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A common measure of prey immune function is not constrained by the cascading effects of predators

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Abstract

Simultaneously defending against predators, stymieing competitors, and generating immune responses can impose conflicting demands for host species caught in the entanglement of a food web. Host immunity is not only shaped by direct interactions among species, but also many indirect cascading effects. By reducing competition, predators in particular can affect resource acquisition necessary for hosts to mount energetically costly immune responses. However, identifying the links between predators and host immune responses determined by resource acquisition is a complex affair, because predators can (1) reduce host density and thus competition among hosts, (2) exert non-consumptive trait-mediated effects on host resource acquisition behavior, and (3) generate natural selection on host resource acquisition behavior. To examine the relative contributions of these potential predator driven density- and trait-mediated effects on a key aspect of immune function (total phenoloxidase activity, total PO), we conducted mesocosm and field experiments with larval damselflies (*Enallagma signatum*) and their dominant fish predator (*Lepomis macrochirus*). Although we expected to observe declines in total PO activity with increases in damselfly density, we found no relationship between density and total PO activity. We also found no support for the prediction that total PO activity would vary as a result of either non-consumptive trait-mediated effects or selection on damselfly foraging activity underlying resource acquisition. Despite the lack of trait- or density-mediated effects, we did find that total PO activity increased with damselfly prey density among lakes, implying resource limitation for this aspect of immune function. These unexpected results point to the need to better understand the ecological conditions whereby predators and competitors constrain immune functions necessary for species to defend themselves in complex food webs.

Keywords Competition · Density · Direct effects · Immune function · Predation · Trait-mediated effects

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Introduction

Immune defenses are critical components of host defense against parasites and pathogens (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; Schmid-Hempel 2005; Siva-Jothy et al. 2005; Sadd and Schmid-Hempel 2009). However, immune defenses are energetically costly and depend on the ability of hosts to acquire and utilize resources (Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; González-Santoyo and Córdoba-Aguilar 2012). Yet resources are often limited, and resource acquisition is further constrained by the ecological milieu (e.g., interactions with predators, competitors, mutualists) species exist in. Consequently, identifying the ecological factors and phenotypic traits influencing resource acquisition can provide insight into understanding how host immune defenses can be shaped by complex food webs. Here we ask how the cascading effects of predators on host competitive interactions shapes a common component of immune function.

We focus on predators because they can have a role in shaping the interplay between host competition and resource acquisition, and therefore immune function via several paths (Fig. 1). First, the direct consumptive effects of predators lower host population densities, which can reduce intraspecific competition (Chase et al. 2002; Chesson and Kuang 2008). Because increasing competition reduces resource acquisition (McPeek and Crowley 1987; McPeek 1998; Kobler et al. 2009), predator-mediated reductions in competition should increase resource access, enhancing host immune function (Siva-Jothy and Thompson 2002; Kristan 2008; Budischak et al. 2018). Second, predators can generate indirect (non-consumptive) trait-mediated effects that influence competition (Werner and Peacor 1993; Preisser et al. 2005). For example, many prey respond to the presence of predators by reducing activity rates (e.g., foraging) and such reduced activity can decrease competition (Werner and Peacor 1993; McPeek 2004; Strobbe and Stoks 2004; Ousterhout et al. 2018; Siepielski et al. 2020). These indirect effects of predators could then act to increase resource access and enhance immune function. Alternatively, the indirect effects of predators might be expected to decrease immune function, as reductions in foraging rates could instead limit resource acquisition and, by extension, immune function. Finally, natural selection exerted by predators, which couples the consumptive and trait-based effects of reduced activity, can also reduce prey resource consumption (Strobbe et al. 2011; Ousterhout et al. 2018), and thus the strength of competition (Siepielski et al. 2020). Predators can also indirectly affect prey immune function via stress responses (e.g., Duong and McCauley 2016; Adamo 2017), but such effects are beyond the scope of this study. While these disparate paths between predators and host competitive interactions imply that the direct and indirect effects of predators should strongly affect host immune function, revealing these paths is a complex affair.

A wealth of studies in *Enallagma* damselflies have uncovered many of the phenotypes and mechanisms linking predation and competition to understand how *Enallagma* persist in food webs, acquire resources, and complete their life cycle (McPeek 1990, 1998, 2017; Stoks and McPeek 2006; Siepielski et al. 2010, 2011, 2020; Siepielski and McPeek 2013). *Enallagma* are aquatic insects that inhabit the littoral zone of waterbodies for most of their life cycle. Species found in lakes with fish as the top predator are adapted to coexisting with these predators by being relative inactive, as reduced activity helps them avoid detection (Strobbe et al. 2011; Swaegers et al. 2017; Ousterhout et al. 2018). Consequently, selection by fish favors less active individuals, which consume fewer prey items and may therefore experience greater resource limitation (Strobbe et al. 2011; Ousterhout et al. 2018; Siepielski et al. 2020). Indeed, damselflies in fish lakes are food limited and show consistent

