Notes

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Predation risk increases immune response in a larval dragonfly (*Leucorrhinia intacta*)

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Abstract. Predators often negatively affect prey performance through indirect, non-consumptive effects. We investigated the potential relationship between predator-induced stress and prey immune response. To test this, we administered a synthetic immune challenge into dragonfly larvae (*Leucorrhinia intacta*) and assessed a key immune response (level of encapsulation) in the presence and absence of a caged predator (*Anax junius*) at two temperatures (22°C and 26°C). We hypothesized that immune response would be lowered when predators were present due to lowered allocation of resources to immune function and leading to reduced encapsulation of the synthetic immune challenge. Contrary to our expectations, larvae exposed to caged predators had encapsulated monofilaments significantly more than larvae not exposed to caged predators. Levels of encapsulation did not differ across temperatures, nor interact with predator exposure. Our results suggest that the previously observed increase in mortality of *L. intacta* exposed to caged predators is not driven by immune suppression. In situations of increased predation risk, the exposure to predator cues may induce higher levels of melanin production, which could lead to physiological damage and high energetic costs. However, the costs and risks of increased allocations to immune responses and interactions with predation stress remain unknown.

Key words: caged predator; encapsulation; melanization; Odonata; temperature; trade-offs.

Introduction

Predators can have indirect, non-consumptive effects (NCEs) on prey, inducing changes in behavior, morphology, physiology, or a combination of traits (Preisser et al. 2005). Plastic responses to predation risk are often adaptive and can decrease the risk of prey detection, capture, and/or consumption (Tollrian and Harvell 1999). However, such responses can be costly and lead to reduced growth, body size, survival, and fecundity (Luttbeg and Kerby 2005, Preisser et al. 2005). Growing evidence suggests that the eco-physiological effects of predation risk stress scale up from the individual level to impact population dynamics, community structure, and ecosystem level energy transfers (Hawlena and Schmitz 2010, Sheriff and Thaler 2014).

Odonate (dragonflies and damselflies) larvae have been used extensively in studies of predator-induced plasticity and NCEs (McPeek 1990, McPeek et al. 2001 and the costs of these anti-predator responses can be high. For example, McCauley et al. (2011) found that the presence of non-consumptive predators resulted in higher rates of metamorphic failure and larval mortality in larval dragonflies (*Leucorrhinia intacta*). However, the proximate source of mortality in response to non-consumptive predators was unknown. It was hypothesized that increased mortality could arise from a trade-off between predator stress responses and immune response. Odonates are susceptible to a number of parasites and pathogens including mites, platyhelminths, gregarines, and bacterial infections (Corbet 1999) and reduced immune responses may increase the risk of mortality from these infections. Trade-offs between investment in anti-predator responses and immune function could underlie the observed pattern of increased mortality when larval odonates are exposed to non-consumptive predators.

A common method of assessing immune responses in arthropods is by measuring the level of melanization (the darkening and hardening of hemocytes (Gillespie et al. 1997) or encapsulation (the aggregation of melanized hemocytes around foreign objects) in response to natural parasites or synthetic objects (e.g., Sephadex beads, nylon filaments; Zahedi et al. 1992, Rantala and Roff 2007). When pathogens and parasites are covered by melanized hemocytes, they undergo separation from the hemolymph and are asphyxiated, starved, or destroyed through the cytotoxic and anti-microbial properties of melanin (Christensen et al. 2005, Nappi and Christensen 2005). Measuring the extent of melanization of foreign bodies introduced into the body allows for the direct measure of immunocompetence in insects, including odonates (Rantala et al. 2000, Rantala and Roff 2007, Nagel et al. 2011).While melanization is crucial for reducing damage

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to the host from pathogens and parasites, it can also be energetically costly and is often suppressed when an individual is under acute stress (Adamo 2009). Balancing infection risks with the costs of melanization can result in trade-offs affecting traits such as body size and developmental time (Rantala and Roff 2005) as well as longevity (Krams et al. 2013). Melanization has also been observed to be reduced in animals engaged in reproduction (Siva-Jothy et al. 1998) and foraging (Konig and Schmid-Hempel 1995), suggesting that there are energetic trade-offs between performing these activities and the melanization response. Therefore measuring levels of encapsulation can reveal trade-offs between this immune response and other functions including anti-predator responses.

