their dry mass, despite the decline in lipid stores. Possibly the digestive system of the low-nutrition caterpillars increased in mass as it attempted to extract resources from the low-nutrition food. Such an increase occurs in third-instar larval *M. sexta* when food consumption is reduced, although only under conditions of high predation risk (Thaler et al., 2012).

Absent-nutrition caterpillars were not exhibiting 'pathological' dysregulated functions, despite the low levels of some measures (Table 1). Although 2 days of starvation produced substantial physiological effects, caterpillars will develop normally if re-fed at this point (Cymborowski et al., 1982). Moreover, the caterpillars typically lived approximately 1 week on the cellulose diet. Therefore they were not 'dying' on fifth instar-day 2. Furthermore, food-limited caterpillars were not simply developmentally delayed. Caterpillars fed the low- or absent-nutrition diet showed physiological differences (e.g. lower blood sugar levels) from younger (i.e. fifth instar-day 1) caterpillars (e.g. Figs 2, 3, Table 1).

### Effects on disease resistance

Food limitation led to mixed effects on disease resistance in this study (Fig. 6) and mixed effects have been found in other insects (Ponton et al., 2013), including an increase in disease resistance to some pathogens (e.g. *D. melanogaster*, Ayres and Schneider, 2009; tent caterpillars *Malacosoma pluviale californicum*, Myers et al., 2011; crickets, *Gryllus texensis*, Kelly and Tawes, 2013; *Galleria mellonella*, Kangassalo et al., 2015). Pathogens differ in their sensitivity to different components of the host's immune system (Chambers et al., 2012). Therefore, as immune system networks realign, resistance against specific pathogens will also change. Presumably the configuration of well-fed caterpillars represents the optimal configuration for their present pathogen environment. However, our host resistance tests might underestimate the ability of food-limited caterpillars to resist disease. It takes between 2 and 9 days for the pathogens used in this study to kill their hosts. During this time, resources may become increasingly scarce for the food-limited caterpillars. Therefore, the host resistance tests assess the effectiveness of the immune configuration at the time of injection, as well as the configuration as it changes due to age (e.g. Booth et al., 2015) or continued nutrient loss (i.e. the food-limited groups).

### Immune system network shifts

Although our data are consistent with the reconfiguration hypothesis, this requires further testing. The PO system, for example, consists of many activators and inhibitors (Kanost and Gorman, 2008). For this reason it is difficult to predict the effect of changes in the expression of a single activator and inhibitor on the PO cascade without knowing the effects on the other components. A similar issue exists with respect to changes in immune gene expression, as many more genes are involved in immunity than were measured in this study (e.g. see He et al., 2015; Cao et al., 2015). Additionally, recent studies show that genes and molecules not traditionally considered 'immune genes' are also important for the immune response (Adamo et al., 2008; Unckless et al., 2015) and a wider view is needed to develop a comprehensive understanding of the immune system network. We also assume that without the postulated reconfiguration, the food-limited caterpillars would be even more susceptible to pathogens given the physiological effects of food limitation. However, this assumption needs to be tested empirically.

We speculate that low-nutrition caterpillars were losing their ability to mount an inducible response, consistent with the reduction in metabolism that occurs during food limitation (Jiao et al., 2015). However, they had not yet switched to the absent-nutrition configuration. Using more diet levels, it may be possible to determine whether there is an energy factor score threshold that triggers an immune system conformational switch, or whether changes occur gradually. Discrete changes in network configuration may provide an explanation for the threshold-like effect of food deprivation on disease resistance seen in some studies (e.g. see discussion in McKean et al., 2008).

## Network reconfiguration in other systems

Most studies have found a decline in PO activation with food limitation (e.g. *Tenebrio molitor*, Siva-Jothy and Thompson, 2002; *Lestes viridis* damselflies, DeBlock and Stoks, 2008; tent caterpillars *Malacosoma pluviale californicum*, Myers et al., 2011), although Yang et al. (2007) found an increase in the geometrid *Epirritia autumnata*. In contrast, most studies have found no effects of food limitation on cell-mediated immunity (e.g. encapsulation, Siva-Jothy and Thompson, 2002; Rantala et al., 2003; phagocytosis, Ayres and Schneider, 2009; or hemocyte number, Myers et al., 2011), although some studies reported a decline (e.g. DeBlock and Stoks, 2008) and others an increase (e.g. Krams et al., 2015). Food limitation levels varied across studies, making them difficult to compare. Our study found that different levels of food limitation produce different immune response patterns.

Food limitation also alters vertebrate immunity (e.g. Klasing, 2007), but the complexity of the vertebrate immune system has made it difficult to determine the functional significance of these alterations at a network level (Afacan et al., 2012). Moreover, this complexity probably allows for multiple possible immune system network configurations in response to food limitation (i.e. network degeneracy; Tieri et al., 2010), making it difficult to discern patterns. The hypothesis that early constitutive immune responses will have priority over inducible responses when food is limited (Lochmiller and Deerenberg, 2000) is supported by work on birds (e.g. *Calidris canutus*; Buehler et al., 2010). When access to food was limited, first-line defense constitutive responses were maintained, but at least one aspect of the inducible response (the acute phase response) was reduced (Buehler et al., 2010), similar in outline to our results in *M. sexta*.

Our study provides evidence that food limitation can produce a shift in the configuration of the immune system. These shifts alter the response patterns of individual immune components, leading to a re-prioritization of different immune functions (e.g. Fig. 1). Such changes will lead to different immunological strengths and vulnerabilities for the organism. Changes in the expression and/or activity of individual immune components during environmental stressors such as food limitation may sometimes be better interpreted as an example of immune reconfiguration, as opposed to immunosuppression.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

S.A.A., G.D., R.E., I.K. and K.F.T. helped design and perform experiments. S.A.A. developed the hypothesis, analyzed the data and wrote the paper. R.E., I.K. and K.F.T. read and critiqued the manuscript.

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