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RESEARCH ARTICLE

Predator exposure-induced immunosuppression: trade-off, immune redistribution or immune reconfiguration?

Shelley A. Adamo[1,‡], Russell H. Easy[2], Ilya Kovalko[1], Jenna MacDonald[1], Ashleigh McKeen[1], Taylor Swanburg[1], Kurtis F. Turnbull* and Catherine Reeve[1]

ABSTRACT

Although predator exposure increases the risk of wound infections, it typically induces immunosuppression. A number of non-mutually exclusive hypotheses have been put forward to explain this immunosuppression, including: trade-offs between the immune system and other systems required for anti-predator behaviour, redistribution of immune resources towards mechanisms needed to defend against wound infections, and reconfiguration of the immune system to optimize defence under the physiological state of fight-or-flight readiness. We tested the ability of each hypothesis to explain the effects of chronic predator stress on the immune system of the caterpillar *Manduca sexta*. Predator exposure induced defensive behaviours, reduced mass gain, increased development time and increased the concentration of the stress neurohormone octopamine. It had no significant effect on haemocyte number, melanization rate, phenoloxidase activity, lysozyme-like activity or nodule production. Predator stress reduced haemolymph glutathione concentrations. It also increased constitutive expression of the antimicrobial peptide *attacin-1* but reduced *attacin-1* expression in response to an immune challenge. These results best fit the immune reconfiguration hypothesis, although the other hypotheses are also consistent with some results. Interpreting stress-related changes in immune function may require an examination at the level of the whole organism.

KEY WORDS: Ecoimmunology, Chronic stress, Stress hormones, Attacin, Phenoloxidase, Glutathione, Octopamine, Predation

INTRODUCTION

Predators induce fight-or-flight stress responses in their prey, but chronic activation of these responses results in immunosuppression in mammals (Sapolsky et al., 2000; Dhabhar, 2009). Similarly, in insects, exposure to predators activates a stress response (Adamo and Baker, 2011), which increases survival from predator attack (Adamo et al., 2013) but can reduce disease resistance (Adamo and Parsons, 2006). Chronic exposure to predators is a common problem for many species (Boonstra, 2013). Because injury is more likely when predators are present, immunosuppression during chronic predator exposure seems maladaptive. Wounds greatly increase the risk of infection, suggesting that there is an increased, not decreased, need for immune protection when predators are nearby (Dhabhar, 2014). Historically, stress-induced immunosuppression has been interpreted as pathological immune dysregulation (e.g. Webster Marketon and Glaser, 2008). However, these immunosuppressive effects are often driven by intricate intracellular signalling systems that are activated by stress hormone receptors (e.g. Huang et al., 2012). Given that this phenomenon exists in animals across phyla, and is often mediated by conserved molecular pathways (Ottaviani and Franceschi, 1996; Adamo, 2008), it seems likely that the immune response to chronic predator stress has an adaptive component. Some researchers have undertaken a re-examination of the immunosuppressive effects of chronic stress (e.g. chronic predator exposure) from an evolutionary perspective (Råberg et al., 1998; Boonstra, 2013).

Recent interpretations of the effects of chronic stress on the immune system have focused on a resource trade-off argument – i.e. immune system responses are curtailed in order to fuel the chronic activation of fight-or-flight-related phenomena (e.g. hypervigilance; Sapolsky et al., 2000; Hawlena and Schmidt, 2010). Another hypothesis, the immune system redistribution hypothesis, posits that fight-or-flight stress leads to a shift in resources within the immune system in order to protect against wound infections (Dhabhar, 2014). According to this scenario, although some central immune functions may decline, resistance to wound infections will be enhanced. This strategy, however, is thought to operate primarily in response to acute stress (e.g. brief exposure to a predator; Dhabhar, 2014).

In this paper, we suggest a third possibility, i.e. that insects reconfigure their immune systems when the risk of predation is chronically high. In other words, they change the magnitude, duration and/or dynamics of different immune components relative to baseline conditions. These changes maximize immune function given the animal's physiological state of fight-or-flight readiness. Therefore, some immune components may be upregulated, despite the need for resources for the preparation for fight-or-flight behaviours. For example, during short-term fight-or-flight behaviours, molecular resources are shifted away from the immune system (i.e. a resource trade-off; Adamo et al., 2008), but at the same time, stress hormones upregulate other immune functions; these changes appear to compensate for the loss of molecular resources (Adamo, 2014). Similarly, during starvation, insect immune systems appear to be able to adaptively reconfigure in order to maintain immune function even though resource availability is reduced (Adamo et al., 2016a,b). In insects, chronic exposure to predators (i.e. exposure lasting days to weeks) induces a number of physiological changes (e.g. increased jumping ability in grasshoppers; Hawlena et al., 2011: and increased flight stamina in crickets; Adamo and Baker, 2011) that plausibly increase an animal's chance of preventing predation, but are also likely to increase resource use by muscle. These changes appear to co-occur with chronic activation of stress responses (Adamo and Baker, 2011). Therefore, the physiological changes needed to

[1]Department of Psychology and Neuroscience, Dalhousie University, Halifax, NS, Canada B3H 4R2. [2]Department of Biology, Acadia University, Wolfville, NS, Canada B4P 2R6.
*Present address: Department of Biology, University of Western Ontario, London, ON, Canada N6A 3K7.