Environmental temperatures can also affect the immune response. In mosquitoes, the encapsulation of Sephadex beads decreased when temperature increased from 24°C to 30°C (Suwanchaichinda and Paskewitz 1998). Prokkola et al. (2013) found a decrease in encapsulation and development time for meal-worm beetles (*Tenebrio molitor*) exposed to 28°C relative to those at 18°C. Recent work in odonates suggests that anti-predator responses may interact with physiological responses to temperature (Culler et al. 2014). However, interactions between anti-predator response, temperature, and immune response have not been previously examined. Therefore, we evaluated the effects of predation risk on immunity at two ecological realistic temperatures to test for interactions among these factors.

We investigated how predation risk, temperature, and their interaction affected immune response and activity levels in *L. intacta* larvae. Odonate species in the genus *Leucorrhinia* (Anisoptera: Libellulidae) have been a focal group for studies of predator-induced plasticity. In two species of *Leucorrhinia*, the presence of caged predators have been found to induce the growth of longer abdominal spines (*L. dubia* [Johansson and Samuelsson 1994] and *L. intacta* [McCauley et al. 2008]) and in *L. intacta* increased mortality in larvae exposed to non-consumptive predators (McCauley et al. 2011). We hypothesized that trade-offs between immune function and anti-predator responses would result in decreased immune responses for larvae exposed to caged predators compared with larvae not exposed to predation risk cues. Immune responses were evaluated in 3-d trials at two temperatures to assess whether temperature would affect melanization or interact with predation risk cues. We also assessed how predation risk and temperature affected activity level in *L. intacta* larvae to evaluate whether behavioral responses to these conditions might ameliorate the costs of immune responses.

Methods

Immunity experiment

Eggs were collected from adult *L. intacta* between May and July of 2013 at the Koffler Scientific Reserve (King City, Ontario, Canada). Eggs were placed in water-filled plastic containers varying in volume from 190 to 378 L. Larvae hatching from these eggs were reared collectively outdoors in large (378-L) stock tanks in the absence of predators and supplied with sufficient amounts of zooplankton and mosquito larvae (*Daphnia* spp. and Culicidae) for larvae to feed ad libitum. Beginning in September 2013, larvae of adequate size (head plus abdomen length >1 cm) were collected and stored in individually in 470-mL plastic cups at room temperature (~20°C) for at least 2 weeks before trials began. Larvae were fed *Daphnia* spp. two to three times a week.

The experiment was a randomized full 2 × 2 factorial design crossing predator exposure and temperature (absence/presence of predator and 22°/26°C). These temperatures were chosen to represent a range of ecologically realistic summer temperatures for the littoral zone of ponds in this region where these larvae develop. Each 19-L aquaria contained dechlorinated water, artificial vegetation, an aquarium heater, a clear acrylic container weighed down by rocks and a focal *L. intacta* larva (head width 4.06 ± 0.45 mm, mean ± 1 SD). A standard aliquot of zooplankton was added to each aquaria at the start of the trial, allowing focal *L. intacta* to feed ad libitum. Acrylic containers were left empty in predator-absent treatments. In predation risk treatments, large dragonfly larvae (*Anax junius*) were placed in the acrylic container within the aquaria. In these tanks *A. junius* were fed one *L. intacta* two hours prior to the experimental period in order to produce chemical stimuli indicating predation risk. Predators consuming conspecifics generate the strongest cues indicating predation risk (Schoeppner and Relyea 2009).

To test immune response in *L. intacta*, a 1-mm nylon monofilament (0.2 mm in diameter and sanded to increase retention within the larva) was injected dorsolaterally into the seventh abdominal segment of each larva, perpendicular to the cuticle suture, prior to the experimental period. Injections were administered using a single-shot tag injector (Northwest Marine Technology, Shaw Island, WA, USA). Following the injections, larvae were randomly assigned to treatments and placed individually into tanks. Each tank represented a replicate; there were five replicates per treatment in the first three trials. A fourth trial was conducted with 10 larvae ($N = 4$ for no predator, 26°C and $N = 2$ for all other treatments). One larvae from the no predator, 26°C treatment died during the 4th trial for unknown reasons but all other larvae from all trials survived the experiment ($N = 17$ for no predator, 26°C and $N = 15$ for all other treatments).

