

Atrazine Exposure Influences Immunity in the Blue Dasher Dragonfly, *Pachydiplax longipennis* (Odonata: Libellulidae)

Coy R. St. Clair¹ and Claire A. Fuller^{1,2}

¹Department of Biological Sciences, Murray State University, 2112 Biology Building, Murray, KY 42071 and ²Corresponding author, e-mail: cfuller@murraystate.edu

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Abstract

Agricultural runoff containing herbicide is known to have adverse effects on freshwater organisms. Aquatic insects are particularly susceptible, and herbicide runoff has the potential to affect immunity in this group. Here we examined the effect of ecologically relevant levels of atrazine, an herbicide commonly used in the United States, on immune function in larvae of the blue dasher dragonfly (Odonata: Libellulidae, *Pachydiplax longipennis* Burmeister 1839) during a long-term exposure at ecologically relevant concentrations. Larvae were exposed to concentrations of 0, 1, 5, and 10 ppb atrazine for 3 or 6 wk. Hemocyte counts, hemolymph phenoloxidase (PO) activity, cuticular PO, and gut PO were measured at the end of each trial period as indicators of immune system strength. Atrazine concentration had a significant effect on hemocyte counts after controlling for larval size. There was a significant interaction between time and concentration for hemolymph PO, cuticular PO, and a marginal interaction for gut PO. The effect of atrazine on the measured immune parameters was often nonmonotonic, with larger effects observed at intermediate concentrations. Therefore, atrazine affects both hemocyte numbers and PO activity over time in *P. longipennis*, and the changed immune function demonstrated in this study is likely to modify susceptibility to pathogens, alter wound healing, and may decrease available energy for growth and metamorphosis.

Key words: atrazine, immunity, Odonata, hemocyte, phenoloxidase

Insect immune systems are of paramount importance in warding off pathogens such as bacteria and fungi (Moreno-Garcia et al. 2012), and lowered immune function can have a direct effect on fitness by increasing incidence of infections (Eslin and Prevost 1998, Kraaijeveld et al. 2001, Cerenius et al. 2008, Butt et al. 2016). Additionally, immune function can be energetically costly, resulting in indirect effects on fitness via trade-offs (Moret 2003, Cotter et al. 2004, Moreno-Garcia et al. 2012). For example, increased energy channeled to immunity could result in decreased energy available for reproduction. Insect investment in immunity can change in response to a number of environmental factors such as temperature (Fedorka et al. 2013a, Fuller and Postava-Davignon 2014, Kutch et al. 2014), season (Fedorka et al. 2013b), and previous exposure to pathogens (Mikonranta et al. 2014). Individuals with increased investment in immunity may have less energy to devote to reproduction (Schwenke et al. 2016) or behaviors such as dispersal (Suhonen et al. 2010), predator avoidance (Rantala et al. 2010), or predation (St. Clair and Fuller 2014). Immune response is also subject to natural stressors such as copulation, oviposition, and territory competition (Siva-Jothy et al. 1998, Koskimaki et al. 2004).

Immune stress from environmental contaminants is likely to place additional strain on this cost/benefit system (Guedes et al. 2016). A number of authors have documented the impact of

pesticides on economically important terrestrial species (Chen et al. 2017, Kalita et al. 2017, Zhu et al. 2017). However, fewer studies have focused on immunological impacts of pesticides on nontarget insect species, particularly in the aquatic environment. Atrazine is the second most commonly used herbicide in North America (Atwood and Paisley-Jones 2017); it reaches freshwater via runoff from agricultural fields and can persist in surface water for months at relatively high levels due to low hydrolysis and aqueous photolysis rates (Solomon et al. 1996). While short-term concentrations can be exceedingly high (e.g., >130 ppb; Cope et al. 2004), long-term concentrations, although lower on average, may also be high enough to cause ecological impacts. The U.S. Environmental Protection Agency monitored 20 watersheds across five midwestern states for atrazine in 2007 and 2008, and detected a range of 0.30 to 9.12 ppb of annual mean atrazine concentration, with a mean value for all watersheds of 2.44 ppb (U.S. EPA Atrazine Monitoring Program; data available at: <https://www.epa.gov/ingredients-used-pesticide-products/atrazine-monitoring-program-data-and-results>; accessed 27 September 2018). Atrazine has been shown to impact the survival, life history, physiology, and development of freshwater organisms (Hayes 2004, Storrs and Kiesecker 2004, Koprivnikar et al. 2006, Bara et al. 2014, McMahon et al. 2017),

and many of these effects have been demonstrated at concentrations of 3 ppb or below. Additionally, atrazine has been shown to have adverse effects on immunity in fish and amphibians (Forson and Storfer 2006, Rohr and McCoy 2010, Sifkarovski et al. 2014). For example, atrazine exposure increased susceptibility to ranavirus in the tiger salamander (Forson and Storfer 2006), and several studies have demonstrated that fish and amphibians exhibited decreased immune function and were more likely to contract infections under the influence of atrazine (reviewed in Rohr and McCoy 2010). As insect immune systems share some similarities with those of other taxa, it is reasonable to expect that atrazine has an effect on insect immune function (Kavanagh and Reeves 2007).

Because immune function is inextricably tied to other behavioral and physiological traits, effects of a ubiquitous agrochemical such as atrazine may be especially important. Dragonfly larvae play a fundamental role in freshwater ecosystems as predator to smaller invertebrates and smaller dragonflies, as well as prey for many larger species (Corbet 1999, Oertli 2008, Samways 2008). In fishless ponds they are often top predator (McPeck 1990). Here we examine the nonlethal immunological effects of exposure to atrazine in larvae of the blue dasher dragonfly, *Pachydiplax longipennis*.