‡Author for correspondence (sadamo@dal.ca)

S.A.A., 0000-0002-5973-571X

Received 16 November 2016; Accepted 14 December 2016

Journal of Experimental Biology

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enhance anti-predator behaviour over the long term are likely to require changes in the immune system network in order to reduce immunosuppression. Such a perspective may help make sense of the mix of increased and decreased immune functions found in larval insects exposed to chronic predator stress (e.g. Joop and Rolff, 2004; Slos et al., 2009; Duong and McCauley, 2016; Op de Beeck et al., 2016).

We examined the ability of these three, non-mutually exclusive, perspectives to account for the changes observed in the caterpillar *Manduca sexta* when it is exposed to mock predator attacks for the first 3 days of its final larval instar. Three days represents more than 1/3 of the normal time period for that life stage. Therefore, these mock attacks are not a brief phenomenon for the caterpillar, but represent a sustained increase in the apparent predation risk. *Manduca sexta* show a strong physiological response to predation threat (Thaler et al., 2012, 2014), and enter a 'hypervigilant state' with heightened anti-predator behaviour in response to chronic mock predator attack (Walters et al., 2001). The natural history of *M. sexta* also makes it a good organism for this type of test (Thaler et al., 2012). It typically lives its entire life on a single plant (Bernays and Woods, 2000). Unlike other insects that can move to different locations to avoid predators (e.g. wood crickets, *Nemobius sylvestris*; Bucher et al., 2015), it must rely on internal mechanisms to deal with an increased predation risk. We assessed the effects of predator stress on key aspects of its immune system, including cellular immunity (haemocyte count and nodulation), humoral immunity (phenoloxidase and lysozyme-like activity), clot formation and immune gene expression (see Jiang, 2008; Kanost and Gorman, 2008; Strand, 2008; Zhang et al., 2011; Chevignon et al., 2015). Like vertebrates, insects have both constitutive and inducible immune responses (Schmid-Hempel, 2011). Constitutive immune components are those that are consistently present, regardless of pathogen exposure. Inducible components are produced only after an immune challenge. Target genes were chosen to span the different types of immune responses. Attacin-1 is an antimicrobial peptide that is induced by infection (An et al., 2010). Lysozyme is an antimicrobial protein that is expressed constitutively, but is also upregulated upon infection (He et al., 2015). Plasmatocyte spreading peptide (PSP) is an insect cytokine capable of altering cell-mediated immunity (Eleftherianos et al., 2009). Pro-phenoloxidase activating proteinase 3 (PAP-3) is an enzyme that is critical for the activation of phenoloxidase (Kanost and Gorman, 2008); serpin-3 is an inhibitor of phenoloxidase activation (Kanost and Gorman, 2008). Finally, we measured glutathione (GSH) levels in the haemolymph because predator exposure induces oxidative stress damage in insects (Janssens and Stoks, 2014). GSH is a major antioxidant in insects and plays a role in immune function (Clark et al., 2010). Moreover, ants exposed to chronic social stress show a reduction in GSH (Schneider et al., 2016).

If chronic predator exposure produces a redirection of resources towards fight-or-flight systems (i.e. a trade-off), we should find a downregulation of at least some immune functions. Whether constitutive or inducible responses are the most energetically expensive type of immune response is unclear; however, inducible responses are thought to be less costly overall because of reduced running costs, and are likely to be favoured when resources are low (Westra et al., 2015). Therefore, we would expect a decrease in constitutive immune mechanisms in particular. If predator stress results in immune redistribution, we would expect an increase in immune functions needed for wound protection, including increased phenoloxidase activity, an increase in clotting and an increase in haemocyte cell number (Strand, 2008). If predator stress induces immune reconfiguration, we would expect a mix of immune effects with an increase in some functions and a reduction in others. Specifically, we predict that constitutive immune gene responses will be upregulated to compensate for the declines in some immune functions that occur during fight-or-flight behaviours (e.g. pathogen recognition; Adamo et al., 2008; Adamo, 2014).

MATERIALS AND METHODS

Manduca sexta eggs were obtained from Great Lakes Hornworm (Washington Township, MI, USA) or from our colony established from these eggs. Caterpillars were housed in individual cups after the 2nd instar. Food (*M. sexta* artificial wheat-germ based diet, supplied by Recorp, Georgetown, ON, Canada) was supplied *ad libitum*. They were kept on a 12 h:12 h light:dark cycle at 21±1°C and between 40% and 70% relative humidity. Only animals that weighed within 2 s.d. of the mean mass for 5th instar-day 0 (hereafter 5th-day 0) in our lab (Adamo et al., 2016a,b) were included in the study. We were concerned that very light or very heavy individuals might be physiologically unusual, resulting in outliers. Fewer than 1 in 10 caterpillars were excluded from the study for this reason.

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

All chemicals were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted.