After 72 h in the experimental aquaria, larvae were collected and preserved in 95% ethanol. The length of this experiment presented larvae with an acute predation-risk stress. Larval head width was measured using digital calipers (accuracy to ±0.01 mm) and the monofilament was dissected from the insect and stored in 95%

ethanol. Monofilaments were examined using an Olympus SZ microscope (Richmond Hill, ON, Canada) and photographed using an Infinity 1 microscope camera(Richmond Hill, ON, Canada). Two monofilaments were lost during the third trial photography procedure. Photographs were saved as .tiff files and converted into 16-bit grayscale images in ImageJ (Schneider et al. 2012). Since melanization was not uniform around the monofilament, two photos were taken at 180° rotations. Melanization was assessed by isolating the monofilament within the image and analyzing pixel values. ImageJ uses a pixel value scale of 255 (white) to 0 (black) and produces a summary histogram of pixel values. To convert this measure into a measure of melanization as the darkness of the pixel value we used 255—mean pixel value (calculated from both sides photographed) as our measure of melanization.

Behavioral response trials

In September 2015 larval *L. intacta* (head width 4.42 ± 0.45, mean ± 1 SD) were collected from the same pond at which adults were captured for our immunity experiment. These larvae were housed together in an outdoor mesocosm for 5 d prior to the behavioral trials and were brought into the lab 18 h before observations began. Throughout this period, larvae were fed a mix of zooplankton and larval *Chaoborus* to allow for ad libitum feeding.

Behavioral observations were conducted in 20, 2-gallon aquaria placed on a rack with four aquaria per shelf. Observations were conducted under the same four treatment conditions as in the immunity trials, a 2 × 2 cross of temperature (22° or 26°C) and predator exposure (caged *A. junius* or an empty cage). Aquaria on each shelf were randomly assigned to one of the four treatments. Aquarium heaters were placed in each tank to maintain water temperatures. A six-cell grid marked on white paper was taped to the back of each aquarium to provide a visual template for assigning larval position in observations. Predators (10 larval *A. junius*) were placed in water-filled cages on a lab bench 14 h prior to trials commencing and each was given one larval *L. intacta* to consume. Empty cages were filled with water and left on the same lab bench to standardize the condition of the water added to each aquarium. To homogenize predator chemical cues, water from *Anax* cages was combined and a standard volume (175 mL) added to each aquaria 2.5 h before observations began. To provide visual cues of predation risk, cages with *Anax* were then added to aquaria in the predator treatment. Water from the empty cages was similarly combined and added to the no predator aquaria, and one empty cage was placed in each no predator replicate.

A single *L. intacta* larva was added to each aquaria 2 h before observations began. Instantaneous observations were made every 5 min recording larval position (square 1–6 in which the head of larva was located) and whether the larva was active or inactive. This was done for 85 min in each trial for a total of 17 observation points per trial. Two trials with different *L. intacta* larvae were conducted with the same set-up and replication in each. This method of assessing activity levels has been widely used in odonates and is capable of distinguishing effects of predators on activity (Brodin et al. 2006).

Statistical analysis

The effects of predation, temperature, and the interaction between these factors on the level of melanization were assessed using a univariate general linear model (GLM) with mean pixel value as the response variable and predator exposure and temperature as explanatory factors and trial as a random factor. To assess whether larval size affected melanization levels, the relationship between head width and mean pixel value was examined using a bivariate correlation. To assess the robustness of mean pixel values as a metric, the median of pixel values from combined measures of the two sides of the monofilament were also analyzed and similar results (not presented) were found.

The effects of predator presence and temperature on larval activity level was evaluated by comparing movement frequency between treatments. Observations of larvae found very few instances of them being recorded as active at the moment of observation and so no analyses were conducted on these data. Counts of the number of moves made by each larva were square-root transformed (square root of moves + 0.5). A GLM compared activity levels between larvae in all four treatments, trial was included as a random factor. Analyses were conducted in SPSS 22.0 (IBM, Armonk, NY, USA).

Results

Larvae exposed to caged predators melanized the monofilaments significantly more than larvae not exposed to caged predators ($F_{1,60}$ = 4.58, P = 0.04, Fig. 1). Temperature had no significant effect on melanization levels ($F_{1,60}$ = 0.04, P = 0.84, Fig. 1). The interaction between predation and temperature was also not significant ($F_{1,67}$ = 1.48, P = 0.23). Trials differed significantly ($F_{3,60}$ = 8.41, P < 0.01) with lower levels of melanization in the last trial. There was no significant correlation between head width and mean pixel value (Pearson r_{67} = 0.06, P = 0.62) and the same pattern was found within all treatments.