We examined the response of four indicators of immune function: hemocyte counts, hemolymph phenoloxidase (PO) activity, cuticular PO activity, and midgut PO activity. Hemocytes are the circulating immune-effector cells of insects, serving four basic functions: the encapsulation of large foreign objects, phagocytosis of small particles, hemolymph coagulation, and nutrient storage and distribution (Gullan and Cranston 2000). Hemocytes also produce PO for the hemolymph PO cascade. Hemocyte load has been correlated with insect ability to produce an effective immune response, thus serves as an indicator of immune function (Rolff and Siva-Jothy 2004, Moreno-Garcia et al. 2012). The PO enzyme cascade is a versatile chemical defense involved in the production of melanin, which is toxic to pathogens (Rolff and Siva-Jothy 2004, Cerenius et al. 2008). The PO cascade is initiated by physical damage, infection, or nonself invasions, comprises a significant portion of insect immunological defense, and is often used in the assessment of insect immune function (e.g., Moret 2003, Schmid-Hempel and Ebert 2003). The cuticle and midgut are points of entry for pathogens. Phenoloxidase has been shown to be important in defense against pathogens in the cuticle (Bailey 2011, Dubovskiy et al. 2013, Lee et al. 2015, Tyurin et al. 2016), the midgut (Castro et al. 2012), and in the hemolymph in a number of insects (reviewed by Schmid-Hempel 2005, Cerenius et al. 2008, Moreno-Garcia et al. 2012).

The purpose of this study was to determine the immunological effects on *P. longipennis* of atrazine exposure. We hypothesized that ecologically relevant levels of atrazine negatively affect *P. longipennis* immunity. It has been previously suggested that exposure time may play a vital role in determining effects (Stuijzand et al. 1999). Therefore, we predicted that the impact of atrazine increases with exposure time (3 and 6 wk).

Methods

Pachydiplax longipennis larvae were obtained from cattle tanks at Hancock Biological Station and several small ponds in the Land Between the Lakes National Recreation Area (LBL) in Calloway and Trigg counties, KY. The cattle tanks were filled in February 2009 as part of another experiment and allowed natural colonization by invertebrates. They were not being used for experimental purposes at the time of larvae collection and mimicked the natural environment of an ephemeral pond. Collection occurred in late April and

early May 2010. Pesticide use is restricted and atrazine use was banned in LBL in 2003 (U.S. Department of Agriculture 2007), and the ponds used for collection were not subject to agricultural runoff from areas outside LBL; therefore, it is unlikely that larvae were previously exposed to agricultural contaminants.

Larvae were maintained in an environmental chamber throughout the experiment (22°C; 12:12 h light-dark cycle). They were fed zooplankton from Kentucky Lake and cultured *Daphnia magna* weekly for the first 3 wk of the experiment. As the dragonfly larvae grew during the course of the experiment, caddisfly larvae obtained from a local stream were added to the feeding regimen. These were fed weekly in addition to the zooplankton.

Larvae were placed in small, flat-bottom glass containers (diameter 8 cm, depth 2.5 cm) with 80 ml of one of four atrazine levels (0, 1, 5, or 10 ppb atrazine). Solutions were mixed using Nanopure water and agricultural-grade Aatrex herbicide (42.6% atrazine, 56.5% inactive ingredients). Pure atrazine was not used in order to better reflect conditions encountered in agricultural areas. The four atrazine levels were based on actual atrazine content in the solutions. Atrazine levels were checked from randomly selected representative samples (three samples from each concentration) once every 2 wk using a Triazine Plate Kit (Strategic Diagnostics, LLC, Newark, DE), at which time water was changed with new solution in all replicates. This was precautionary, as there was little degradation of atrazine within any of the 2-wk periods (<8%). The results of the plate kit readings agreed with previous findings that atrazine degrades at approximately 4.5% per week in standing water (Campero et al. 2007). Solutions were refreshed primarily to provide fresh water for the larvae.

The immune parameters were measured after 3 and 6 wk of exposure to atrazine. We chose these time points because larvae are exposed repeatedly in nature as atrazine is applied to field that border streams and ponds. A different set of individuals was used for each time point, as obtaining cuticular and gut PO required the sacrifice of the larva. Target sample size was 10 for each concentration at each time point. One data point is missing from hemolymph PO because enough hemolymph could not be collected.

Hemocyte Counts

Five microliters of hemolymph were collected by cutting one hind leg at the joint connecting the femur and thorax and using a 2- μ l calibrated Microcap pipette to collect hemolymph from the wound. The hemolymph was placed in 20 μ l of iced anticoagulant (Siva-Jothy et al. 2001; 98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid, 5% sucrose, pH 7.2). The mixture was held on ice for less than 2 h to prevent cell lysis. Collection of hemolymph for hemolymph PO and collection of the gut and cuticle occurred simultaneously with collection for hemocyte counts. However, because the cells begin to lyse at 2 h, hemocytes were counted before other samples were assayed. Ten microliters of the hemolymph anticoagulant mixture were placed on a hemocytometer and visible hemocytes were counted using standard methods for blood cell counts (adapted from Koskimaki et al. 2004).