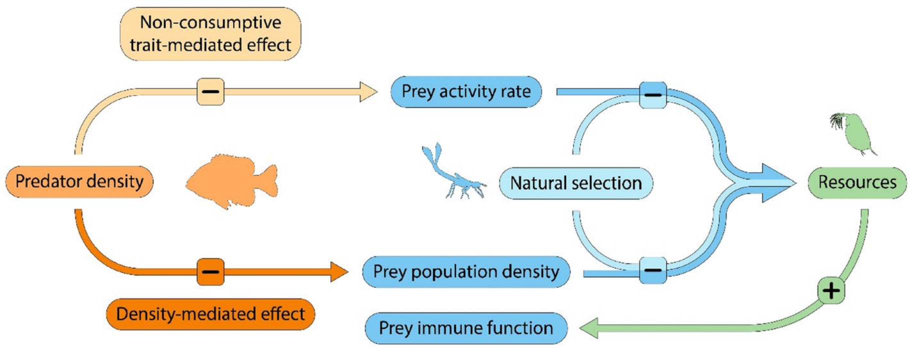

Fig. 1 Conceptual diagram of the hypothesized relationships between predators, prey (host) population density, prey activity rates, resource acquisition rates, and prey immune function. Predators reduce both prey population density (via direct, density-mediated consumptive effects) and prey activity rates (via indirect, non-consumptive trait-mediated effects). Natural selection lowers both the average activity rate and density of a given prey population, as predators disproportionately consume the more active individuals. Predator-mediated selection decreases competition for resources, increasing the per capita resource acquisition rate. Increases in resource acquisition rate then drive increases in prey immune function

declines in growth rates with increasing densities (Anholt 1990; McPeek 1990, 1998; Siepielski et al. 2010, 2020; Ousterhout et al. 2019). This negative density-dependent response results from both direct interference competition (e.g., stress responses to conspecifics, McPeek et al. 2001) and indirect resource-based competition (McPeek 1990, 1998; Siepielski et al. 2020).

Numerous studies have also examined how some of these same ecological mechanisms affect damselfly immune function (Contreras-Garduño et al. 2006; Mikolajewski et al. 2008; Jiménez-Cortés et al. 2012). In particular, several studies have investigated the ecological basis for population-level variation in a vital, resource-limited component of their immune system—the phenoloxidase (PO) cascade (Marmaras et al. 1996; González-Santoyo and Córdoba-Aguilar 2012). This enzymatic cascade begins with the activation of PO and produces melanin as its end product, which encases and kills foreign bodies such as parasites and parasitoids (reviewed in González-Santoyo and Córdoba-Aguilar 2012). Total PO (PO measured in the absence of an immune challenge) has been well-investigated in the context of understanding interactions between damselfly hosts and ectoparasitic mites. These studies have found that competitors and predators can limit the strength of this aspect of their immune function, either by diverting resources for use in competition at the adult life stage (Contreras-Garduño et al. 2006), by prioritizing growth under predation risk (Stoks et al. 2006), or by reducing access to resources (Jiménez-Cortés et al. 2012). Indeed, resource-limitation directly reduces total PO in damselflies (Campero et al. 2008; De Block and Stoks 2008). All of these effects on total PO during the larval stage have the potential to then translate to effects on the interactions with ectoparasitic mites at the adult stage, as any reduction in total PO could result in a subsequent increase in parasitism. As of yet, though, how the direct, indirect, and combined effects of predators manifested through natural selection influence host competition and immune function has not been explored. By revealing the paths between predators, competing prey, and immune function, we can further our understanding of the direct and indirect links between defenses against parasitism and other species interactions in ecological communities.

To begin unraveling these complexities, we previously used a series of mesocosm and field experiments to investigate how a fish predator (*Lepomis macrochirus*) shaped the strength of intraspecific competition in a larval damselfly species (*E. signatum*) via direct, indirect, and combined effects of natural selection (Siepielski et al. 2020). In that study we did not examine how these effects shaped damselfly immune function. However, those experiments provided an ideal opportunity to investigate this. Below, we present the major results of those experiments and use them to frame and generate two sets of predictions for how a key component of damselfly immune function (total PO) should vary in response to the effects of predation and competition.