We found no significant effect of temperature (GLM, $F_{1,36}$ = 0.12, P = 0.74) or predator presence ($F_{1,36}$ = 0.32, P = 0.58) on larval *L. intacta* activity levels, nor was activity affected by an interaction between temperature and predator presence ($F_{1,36}$ = 0.000083, P = 0.99, Fig. 2). The effect of trial was not significant ($F_{1,36}$ = 1.09, P = 0.31).

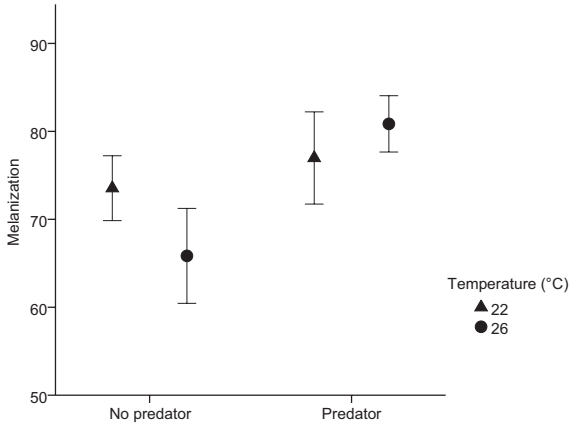

FIG. 1. Melanization of monofilaments was higher when *Leucorrhinia intacta* larvae were exposed to caged predators ($P < 0.05$). Triangles represent mean melanization levels for monofilaments removed from larvae held at 22°C and circles from larvae held at 26°C. There was no significant effect of temperature on melanization levels, nor any temperature × predator exposure interaction. Melanization was measured using ImageJ, which uses a 0–255 scale (255, white; 0, black). See *Methods: Immunity experiment* for an explanation of the calculation of melanization. Symbols represent mean values ± SE.

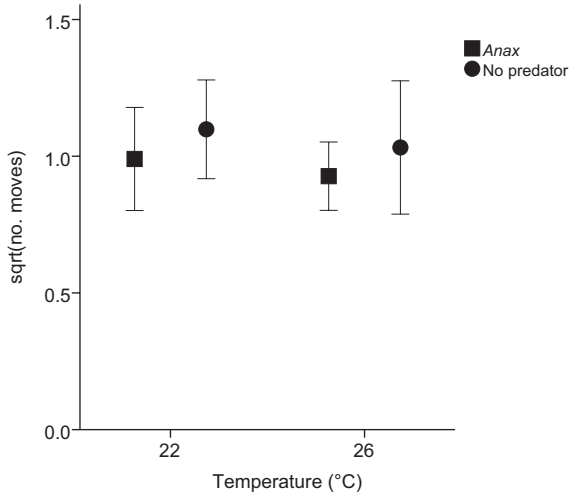

FIG. 2. Activity levels of larval *Leucorrhinia intacta* (measured as mean number of moves; square-root transformed [sqrt]) at two temperatures (°C) and under two predation risk treatments. Behavior was measured in the presence of visual and chemical cues indicating the presence of a predator (*Anax*, gray squares) or in conditions with no predation risk (no predator, black circles). Neither predation risk nor temperature had a significant effect on activity levels (all $P > 0.05$). Symbols represent mean values ± SE.

DISCUSSION

Dragonfly larvae in the presence of caged predators had a stronger melanization response to an injected monofilament, an immune challenge, than larvae not exposed to predators. Temperature did not affect this relationship nor was there a significant interaction between temperature and predator exposure. Contrary to our initial predictions, these results suggest that rather than suppressing the immune response in the presence of predation risk, dragonfly larvae increase melanization in this context. These results suggest that increased vulnerability to pathogens is not the mechanism underlying previously observed increases in mortality of larvae exposed to predator cues (McCauley et al. 2011).