Hemolymph PO

For hemolymph PO assays, 2 μ l of hemolymph were collected by cutting one hind leg as above and using a 2- μ l Microcap pipette to collect the hemolymph from the wound. This hemolymph was added to 10 μ l of PBS and frozen to lyse hemocytes, releasing their PO into the hemolymph. The hemolymph in PBS solution was then incubated for 1 h at room temperature in 70 μ l of chymotrypsin

(0.02 g chymotrypsin in 1.5 ml PBS) to catalyze the inactive form (pro-PO) to the active form (PO). After incubation, 80 μ l of each sample was combined with 100 μ l of a 4 mM L-Dopa solution in a microtiter plate well. To create control samples, 12 μ l of PBS in 70 μ l of chymotrypsin was incubated for 1 h; L-Dopa was added to these samples in wells. Readings were then taken at ambient temperature using a LabSystems microtiter plate reader at 492 nm every 5 min for 45 min. These values were plotted and the slope found. The slope of each sample indicated PO activity (adapted from Barnes and Siva-Jothy 2000, Rolff 2001).

Cuticular PO and Gut PO

For cuticular PO, the entire abdominal cuticle was used for assays after all internal organs were removed. For gut PO, the midgut was collected. The cuticle or gut was placed in 2% formaldehyde and 0.5% glutaraldehyde in phosphate buffer (30.5 mM NaH_2HPO_4 , 19.5 mM Na_2HPO_4) for 1 h. It was then placed in phosphate buffer for 1 h to rinse. Rinsing was repeated twice more, for a total of three phosphate buffer washes. The cuticle was then incubated in 500 μ l chymotrypsin for 1 h to activate PO, and then incubated in 200 μ l dopamine for 30 min. Two hundred microliters of the liquid were then placed in microtiter plate wells and read once at 492 nm (Cotter and Wilson 2002).

Data Analysis

Data were transformed using a log (hemocyte counts) or reciprocal transformation (cuticular and gut PO activity), depending on which transformation best normalized the data. Hemolymph PO did not require transformation. Two values were extremely low for hemocyte counts and one value was very low for cuticular PO, indicating poor samples. These values were outside the 95% confidence intervals; thus, the removal of these data points was justified.

Analysis of Covariance (ANCOVA) was used to analyze data from each assay, with head width (HW) as a covariate to account for size. We measured HW (an indicator of size) at the beginning and at the end of the experiment (initial and final HW). These two variables are highly correlated ($r = 0.91$, $df = 80$, $P < 0.0001$); we determined which to use in each ANCOVA by first running Akaike Information Criterion (AIC) models (Table 1). The full model for each variable included concentration (0, 1, 5, and 10 ppb), time (3 and 6 wk), an interaction term (concentration * time), and either initial or final head width as the covariate. If main or interaction effects were significant, we used the Tukey HSD post hoc test to identify significant differences among groups.

Results

Atrazine did not affect growth (HW: all $F < 2.85$, $P > 0.1$) or final HW (all $F < 1.33$, $P > 0.25$). Head width covariates were significant for all models (see Table 2); in each analysis, HW was positively related to the immune parameter being measured.

Table 1. AIC (corrected) values for competing models

Variable	Initial HW	Final HW
Hemocyte count	48.4	50.4
Hemolymph PO	632.8	630.6
Cuticular PO	237.8	243.6
Midgut PO	457.4	456.7

Bold values indicate variable used as covariate in ANCOVA models.

Hemocyte Counts

Both concentration of atrazine and time significantly affected hemocyte counts (Table 2; $F_{\text{conc}} = 3.45$; $df = 3,69$; $P = 0.021$; $F_{\text{time}} = 4.34$; $df = 1,69$; $P = 0.04$) but the interaction was not significant. Tukey post hoc tests showed that hemocyte counts of the 1 ppb group were significantly higher than the control group ($P = 0.05$; Fig. 1A) and overall hemocyte counts at 3 wk were significantly lower than at 6 wk ($P = 0.04$; Fig. 1B).

Hemolymph PO Activity

The interaction term was significant for hemolymph PO (Table 2; Fig. 2; $F_{\text{inter}} = 4.16$; $df = 3,70$; $P = 0.009$). Tukey HSD tests showed that at 3 wk, the control ($P = 0.039$) and the 10 ppb ($P = 0.03$) groups had significantly lower PO activity than the control group at 6 wk; the 1 ppb treatment group at 6 wk also had significantly higher PO activity than the control group at 6 wk ($P = 0.038$).

Cuticular PO Activity

The main effects and the interaction term were significant for cuticular PO activity (Table 2; Fig. 3; $F_{\text{conc}} = 5.27$; $df = 3,69$; $P = 0.002$; $F_{\text{time}} = 24.86$; $df = 1,69$; $P < 0.0001$; $F_{\text{inter}} = 3.49$; $df = 3,69$; $P = 0.02$). Note: data are presented in Fig. 3 as reciprocally transformed values; thus, higher bars indicate lower activity. The Tukey HSD test revealed significant differences within time points and within levels: the 1 ppb group at 3 wk had higher cuticular PO activity than the 1 ppb group at 6 wk ($P = 0.003$); the 1 ppb group at 6 wk had lower activity than the 10 ppb group at 6 wk ($P < 0.0001$) and the 5 ppb group at 3 wk had higher activity than the 5 ppb group at 6 wk ($P = 0.017$). For other significant Tukey HSD results, see Fig. 3 caption.