Mock predator paradigm

Once caterpillars finished their moult to the final larval instar (5th-day 0), they were weighed. Caterpillars were assorted randomly by mass into two different groups: stressed and control. Stressed caterpillars were given a simulated predator attack starting on the 1st day of the 5th instar (5th-day 0). The attack consisted of a series of gentle squeezes on the right proleg of segment A6. The squeezes were made with dull-tipped forceps and were sufficient to deflect mechanosensory hairs on the leg. Caterpillars were given eight consecutive squeezes within approximately 30 s. The series of eight squeezes were repeated 4 times within an hour for a total of 3 h. Caterpillars that did not make at least one defensive strike during the 3 h were excluded from the study (27/378). The caterpillars were handled in such a way that self-damage from their own defensive strike was avoided; those that were affected were excluded from the study (5/378). Caterpillars were also carefully inspected for any sign of damage (e.g. discoloration of the leg), and those caterpillars were also excluded from the study (2/378). This procedure was repeated on the following 2 days (5th-day 1 and 5th-day 2). This procedure appeared to produce a less-intense response compared with other studies (e.g. Walters et al., 2001; Bura et al., 2012). For example, regurgitation and defecation were not typically observed during a mock predator attack. Control caterpillars were unmanipulated. After the final mock predator trial, but prior to sample collection, caterpillars (5th-day 2) were re-weighed. In a subset of caterpillars (n=27 stressed, n=25 control), a cube of food (approximately 2 cm^3) was weighed and placed with the caterpillars 24 h prior to their final mock predator trial. This time period was chosen in order to reduce acute stress effects. The cube was re-weighed immediately prior to the final trial.

Octopamine measurements

Haemolymph (i.e. insect blood) was removed 15 min after the final series of mock predator attacks from 5th-day 2 by snipping the

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caterpillar's dorsal horn with ice-cold disinfected scissors. Haemolymph was collected in ice-cold centrifuge tubes, and added to an equal volume of ice-cold sodium acetate buffer (9.96 g l^{-1}, pH 5.0) and vortexed. The mixture was then spun at 14 ***g*** for 30 min at 4°C. The supernatant was transferred to 10K MWCO filters (VWR, Radnor, PA, USA) and spun at 14 ***g*** for 30 min at 4°C. The filtered fluid was placed immediately at −80°C and sent on dry ice to the Center for Microelectrode Technology HPLC Services, University of Kentucky, for measuring octopamine concentration using HPLC-ED (high performance liquid chromatography with electrochemical detection). Haemolymph was collected from control caterpillars at the same time as the stressed caterpillars.

Immune assays

One hour after the final mock predator trial, haemolymph was collected as described above. The collected haemolymph was used for the following assays (methods taken from Adamo et al., 2016a,b): haemocyte number, lysozyme-like activity, phenoloxidase activity, total protein levels, GSH concentration, melanization rate and nodulation production.

Haemocyte number

Total haemocyte count was assessed by diluting haemolymph in ice-cold anti-coagulant (1:10) consisting 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. Diluted haemolymph was placed on a Fuchs–Rosenthal haemocytometer. Cells were counted using phase contrast microscopy (from Adamo et al., 2016a,b).

Lysozyme-like activity

Haemolymph samples were diluted 1:4 in ice-cold PBS and vortexed. For each sample, 10 µl of the haemolymph-PBS mixture was transferred to a microplate well containing 180 µl of a *Micrococcus luteus* cell wall suspension in PBS (12.5 mg per 25 ml). Microplate wells were mixed for 5 s and the change in absorbance at 450 nm was recorded for 10 min at room temperature. Lysozyme standards in the linear range of the assay were run concurrently. Two outliers were removed using the Hoaglin–Iglewicz rule (Hoaglin and Iglewicz, 1987; from Adamo et al., 2016a,b).

Total phenoloxidase activity

Total phenoloxidase activity in the haemolymph was quantified using a method modified from Hall et al. (1995). Haemolymph was diluted 1:20.6 in ice-cold PBS and vortexed. For each sample, 30 µl of the haemolymph-PBS mixture was added to 180 µl of 20 mmol l^{-1} L-3,4-dihydroxyphenylalanine (L-DOPA) and left to incubate for 5 min at room temperature. To activate the zymogen prophenoloxidase, 2 µl of 10% cetylpridinium chloride (CPC) 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 phenoloxidase (Hall et al., 1995). 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 96-well plate using mushroom tyrosinase that captured the linear range of the reaction. Mushroom tyrosinase catalyses the formation of the same dopachrome as phenoloxidase (Xie et al., 2003). One outlier was removed using the Hoaglin–Iglewicz rule (Hoaglin and Iglewicz, 1987; from Adamo et al., 2016a,b).

Total haemolymph protein

Total haemolymph protein was measured using a Bradford assay. Haemolymph was diluted 1:9 in ice-cold PBS and vortexed. The haemolymph-PBS mixture 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 in a 96-well plate. After a 10–15 min incubation period at room temperature, the samples were read in a plate reader set at 590 nm. Bovine albumin was used for standards. Samples were run in duplicate and standards were run in triplicate (from Adamo et al., 2016a,b).

GSH concentration

GSH levels were assessed by measuring both reduced and oxidized GSH species (GSH/GSSG; Cayman Chemical Company, Ann Arbor, MI, USA). Haemolymph 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}). After incubation 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 (Glutathione kit, Cayman Chemical Company). Absorbance was measured at 405 nm. Samples and standards were run in triplicate. One outlier was removed using the Hoaglin–Iglewicz rule (Hoaglin and Iglewicz, 1987; from Adamo et al., 2016a,b).