Larval *L. intacta* activity levels were not significantly affected by either exposure to predator cues or by temperature across the two temperatures we examined (22° or 26°C). Previous measures of activity levels in *L. intacta* found similar patterns with no significant effect of predatory cues from *A. junius* on movement frequency or distance (McCauley 2005) and no difference in movement frequency for larvae held at 23° and 27°C (D.N. Frances and S.J. McCauley, *unpublished data*). While previous studies in odonates have found interactions between temperature and predation risk (Culler et al. 2014) the lack of an activity response to these factors in *L. intacta* in this and previous work, other traits may mediate interactions between these factors. Activity level and foraging rate are tightly linked (Werner and Anholt 1993) and our results suggest that neither predation risk nor temperature altered behavior in a way that would affect foraging rates or facilitate compensation for the costs of up-regulating the immune response.

The increase in the melanization response when exposed to predation risk cues that we observed in this study may be an anticipatory response to an increased risk of cuticular wounding. Some insects use environmental or life-history cues as predictors of immune challenges to adjust or regulate their immune response accordingly (Siva-Jothy 2009). This includes some evidence of this immune "anticipation" in odonates in response to increased risk of parasitism and wounding. Damselfly larvae that encounter higher densities of mite parasites before emergence increase their immune function immediately after emergence to prevent infection during metamorphosis (Nagel et al. 2010). Mature female dragonflies have higher phenoloxidase activity, a measure of insect immunity, than male dragonflies potentially as an anticipatory response to the risk of injury during copulation (Rolff 2001). The aeshnid dragonfly larvae (*A. junius*) used as a predator in this study uses an extendable labium to strike and grasp prey. This feeding mode can result in non-lethal strikes that cause open cuticular wounds and melanin plays a central role in healing wounds of this type. Consistent with the anticipatory response being targeted to the risk of injury, Joop and Rolff (2004) found female damselflies (*Coenagrion puella*) exposed to a combination of mites and aeshnid predators so that both parasitism and wounding risk are increased, had higher levels of phenoloxidase activity, a

key immune response. In contrast, in another damselfly (*Lestes viridis*) exposure to predation risk from fish, an engulfing predator unlikely to cause cuticular wounds, was associated with decreased phenoloxidase activity (Stoks et al. 2006).

The costs of an up-regulated immune response may be energetic, but melanin also has cytotoxic effects detrimental to parasites and pathogens as well as to the host's cells. Sadd and Siva-Jothy (2006) suggest that this "auto-reactive" self-harm is a cost of insect immunity. The biosynthesis of melanin, through the phenoloxidase cascade, also results in reactive oxygen species (ROS) a free radical produced as a by-product of redox reactions (Dowling and Simmons 2009). Unprocessed or elevated levels of ROS can lead to oxidative stress and result in oxidative damage to DNA, RNA, or proteins within the host's cells (Dowling and Simmons 2009). It is possible that in the presence of predators there are elevated levels of ROS, which are not processed quickly enough to avoid cellular damage. This can then lead to physiological damage that decreases an insect's ability to maintain or regulate basic functions.

We found no significant effect of temperature on melanization, although a wider range of temperatures or a longer time exposure to these temperatures may reveal novel insights into how temperature affects the immune response. The narrow temperature range (4°C) in our experiment may limit our ability to detect of the effects of temperature on melanization and larvae were exposed to this temperatures for only 3 d. Investigations of the effects of temperature and immune response in insects have typically used a range from 15° to 31°C (Lynn and Vinson 1977, Robb and Forbes 2006, Pandey et al. 2007) and found an increase in immune response with increasing temperatures followed by a sudden drop, resulting from immune suppression in response to temperature stress. However, these responses can vary across taxa and there is evidence that warmer temperature can increase the melanization response in odonates (Robb and Forbes 2005).

Our results provide evidence to reject the hypothesis that exposure to predation risk decreases the melanization response, making larvae more vulnerable to pathogens. Whether an increased immune response when exposed to predation risk is adaptive is currently unknown. This response may be advantageous for wound healing or preventing pathogen and parasite invasions following wounding but may also be energetically costly and detrimental to maintain unnecessarily (Lochmiller and Deerenberg 2000, Adamo 2009). If these costs are sufficiently high, this investment in immune response in conjunction with the direct negative effects of melanin on host cells could contribute to the observed mortality patterns observed when larvae are in the presence of caged predators (McCauley et al. 2011). If the risk of injury when predators are present increases investment into immune function, this would impose significant energetic costs on larvae already taxed by other anti-predator responses with potential survivorship consequences.

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