Gut PO Activity

For gut PO activity, the main effect time was significant (Table 2; Fig. 4A and B; $F_{\text{time}} = 5.23$; $df = 1,71$; $P = 0.025$) and the interaction term was marginally nonsignificant ($F_{\text{inter}} = 2.45$; $df = 3,71$; $P = 0.07$). Three-week groups showed significantly higher PO activity than at 6 wk (Fig. 4B).

Table 2. ANCOVA analyses of immunity and immune-related variables

Factor	F	df	P
Hemocyte counts			
Concentration	3.45	3,69	0.021
Time	4.34	1,69	0.040
Concentration * time	0.96	3,69	NS
Initial head width	11.18	1,69	0.001
Hemolymph PO			
Concentration	1.36	3,70	NS
Time	2.64	1,70	NS
Concentration * time	4.16	3,70	0.009
Final head width	12.02	1,70	0.0009
Cuticular PO			
Concentration	5.27	3,69	0.002
Time	24.86	1,69	<0.0001
Concentration * time	3.49	3,69	0.02
Initial head width	47.66	1,69	<0.0001
Gut PO			
Concentration	0.45	3,71	NS
Time	5.23	1,71	0.03
Concentration * time	2.45	3,71	NS (0.07)
Final head width	5.98	1,71	0.02

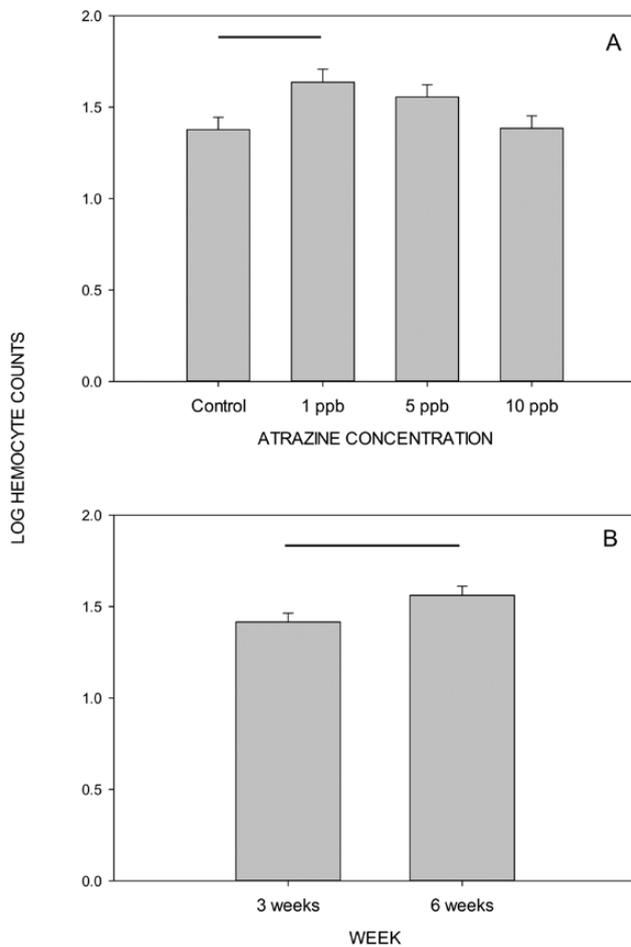


Fig. 1. Least square mean hemocyte counts + SE by treatment (A) and week (B). $N = 10$ for each group except 1 ppb at 6 wk ($N = 8$). Hemocyte counts of the control group were significantly lower than the 1 ppb group ($P = 0.05$) and hemocyte counts at 3 wk were significantly lower than at 6 wk. Endpoints of bars above lines indicate groups that are significantly different from each other.

Discussion

We hypothesized that ecologically relevant levels of atrazine would have negative effects on *P. longipennis* immunity and that these effects would be exacerbated over time. Atrazine did impact hemocyte counts (significant main effect), hemolymph PO (significant interaction), and cuticular PO (significant main effect and interaction) but did not seem to affect midgut PO. Rather than a linear decrease in response with increasing sublethal doses of atrazine, much of our data show a nonmonotonic response in which intermediate exposure levels caused either higher or lower responses than controls or high levels; such nonmonotonic effects are common (Kendig et al 2010, Vandenberg et al. 2012, Jager et al 2013, Guedes and Cutler 2014). Hemocyte counts at both 3 and 6 wk (Fig. 1A) clearly show this type of response as does hemolymph PO at 3 wk (Fig. 2). Cuticular PO at 6 wk (Fig. 3) and midgut PO at 3 wk (data not shown) show a similar pattern but in reverse. However, this pattern is not consistent across all time points.

Time had an effect on hemocyte counts (main effect), hemocyte PO (significant interaction), cuticular PO (significant main effect and interaction), and midgut PO (main effect). In general, hemocyte counts and hemolymph PO showed increases over time while cuticular and gut PO decreased. Because analyses took size into account, our data suggest that larvae place more emphasis on cellular and