Melanization procedure

Haemolymph was collected from control and stressed caterpillars 15 min after the final mock predator trial. Haemolymph samples were added to an equal volume of ice-cold PBS, vortexed for 10 s, and then 100 µl was added to a 96-well plate; 100 µl of PBS was used as the blank. The absorption was measured at 450 nm for 3 h, with readings taken every 3 min. Both the rate of change in absorbance and the lag time (i.e. latency) to the response were assessed. All samples and blanks were run in triplicate.

Nodulation counts

Three hours after the final mock predator trial, control and stressed caterpillars were chilled to anaesthetize them. Animals were killed by removing their head using disinfected scissors. The animal was pinned open and the gut was removed. Only nodules between the level of the second abdominal and terminal ganglion of the central nervous system were counted. Nodules were counted as a single entity, regardless of size.

One group of control and stressed caterpillars were immune challenged 3 h prior to dissection, 5 min after the last mock predator trial (i.e. 5th-day 2). To induce this immune challenge, control caterpillars were injected with 100 µl of heat-killed *Serratia marcescens* (approximately 1/10 LD_{50} dose; MicroKwik Culture, Carolina Biological). Because stressed caterpillars were approximately 5% lighter in an earlier pilot study, they were injected with 95 µl of heat-killed *S. marcescens*. Caterpillars that bled after injection were excluded. Three hours later, caterpillars were chilled and dissected as described above and the number of nodules was counted (Miller and Stanley, 2000).

A subset of caterpillars had their nodules counted by an additional observer who did not know to which group the caterpillar belonged. Inter-observer reliability was high (Spearman correlation, r=0.85, P=0.001, n=11).

Effect of predator stress on gene expression

One hour after the last mock predator trial (5th-day 2), a sample of the fat body was collected from each caterpillar following MIQE

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guidelines (Taylor et al., 2010). The fat body makes the majority of immune proteins in *M. sexta* (Zhang et al., 2014). Fat body was collected from unmanipulated (control) caterpillars at the same time. To induce an immune response in these caterpillars, they were injected with a 60 µl mixture containing heat-killed Gram negative bacteria (*S. marcescens*, 1/10 LD_{50} dose), Gram positive bacteria (*Bacillus cereus*, 1/10 LD_{50}; MicroKwik Culture, Carolina Biological) and fungus (*Beauveria bassiana*, strain GHA, 1/10 LD_{50}; BotaniGard 22WP, Laverlam, Butte, MT, USA) (Adamo et al., 2016a,b) on 5th-day 1 after exposure to the mock predator. Control caterpillars were injected at the same time. The fat body from stressed and control caterpillars was collected 24 h later (i.e. after the final mock predator trial), at the same time that non-immune-challenged caterpillars had their fat body collected (5th-day 2).

Dissections were performed with sterilized instruments and disinfected surfaces. Tissue was stabilized using RNAlater (Qiagen, Hilden, Germany) and stored at −80°C. RNA was extracted using Qiazol, a TissueRuptor and an RNeasy Lipid Mini Tissue kit (all from Qiagen) following the manufacturer's instructions. The extraction included a DNase 1 treatment (RNase-Free DNase set, Qiagen) to remove genomic DNA contamination. The integrity of the RNA was tested using a denaturing 'bleach gel' electrophoresis (Aranda et al., 2012). The concentration and purity of the extracted RNA samples was determined using a NanoDrop 2000c spectrophotometer (ThermoScientific, Waltham, MA, USA). cDNA was synthesized using iScript (Bio-Rad, Hercules, CA, USA). cDNA concentration was measured using a Qubit 1.0 fluorometer and Qubit dsDNA HS Assay kit (Invitrogen, Waltham, MA, USA). Sample concentration was normalized to 100 ng μl^{-1} and samples were stored at −80°C.

Six potential reference genes were tested for stability across the different treatments. We used NormFinder for R (http://moma.dk/normfinder-software) to determine stable reference genes (Andersen et al., 2004), using the quantitative cycle (C_q) values of five biological samples for each candidate reference gene for each group. *Manduca sexta ribosomal protein S3* (*MsS3*) and *Ubiquitin* produced the most stable reference gene combination (score 0.23).

cDNA levels were measured by quantitative real-time PCR (qPCR). The primers for both reference and target genes have been used previously (Adamo et al., 2016a,b; Table 1). Primers were purchased from integrated DNA technologies (http://www.idtdna.com/site).

Primer specificity was confirmed in a previous study (Adamo et al., 2016a,b). Mean efficiencies of target and reference gene primers were calculated by construction of a standard curve using serial dilutions of fat body cDNA. The efficiencies for the target and reference genes ranged from 0.95 to 1.0.

Reactions took place in a 96-well plate run using an iCycler CFX-96 C1000 Thermal Cycler (Bio-Rad). 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. The reaction used the following two-plate gene maximization protocol: initial denaturation (95.0°C for 3 min), followed by 40 cycles of denaturation (95.0°C for 30 s), annealing (52.5°C for plate 1 and 57°C for plate 2 for 45 s), and extension (72.0°C for 30 s). Plate 1 held *serpin-3*, *MsS3* and *Ubiquitin* samples; plate 2 held *Pap3*, *attacin-1*, *lysozyme* and *PSP* samples. The annealing temperatures were chosen based on the melting temperatures of the primers on the plate, and thermal gradients were run for each individual primer. PCR reactions were followed by melt curve analysis to ensure a single product and no-template controls were inspected to eliminate primer-dimer errors. C_q values for each sample and gene target were calculated in CFX Manager (Bio-Rad). All samples (biological replicates) were run in duplicate, including no-template control samples. No-template controls were run for each gene target and reference gene.