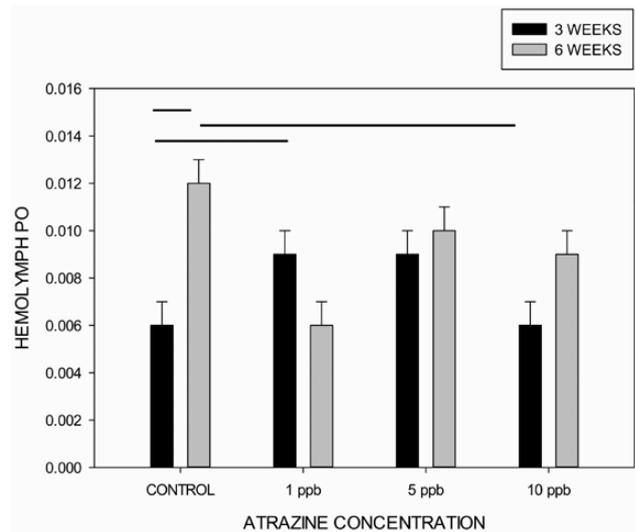


Fig. 2. Least square mean hemolymph phenoloxidase (PO) activity + SE. $N = 10$ for each group except controls at 3 wk ($N = 9$). The control group at 6 wk had significantly higher PO activity than the control group and the 10 ppb groups at 3 wk; the control group at 6 wk also had significantly higher PO activity than the 1 ppb treatment at 6 wk ($P = 0.039$, $P = 0.03$, $P = 0.038$, respectively). Endpoints of bars above lines indicate groups that are significantly different from each other.

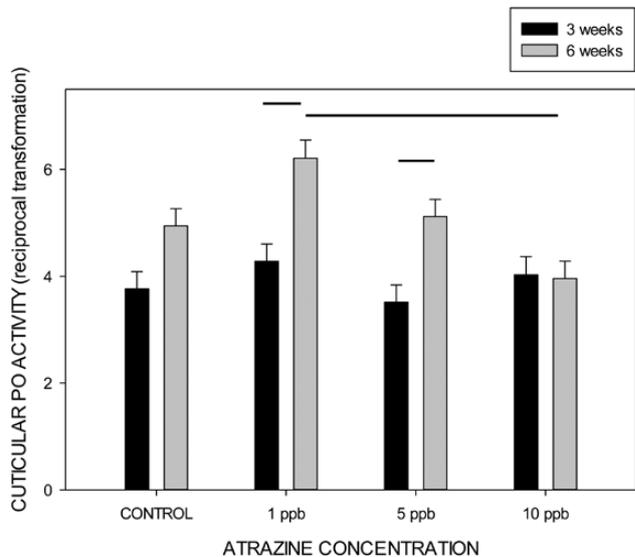


Fig. 3. Mean cuticular phenoloxidase (PO) activity + SE. $N = 10$ for each group except for the 1 ppb group at 6 wk ($N = 9$). Because the interaction between atrazine and time point was significant, we examined each group separately. Note: data are presented as reciprocally transformed values; thus, higher bars indicate lower activity. Significant two-mean comparisons within time points and within levels: the 1 ppb group at 3 wk had higher cuticular PO activity than the 1 ppb group at 6 wk ($P = 0.003$); the 1 ppb group at 6 wk had lower activity than the 10 ppb group at 6 wk ($P < 0.0001$) and the 5 ppb group at 3 wk had higher activity than the 5 ppb group at 6 wk ($P = 0.017$). Additional significant two-mean comparisons: the control at 3 wk had significantly higher activity than the 1 ppb group at 6 wk ($P < 0.0001$); the control at 6 wk had significantly lower activity than the 5 ppb group at 3 wk ($P = 0.049$); the 1 ppb group at 6 wk had significantly lower activity than the 5 ppb ($P < 0.0001$) and the 10 ppb ($P = 0.001$) groups at 3 wk. Endpoints of bars above lines indicate groups that are significantly different from each other.

humoral immunity and less on cuticular and gut PO as they grow. While phenoloxidase in the cuticle and the gut can contribute to immunity, it is also active in melanization (Marmaras et al. 1996).

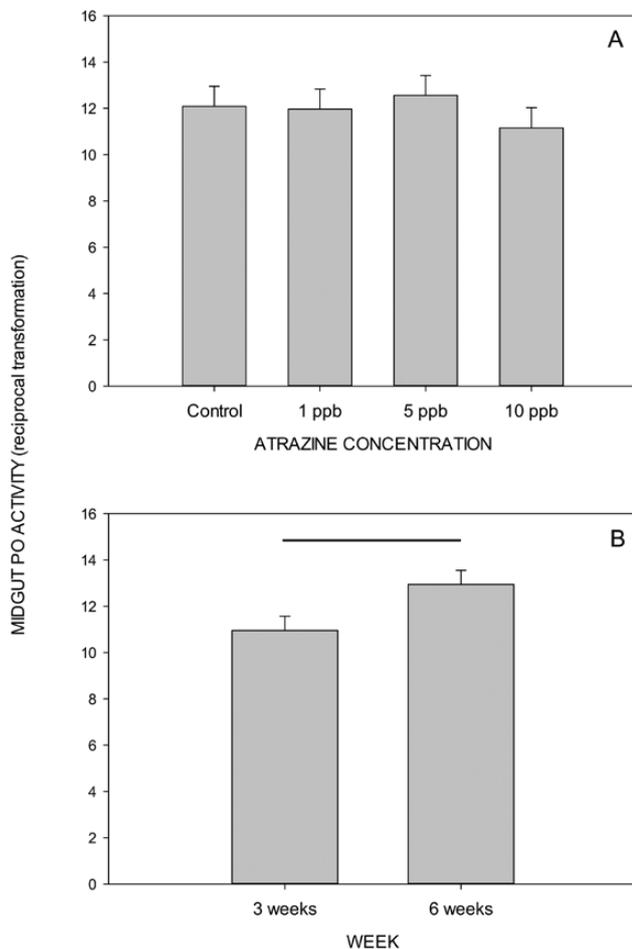


Fig. 4. Mean gut PO activity + SE. $N = 10$ for each group by treatment (A) and week (B). Gut PO activity decreased significantly from 3 to 6 wk ($P = 0.025$). Endpoints of bars above lines indicate groups that are significantly different from each other. Note: data presented as reciprocally transformed values; thus, higher bars indicate lower activity.