Data were calculated as fold-change in expression of target genes in test animals against control (non-treated) animals using the Relative Expression Software Tool (REST, v. 1, 2009) program. The normalized expression ($\Delta\Delta C_q$) was calculated as the relative quantity of the target gene normalized to the quantities of the reference genes.

Data analysis

Data were analysed using SPSS (v. 22.0) and GraphPad Prism (v. 5.0); the qPCR data were analysed using CFX Manager v. 3.1 (Bio-Rad) and the REST program (2009; http://rest.gene-quantification.info). The REST program uses a randomization technique in which the sample values are reallocated to each group 10,000 times to determine how often results as extreme as those observed would occur by chance. Other data were tested for normality and non-normal data were tested using non-parametric statistics. Unless otherwise stated, all values in the text are means±s.d.

RESULTS

Repeated mock predator attack increased haemolymph octopamine concentration in the caterpillars (Fig. 1; t_{26}=3.1, P=0.005, control n=14, stressed n=14). It also resulted in a significant decline in mass gain relative to control caterpillars (control, 1.96±0.69 g per 2 days, n=63; stressed, 1.55±0.72 g per 2 days, n=68, $F_{1,128}$=11.7, P<0.001, start mass used as a covariate). The decline in mass gain did not appear to be due to a reduction in food consumption. Control caterpillars ate 1.97±0.73 g (n=25), while stressed caterpillars ate 1.77±0.69 g (n=27) ($F_{1,49}$=0.57, P=0.45; initial mass of the caterpillar used as a covariate) during the final 24 h of the mock predator exposure. As might be expected from the reduced mass gain, stressed caterpillars required an additional day to achieve dorsal vessel exposure [median (1st, 3rd quartiles): control, 7 days (7,8 days), stressed, 8 days (7.25,8 days), Mann–Whitney U=36.5, P=0.03, n=12 per group].

Chronic exposure to mock predator attack had no measurable effect on some immune measures. There was no effect on the rate of melanization (control, 0.070±0.020 absorbance units min^{-1}, n=16; stressed 0.061±0.027 absorbance units min^{-1}, n=24; $F_{1,40}$=1.28,

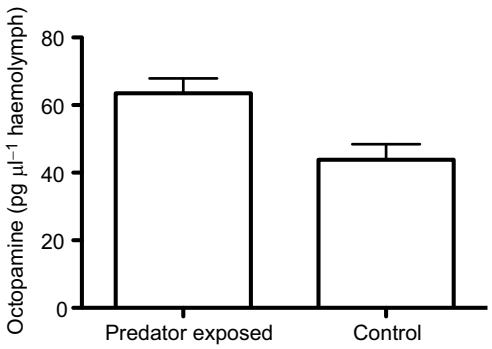

Fig. 1. Effect of mock predator attacks on octopamine concentration. Exposure of *Manduca sexta* to simulated predator attacks increased haemolymph octopamine concentration. Bars denote the mean and error bars represent the s.e.m.

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Table 1. Forward and reverse primer sequences for target genes and reference genes

Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)	Efficiency	References
PSP	ATGAAGTTATTTTTTATAGTT	TCAAAATGTAAGTTTGCATCT	0.95	Eleftherianos et al., 2009
Attacin-1	GCAGGCGACGACAAGAAC	ATGCGTGTTGGTAAGAGTAGC	1.0	An et al., 2009
Lysozyme	GTGTGCCTCGTGGAGAATG	ATGCCTTGGTGATGTCGTC	1.0	An et al., 2009
Serpin-3	GATTCCTCGCGATTCGATGC	CATTTACGTCATTAAGTTTCATG	0.97	Zhu et al., 2003
PAP-3	ATTAAGCTGTTGTGTGGTG	CGGGTGCGGTATTGTCTTC	0.98	Jiang et al., 2003
*Ubiquitin**	AAAGCCAAGATTCAAGATAAG	TTGTAGTCGGATAGCGTGCG	0.98	Kumar et al., 2012
*MsS3**	CGCGAGTTGACTTCGGT-3′	GCCGTTCTTGCCCTGTT-3′	1.0	Zhu et al., 2003

*Reference genes.

P=0.26), haemocyte number (control, 7330±2374 cells µl^{-1} haemolymph, n=28; stressed 7588±2628 cells µl^{-1} haemolymph, n=30; t-test, t_{56}=0.70, P=0.39), total haemolymph protein (control, 7.2±5.5 mg ml^{-1}, n=16; stressed, 6.1±4.1 mg ml^{-1}, n=24, t_{38}=0.72, P=0.47), total phenoloxidase activity (control, 92±16 µg tyrosinase equivalent, n=22; stressed, 120.0±21 µg tyrosinase equivalent, n=21, t_{41}=1.06, P=0.29) or lysozyme-like activity (control, 42.8 ±3.2 µg lysozyme equivalent, n=22; stressed, 46.2±2.4 µg lysozyme equivalent, n=22, t_{42}=0.84, P=0.41). Injecting heat-killed bacteria induced an increase in the number of nodules in both control (n=31) and stressed caterpillars (n=38) compared with unchallenged controls (n=38), or unchallenged stressed caterpillars (n=35) (Fig. 2; Kruskal–Wallis=82.5, P<0.0001; Dunn's multiple comparisons, P<0.05). There was no significant difference in the number of nodules between stressed and control caterpillars with (Dunn's multiple comparison test, P>0.05) or without an immune challenge (Dunn's multiple comparison test, P>0.05).

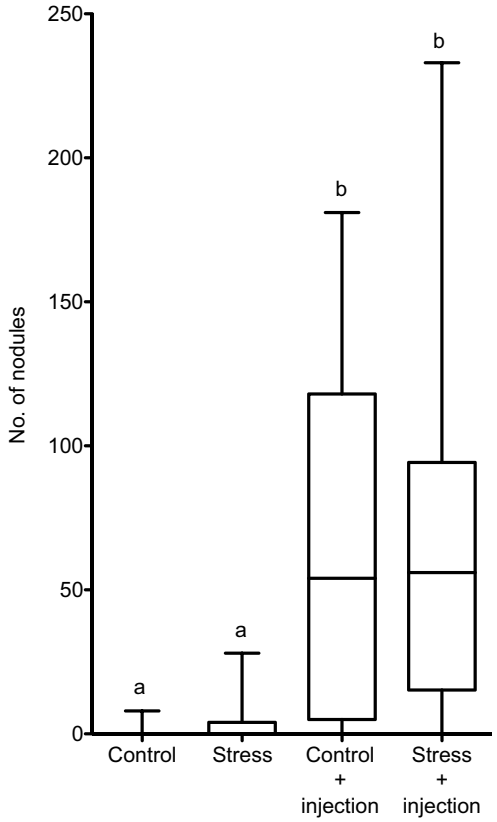

Fig. 2. Effect of mock predator attacks on nodulation. Box and whisker plot of the number of nodules in control and stressed caterpillars with and without induction using heat-killed bacteria. Bars with different letters are significantly different from one another.

Exposure to repeated mock predator attacks reduced the latency to melanization (control, 7018±649 s, n=16; stressed, 5445±729 s, n=22, t_{36}=2.2, P=0.04). Also, caterpillars chronically exposed to mock predator attack had less GSH in their haemolymph (control, 135.0±19.5 µmol l^{-1}, n=11; stressed, 74.7±12.9 µmol l^{-1}, n=8, t_{17}=2.37, P=0.03).

Mock predator attack also altered immune gene expression. Stressed caterpillars (n=13) had greater *attacin-1* gene expression relative to controls (n=9) (Fig. 3A, P=0.001). Expression of the other assessed genes was not significantly different from controls (*serpin-3*, P=0.09; *PAP-3*, P=0.70; *lysozyme*, P=0.91; *PSP*, P=0.72). However, after an immune challenge, stressed caterpillars (n=13) had reduced inducible *attacin-1* gene expression compared with immune-challenged controls (n=13) (Fig. 3B, P=0.04). Immune-challenged control caterpillars (n=13) increased expression of *attacin-1* (P<0.0001), *lysozyme* (P=0.02) and *PAP-3* (P=0.02) relative to non-immune-challenged controls (n=13) (Fig. 3C).

DISCUSSION

Mock predator attacks induced defensive behaviours (i.e. the defensive strike; Walters et al., 2001), decreased mass gain, increased development time and raised the haemolymph concentration of the stress neurohormone octopamine (Fig. 1) in caterpillars. These results demonstrate that mock predator attacks elicit a sustained fight-or-flight response in the caterpillar. It also demonstrates the severity of the fitness effect. A day's delay in reaching metamorphosis could significantly reduce survivorship to adulthood because of the high predation risk during this vulnerable life stage (Bernays and Woods, 2000; Kingsolver et al., 2012). Predator stress is known to increase metabolic rate in younger instars of *M. sexta* (Thaler et al., 2014), and this may explain why mass gain declined without a significant decrease in food consumption.

The energetic trade-off hypothesis does not fully explain the results. Despite a resource shortage (demonstrated by the reduced mass gain), predator stress enhanced constitutive expression of *attacin-1*. In contrast, the immune redistribution hypothesis is supported by the decrease in melanization latency, which would probably promote wound healing. Octopamine, acting as a neurohormone, increases the rate of clotting in lobsters (Battelle and Kravitz, 1978) and may play a similar role in the reduction in melanization latency in stressed caterpillars. However, there was no increase in phenoloxidase activity, no increase in the expression of the activating enzyme *PAP-3*, no decrease in the inhibitor *serpin-3* and no increase in the rate of melanization. Moreover, activation of a stress response does not appear to reduce wound infections in the one insect in which this has been tested (crickets, *Gryllus texensis*; Adamo and Parsons, 2006). Furthermore, the reduction in inducible *attacin-1*would not promote wound protection (Fig. 3B). The immune reconfiguration hypothesis was supported by the increase in constitutive gene expression of *attacin-1*, and the decline in its