Thus, the ramifications of a decrease in PO activity in these areas are unclear in *P. longipennis* larvae. However, that time had an effect in this analysis suggests exposure duration is important when considering the effects of atrazine, and that exposure may have differential effects on larvae at different instars. Additionally, immune function may naturally change as larvae grow, as indicated by the differences in cuticular, gut, and hemolymph PO activity in the control groups. Periods of extended atrazine exposure may change the direction or magnitude of these changes.

The results of this study do not show a clear-cut pattern with regard to the immune response of *P. longipennis* to atrazine exposure. It is unclear if atrazine is perceived by the immune system as an immune challenge, as a pathogen would be, or if another physiological explanation is necessary to account for instances of decreased response, such as decreased energy availability for immunity. Regardless, the data indicate that atrazine clearly influenced the measured immune parameters. These results are important because of the paucity of information on invertebrate immunity and atrazine. While the effects of atrazine on vertebrate immunity have received a great deal of attention (Rohr and McCoy 2010, Sifkarovski et al. 2014) and several authors have documented effects of other agrochemicals on invertebrate immune systems (Chen et al. 2017, Kalita et al. 2017, Zhu et al. 2017), we did not find studies examining the

impact of atrazine on invertebrate immune systems, despite the high levels of use in the United States (Atwood and Paisley-Jones 2017).

It is becoming clear that agrochemicals have population and community-level impacts on organisms (Rohr and Crumrine 2005, Choung et al. 2013, Köhler and Triebkorn 2013). Dragonflies are important members of both aquatic and terrestrial communities. Changes in fitness at the larval stage may follow the dragonflies into adulthood, potentially changing interactions in a terrestrial setting. For example, Thomas and Rudolf (2010) found evidence that insects with dual life cycles can experience effects on immunity across metamorphosis and Benard and Fordyce (2003) showed that the costs of induced defenses in larval *Bufo boreas* were only apparent after metamorphosis. Thus, it is possible that a change in immunity in the larval stage of *P. longipennis* could lower fitness in the adult stage. Life history traits such as reproduction, dispersal, and predation may be altered, which could result in changes to population structure within a single pond, gene flow among metapopulations in different ponds, or loosen predation pressure for terrestrial prey species. Our study shows subtle but significant changes in the immune function of *P. longipennis* that may have widespread ramifications for aquatic and terrestrial communities. Thus, further research is warranted to elucidate the relationship between atrazine and immunity in this ecologically important group.

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References Cited

- Atwood, D., and C. Paisley-Jones. 2017. Pesticide industry sales and usage: 2008–2012 market estimates. US EPA, Washington, DC.
- Bailey, N. W. 2011. A test of the relationship between cuticular melanism and immune function in wild-caught Mormon crickets. *Physiol. Entomol.* 36: 155–164.
- Bara, J. J., A. Montgomery, and E. J. Muturi. 2014. Sublethal effects of atrazine and glyphosate on life history traits of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *Parasitol. Res.* 113: 2879–2886.
- Barnes, A., and M. T. Siva-Jothy. 2000. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proc. Biol. Sci.* 267: 177–182.
- Benard, M. E., and J. A. Fordyce. 2003. Are induced defenses costly? Consequences of predator induced defenses in western toads, *Bufo boreas*. *Ecology.* 84: 68–78.
- Butt, T. M., C. J. Coates, I. M. Dubovskiy, and N. A. Ratcliffe. 2016. Entomopathogenic fungi: new insights into host-pathogen interactions. *Adv. Genet.* 94: 307–364.
- Campero, M., S. Slos, F. Ollevier, and R. Stoks. 2007. Sublethal pesticide concentrations and predation jointly shape life history: behavioral and physiological mechanisms. *Ecol. Appl.* 17: 2111–2122.
- Castro, D. P., C. S. Moraes, M. S. Gonzalez, N. A. Ratcliffe, P. Azambuja, and E. S. Garcia. 2012. *Trypanosoma cruzi* immune response modulation decreases microbiota in *Rhodnius prolixus* gut and is crucial for parasite survival and development. *PLoS One.* 7: e36591.
- Cerenius, L., B. L. Lee, and K. Söderhäll. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* 29: 263–271.
- Chen, X. D., T. A. Gill, K. S. Pelz-Stelinski, and L. L. Stelinski. 2017. Risk assessment of various insecticides used for management of Asian citrus psyllid, *Diaphorina citri* in Florida citrus, against honey bee, *Apis mellifera*. *Ecotoxicology.* 26: 351–359.

- Choung, C. B., R. V. Hyne, M. M. Stevens, and G. C. Hose. 2013. The ecological effects of a herbicide-insecticide mixture on an experimental freshwater ecosystem. *Environ. Pollut.* 172: 264–274.
- Cope, W. G., R. B. Leidy, and E. Hodgson. 2004. Classes of toxicants: use classes, pp. 49–74. In E. Hodgson (ed.), *A textbook of modern toxicology*, 3rd ed. John Wiley and Sons, Inc., Hoboken, NJ.
- Corbet, P. S. 1999. *Dragonflies: behavior and ecology of Odonata*. Cornell University Press, Ithaca, NY.
- Cotter, S. C., and K. Wilson. 2002. Heritability of immune function in the caterpillar *Spodoptera littoralis*. *Heredity*. 88: 229–234.
- Cotter, S. C., L. E. Kruuk, and K. Wilson. 2004. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *J. Evol. Biol.* 17: 421–429.
- Dubovskiy, I. M., M. M. A. Whitten, V. Y. Kryukov, O. N. Yaroslavtseva, E. V. Grizanova, C. Greig, K. Mukherjee, A. Vilcinskas, P. V. Mitkovets, V. Glupov, et al. 2013. More than a colour change: insect melanism, disease resistance and fecundity. *Proc. R. Soc. Lond. B Biol. Sci.* 280: 20130584.
- Eslin, P., and G. Prevost. 1998. Hemocyte load and immune resistance to *Asobara tabida* are correlated in species of the *Drosophila melanogaster* subgroup. *J. Insect Physiol.* 44: 807–816.
- Fedoraka, K. M., V. Lee, and W. E. Winterhalter. 2013a. Thermal environment shapes cuticle melanism and melanin-based immunity in the ground cricket *Allonemobius socius*. *Evol. Ecol.* 27: 521–531.
- Fedoraka, K. M., E. K. Copeland, and W. E. Winterhalter. 2013b. Seasonality influences cuticle melanization and immune defense in a cricket: support for a temperature-dependent immune investment hypothesis in insects. *J. Exp. Biol.* 216: 4005–4010.
- Forson, D. D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. *Ecol. Appl.* 16: 2325–2332.
- Fuller, C. C., and M. Postava-Davignon. 2014. Termites like it hot and humid: the ability of arboreal tropical termites to mediate their nest environment against ambient conditions. *Ecol. Entomol.* 39: 253–262.
- Guedes, R. N., and G. C. Cutler. 2014. Insecticide-induced hormesis and arthropod pest management. *Pest Manag. Sci.* 70: 690–697.
- Guedes, R. N. C., G. Smaghe, J. D. Stark, and N. Desneux. 2016. Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. *Ann. Rev. Entomol.* 61: 1–20.
- Gullan, P. J., and P. S. Cranston. 2000. *The insects: an outline of entomology*, 2nd ed. Blackwell Publishing, Hoboken, NJ.
- Hayes, T. B. 2004. There is no denying this: defusing the confusion about atrazine. *BioScience*. 54: 1138–1149.
- Jager, T., A. Barsi, and V. Ducrot. 2013. Hormesis on life-history traits: is there such thing as a free lunch? *Ecotoxicology*. 22: 263–270.
- Kalita, M. K., K. Haloi, and D. Devi. 2017. Cypermethrin formulation (Ustad-10 EC) induces genotoxicity via apoptosis, affects nutritional physiology, and modulates immune response in silkworm *Philosamia ricini* (Lepidoptera: Saturniidae). *J. Econ. Entomol.* 110: 1010–1024.
- Kavanagh, K., and E. P. Reeves. 2007. Insect and mammalian innate immune responses are much alike. *Microbe*. 2: 596–599.
- Kendig, E. L., H. H. Le, and S. M. Belcher. 2010. Defining hormesis: evaluation of a complex concentration response phenomenon. *Int. J. Toxicol.* 29: 235–246.
- Köhler, H. R., and R. Triebkorn. 2013. Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? *Science*. 341: 759–765.
- Koprivnikar, J., M. R. Forbes, and R. L. Baker. 2006. Effects of atrazine on cercarial longevity, activity, and infectivity. *J. Parasitol.* 92: 306–311.
- Koskimaki, J., M. J. Rantala, J. Taskinen, K. Tynkkynen, and J. Suhonen. 2004. Immunocompetence and resource holding potential in the damselfly, *Calopteryx virgo*. *Behav. Ecol.* 15: 169–173.
- Kraaijeveld, A. R., E. C. Limentani, and H. C. Godfray. 2001. Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Proc. Biol. Sci.* 268: 259–261.
- Kutch, I. C., H. Sevgili, T. Wittman, and K. M. Fedorka. 2014. Thermoregulatory strategy may shape immune investment in *Drosophila melanogaster*. *J. Exp. Biol.* 217: 3664–3669.
- Lee, K. S., B. Y. Kim, and B. R. Jin. 2015. Differential regulation of tyrosine hydroxylase in cuticular melanization and innate immunity in the silkworm *Bombyx mori*. *J. Asia Pac. Entomol.* 18: 765–770.
- Marmaras, V. J., N. D. Charalambidis, and C. G. Zervas. 1996. Immune response in insects: the role of phenoloxidase in defense reactions in relation to melanization and sclerotization. *Arch. Insect Biochem. Physiol.* 31: 119–133.
- McMahon, T. A., R. K. Boughton, L. B. Martin, and J. R. Rohr. 2017. Exposure to herbicide atrazine nonlinearly affects tadpole corticosterone levels. *J. Herpetol.* 51: 270–273.
- McPeck, M. A. 1990. Determination of species composition in the *Enallagma* damselfly assemblages of permanent lakes. *Ecology*. 71: 83–98.
- Mikonranta, L., J. Mappes, M. Kaukoniitty, and D. Freitak. 2014. Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection. *Front. Zool.* 11: 23.
- Moreno-García, M., A. Córdoba-Aguilar, R. Condé, and H. Lanz-Mendoza. 2012. Current immunity markers in insect ecological immunology: assumed trade-offs and methodological issues. *Bull. Entomol. Res.* 103: 127–139.
- Moret, Y. 2003. Explaining variable costs of the immune response: selection for specific versus non-specific immunity and facultative life history change. *Oikos*. 102: 213–216.
- Oertli, B. 2008. The use of dragonflies in the assessment and monitoring of aquatic habitats, pp. 79–95. In A. Córdoba-Aguilar (ed.), *Dragonflies and damselflies: model organisms for ecological and evolutionary research*. Oxford University Press, Oxford, United Kingdom.
- Rantala, M. J., J. Honkavaara, and J. Suhonen. 2010. Immune system activation interacts with territory-holding potential and increases predation of the damselfly *Calopteryx splendens* by birds. *Oecologia*. 163: 825–832.
- Rohr, J. R., and P. W. Crumrine. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. *Ecol. Appl.* 15: 1135–1147.
- Rohr, J. R., and K. A. McCoy. 2010. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. *Environ. Health Perspect.* 118: 20–32.
- Rolff, J. 2001. Effects of age and gender on immune function of dragonflies (Odonata, Libellulidae) from a wild population. *Can. J. Zool.* 79: 2176–2180.
- Rolff, J., and M. T. Siva-Jothy. 2004. Selection on insect immunity in the wild. *Proc. R. Soc. Lond. B Biol. Sci.* 271: 2157–2160.
- Samways, M. J. 2008. Dragonflies as focal organisms in contemporary conservation biology, pp. 97–108. In A. Córdoba-Aguilar (ed.), *Dragonflies and damselflies: model organisms for ecological and evolutionary research*. Oxford University Press, Oxford, United Kingdom.
- Schmid-Hempel, P. 2005. Evolutionary ecology of insect immune defenses. *Annu. Rev. Entomol.* 50: 529–551.
- Schmid-Hempel, P., and D. Ebert. 2003. On the evolutionary ecology of specific immune defence. *Trends Ecol. Evol.* 18: 27–32.
- Schwenke, R. A., B. P. Lazzaro, and M. F. Wolfner. 2016. Reproduction-immunity trade-offs in insects. *Annu. Rev. Entomol.* 61: 239–256.
- Sifkarovski, J., L. Grayfer, F. De Jesús Andino, B. P. Lawrence, and J. Robert. 2014. Negative effects of low dose atrazine exposure on the development of effective immunity to FV3 in *Xenopus laevis*. *Dev. Comp. Immunol.* 47: 52–58.
- Siva-Jothy, M. T., Y. Tsubaki, and R. E. Hooper. 1998. Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiol. Entomol.* 23: 274–277.
- Siva-Jothy, M. T., Y. Tsubaki, R. E. Hooper, and S. J. Plaistow. 2001. Investment in immune function in chronic and acute immune challenge in an insect. *Physiol. Entomol.* 26: 1–5.
- Solomon, K. R., D. B. Baker, P. Richards, K. R. Dixon, S. J. Klaine, T. W. La Point, R. J. Kendall, J. M. Giddings, J. P. Giesy, L. W. J. Hall, et al. 1996. Ecological risk assessment of atrazine in North American surface waters. *Environ. Toxicol. Chem.* 15: 31–76.
- St. Clair, C. R., and C. A. Fuller. 2014. Atrazine exposure increases time until cannibalistic response in the widow skimmer dragonfly (*Libellula luctuosa*). *Can. J. Zool.* 92: 113–117.