Journal of Experimental Biology (2017) 220, 868-875 doi:10.1242/jeb.153320

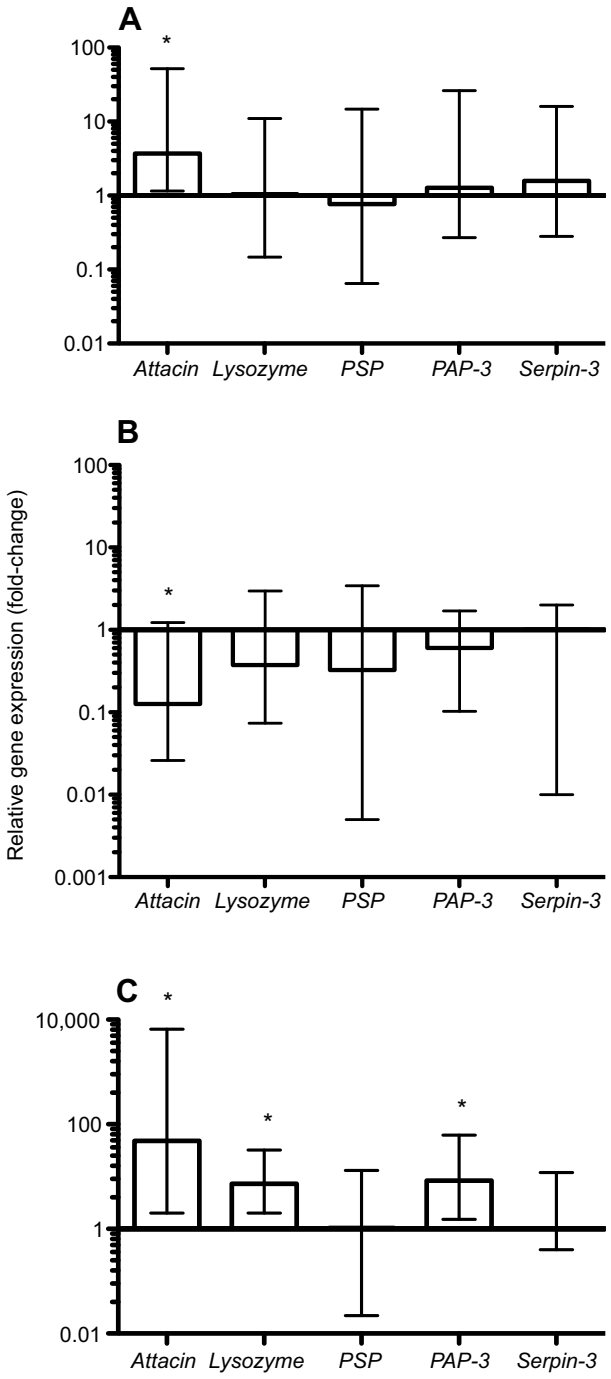

Fig. 3. Effect of mock predator attacks on gene expression. (A) Gene expression of stressed caterpillars (n=13) relative to control caterpillars (n=9). (B) Gene expression of stressed caterpillars 24 h (n=13) after an immune challenge relative to immune-challenged controls (n=13). (C) Gene expression of immune-challenged controls (n=13) relative to non-challenged controls (n=9). Bars are means and error bars are 95% confidence intervals. The asterisk denotes a value that is significantly different from control. PSP, plasmatocyte spreading peptide; PAP-3, pro-phenoloxidase activating proteinase 3.

inducible expression. However, more information is needed about the causes underlying these shifts to determine whether immune reconfiguration is the best explanation for these changes in immune function. For example, we measured the expression of only a few genes; therefore, we cannot conclude that other inducible immune genes were also suppressed.

The increase in constitutive antimicrobial peptide gene expression is consistent with the effect of other stressors, such as starvation or pinching (e.g. *Drosophila melanogaster*: Becker et al., 2010; Tsuzuki et al., 2012; mosquitoes (*Aedes aegypti*): Price et al., 2015; and *M. sexta* 5th instar caterpillars: Adamo et al., 2016a,b). Specific intracellular pathways induce antimicrobial peptide expression and production without the usual activation of pathogen recognition pathways (e.g. Toll receptors; Tsuzuki et al., 2012). The existence of these stress-induced pathways suggests that immune activation during exposure to various stressors has been selected for.