- Storrs, S. I., and J. M. Kiesecker. 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. *Environ. Health Perspect.* 112: 1054–1057.
- Stuijffzand, S., L. Poort, G. Greve, G. van der Geest, and M. Kraak. 1999. Variables determining the impact of a diazinon on aquatic insects: taxon, developmental stage, and exposure time. *Environ. Toxicol. Chem.* 19: 582–587.
- Suhonen, J., J. Honkavaara, and M. J. Rantala. 2010. Activation of the immune system promotes insect dispersal in the wild. *Oecologia.* 162: 541–547.
- Thomas, A. M., and V. H. W. Rudolf. 2010. Challenges of metamorphosis in invertebrate hosts: maintaining parasite resistance across life-history stages. *Ecol. Entomol.* 35: 200–205.
- Tyurin, M. V., V. Y. Kryukov, O. N. Yaroslavtseva, E. A. Elisafenko, I. M. Dubovskiy, and V. V. Glupov. 2016. Comparative analysis of immune responses in Colorado potato beetle larvae during development of mycoses caused by *Metarhizium robertsii*, *M. brunneum* and *M. pempfigi*. *J. Evol. Biochem. Physiol.* 52: 252–260.
- US Department of Agriculture. 2007. Revised environmental assessment: continued maintenance of open lands. Available at: https://www.fs.usda.gov/nfs/11558/www/nepa/11628_FSPLT2_025660.pdf. Accessed 27 September 2018.
- Vandenberg, L. N., T. Colborn, T. B. Hayes, J. J. Heindel, D. R. Jacobs, Jr., D. H. Lee, T. Shioda, A. M. Soto, F. S. vom Saal, W. V. Welshons, et al. 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses. *Endocr. Rev.* 33: 378–455.
- Zhu, Y. C., J. Yao, J. Adamczyk, and R. Luttrell. 2017. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). *PLoS One.* 12: e0178421.