In vertebrates, one hypothesis as to why stress hormones are immunosuppressive is the need to prevent immunopathology (Råberg et al., 1998). We did not examine this suggestion in this paper. Although insect stress hormones (e.g. octopamine and adipokinetic hormone) typically reduce disease resistance when injected, they are largely immunoenhancing at the cellular level (Adamo, 2008, 2012), making it unlikely that they curtail immunopathology. However, immunopathology is a potential problem for insects chronically exposed to predators because this exposure produces increased levels of oxidative stress (Janssens and Stoks, 2014). This increase in oxidative stress is sufficient to cause biologically significant damage (Janssens and Stoks, 2014). The reduction of GSH levels by predator stress may partly explain why it causes oxidative stress damage. Immune responses also produce oxidative stress (e.g. phenoloxidase; Gonzalez-Santoyo and Cordoba-Aguilar, 2012) that can be damaging (Sadd and Siva-Jothy, 2006). The increase in oxidative stress during predator exposure may increase this type of immune-generated damage. Therefore, insects might benefit from immunosuppression during chronic predator-induced stress. However, the immune reconfiguration hypothesis provides a plausible reason as to why an immune component that creates oxidative stress (e.g. phenoloxidase) was not suppressed. If inducible immunity is generally reduced during predator stress, as it is during starvation (Adamo et al., 2016a,b), then constitutive defence becomes critical. One inexpensive method of enhancing constitutive phenoloxidase responses may be to reduce GSH haemolymph concentrations. High GSH concentrations delay melanization (Clark et al., 2010). This enhancement would probably come at the cost of increased immunopathology from activated phenoloxidase. The increased oxidative stress costs are probably not trivial (Janssens and Stoks, 2014), and may explain why antioxidant mechanisms other than GSH are upregulated during predator stress (Slos et al., 2009; but see Slos and Stoks, 2008). However, running the risk of increased oxidative stress (i.e. immunopathology) may be less costly in terms of fitness than having a badly compromised immune system.

Although an immune system network perspective suggests that it could be adaptive to lower GSH concentrations in the haemolymph, an energetic trade-off model can also explain the reduction in GSH. GSH represents a substantial pool of scarce amino acids such as cysteine (Barbehenn et al., 2013) that may be required elsewhere to augment anti-predator behaviour. The two explanations are not mutually exclusive, and both may contribute to selection to reduce GSH during chronic predator stress.

Most studies on the effect of predator stress on larval insect immune systems find no effect, or a positive effect, on constitutive immune function (e.g. haemocyte number, Joop and Rolff, 2004; phenoloxidase activity, Slos et al., 2009; melanization of implants, Duong and McCauley, 2016), with one study (Op de Beeck et al., 2016) finding a negative effect of predator cues on phenoloxidase activity in mosquito larvae (when measured on the whole animal, as opposed to haemolymph levels). The effect on inducible

mechanisms (e.g. gene expression) in larval insects has not been previously examined using a heat-killed challenge. Total haemolymph protein is unaffected during chronic predator stress (this study; Van Dievel et al., 2016), despite the decrease in mass gain, and therefore there is no evidence that protein synthesis in general is reduced. It remains unclear why inducible mechanisms might be more susceptible to suppression.

The effect of chronic fight-or-flight stress in mammals has been better studied, and it has been known for some time that individual immune components respond differently to chronic stress (Sapolsky et al., 2000; Dhabhar, 2009, 2014). However, there is currently no explanation as to why these differences exist. The complexity of the mammalian immune system makes it difficult to discern patterns within the complex changes that occur with various stressors. Although chronic stress impairs defence against microbial, viral and cancer threats, it does not suppress all immune functions (Schmidt et al., 2016). For example, chronic stress promotes pro-inflammatory mechanisms, while simultaneously mobilizing inhibitory mechanisms (e.g. regulatory T cells; Schmidt et al., 2016; Dhabhar, 2009, 2014). Many of these types of changes appear to be driven by specific receptors activated by chemical mediators of the stress response (e.g. Padro and Sanders, 2014). Given the complex intracellular machinery mediating these effects, it seems unlikely that they are mere pathology. Moreover, comparative studies on mammals other than rodents and humans suggest that evolution is capable of removing the negative impact of chronic stress on disease resistance (Boonstra, 2013). Therefore, in both mammals and insects, the mixed negative and positive effects of chronic stress on the immune system may serve an adaptive function at the organismal level. However, these functions may require explanations that span different physiological systems, and possibly even involve changes within the microbiome.

We have shown that chronic, repeated stress and elevated stress hormone levels do not invariably lead to global immunosuppression, but can enhance some immune functions. Enhancements of immune function during chronic stress have been called dysregulation (e.g. Schmidt et al., 2016; Dhabhar, 2014), presumably because they co-occur with declining disease resistance. However, they may also be signs of immune reconfiguration, resulting in an immune system optimized for function in the current physiological state. For example, increased constitutive immune gene expression might help compensate for reductions in immune function due to trade-offs elsewhere (Adamo, 2014). Such a reconfiguration does not mean that the animal's disease resistance is greater than that of controls, but that it is greater than it would be without the reconfiguration. Changes in immune function due to predator-induced stress, or other chronic stressors, may be difficult to interpret if only a small number of immune components are assessed in isolation. Interpreting stress-related changes in immune function may require examination at the level of the whole organism.

Acknowledgements
We thank A. Grabtchak for animal care support, and P. Nalan and L. An for assistance with experiments.

Competing interests
The authors declare no competing or financial interests.

Author contributions
S.A.A. conceived and designed the study, analysed the data and wrote the paper, R.H.E. helped design the molecular studies, I.K. and K.F.T. participated in the design of the molecular studies and performed the qPCR, J.M. helped design the haemocyte counts and performed biochemical and behavioural studies, A.M. and T.S. participated in the design of the nodulation studies and performed them, C.R. helped design the study, performed molecular and behavioural studies, and helped analyse the data. All authors commented on the manuscript.

Funding
This study was funded by a Natural Sciences and Engineering Council of Canada Discovery Grant (RGPIN 203133-2013) to S.A.A.

Data availability
The data sets supporting this article are available upon request from the corresponding author.

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