Background study and derived predictions

First, we used a mesocosm experiment to parse out: (i) the direct consumptive effects of fish predators reducing damselfly density, (ii) the indirect non-consumptive trait mediated effects reducing activity rates, and (iii) the combined consumptive and trait-based effects generated by fish exerting selection favoring less-active individuals (e.g., Fig. 1). We found that reducing damselfly densities had the greatest effect on damselfly growth rates and thus the strength of intraspecific competition. However, this effect depended on the strength of selection on damselfly activity rates, since density-dependent growth in damselflies (i.e., the slope relating growth rates to damselfly density) weakened as selection for less active individuals increased. That is, the strength of intraspecific competition decreased as selection by fish predators on activity rates increased (see Fig. 1 of Siepielski et al. 2020). Less active individuals also had lower attack rates and higher handling times associated with prey capture. Reductions in activity alone (a pure trait-mediated effect) had minimal effects on growth rates. From these combined results, we predict that i) immune function (total PO activity) should increase as damselfly densities decrease, and ii) this effect should be greatest as the strength of selection favoring less-active damselflies increases.

Next, we conducted field experiments to examine how the strength of competition damselflies experienced varied among lakes differing in fish and damselfly prey densities. We found that damselfly activity rates declined as fish densities increased among lakes (Siepielski et al. 2020), presumably as an adaptive response to more intense selection (Ousterhout et al. 2018, see also Benkman 2013). Thus, we expected that the strength of negative density dependence in damselfly growth rates should also decline as fish densities increased—a pattern we found support for (see Fig. 3 of Siepielski et al. 2020). We also found that individual damselfly growth rates (growth in the absence of competitors) increased with natural prey density in these lakes, but the strength of density-dependence in growth rates was not associated with prey densities. That is, damselflies grew faster in lakes with more prey, implying resource limitation (e.g., McPeek 1998), but this effect did not depend on damselfly density. From these results we predict that as fish densities increase, and the strength of competition declines because of reduced activity rates, that total PO should increase. Similarly, we predict that as prey densities increase among lakes, total PO should also increase. Evaluating these predictions provides a key test of how predators can shape host competitive dynamics and in turn influence how a key aspect of immune function is shaped in complex food webs where species face conflicting ecological demands (Rigby and Jokela 2000).

Materials and methods

To test these predictions, we saved and used the same larval damselflies from the above experiments. Because the methods for these experiments (save the PO assays) have been previously published (Siepielski et al. 2020), we only briefly summarize them here.

Prediction 1: effects of predator consumption, trait, and selection on immune function

This first experiment was designed to isolate the effects of predator consumption, non-consumptive trait-mediated effects on damselfly activity rates, and selection on damselfly activity rates on damselfly total PO activity levels. To accomplish this, we used a fully factorial design crossing damselfly density with differences in average damselfly activity rates. A full description of these methods and the experimental design can be found in Siepielski et al. (2020). Below, we include the most salient details. Differences in total PO activity because of damselfly density would reflect changes in the competitive environment driven by predator consumption (e.g., reducing damselfly density). Differences in total PO because of damselfly activity alone, controlling for density, mimic non-consumptive effects of predators. We note that while directly manipulating activity as we did mimics the effects of reduced foraging in the presence of a predator, the possibility exists that individuals with a genetic background for reduced activity (e.g., Swaegers et al. 2017) may have immune compensatory mechanisms that offset the costs of reduced foraging, a point we return to in the *Discussion*. Importantly, we previously found an overall positive correlation in activity levels among individuals with and without predator cues (Siepielski et al. 2020), meaning that more active individuals are more active in the presence of predator cues, yet they still reduce their activity rates. Regardless, by crossing these two factors, the interaction between them captures how selection imposed by predators can alter total PO activity levels (e.g., the effect of depressing density via consumption when combined with changes in activity is equivalent to a covariance between fitness (survivorship) and traits).

To quantify activity rates, we gathered 400 late instar *E. signatum* larvae from two lakes with low fish densities (Charleston and Greenwood) in west-central Arkansas, USA (Ousterhout et al. 2019). Damselflies in these lakes were likely under weak selection by fish (Ousterhout et al. 2018) and thus represented a broad distribution of activity rate phenotypes. Activity rates were quantified using open field tests (Johansson and Rowe 1999; Brodin and Johansson 2004; Start and Gilbert 2017). All assays were conducted in a greenhouse under natural lighting and temperature conditions with a fan constantly circulating air. We assayed the activity rates of individual larvae by placing a single larva in a petri dish (10-cm diameter) filled with filtered pond water. Larvae acclimated for 12–15 h, after which we recorded their position every 20 min for 3 h. Activity rate was quantified as the sum of minimum distances between successive locations, expressed as mm moved/three hours. Importantly, activity rates of individual larvae saved from the previous experiment were a repeatable phenotype (repeatability = 0.56, 95% CI: 0.27, 0.76, Siepielski et al. 2020, see also Start 2018).

Directional selection by predators on activity levels works by generating differences in mean activity rates before relative to after consumption by a predator. Given our extensive knowledge of how selection by fish acts on damselfly activity (Strobbe et al. 2011; Swaegers et al. 2017; Ousterhout et al. 2018; Siepielski et al. 2020), we simulated such selection by ranking the activity rates of the 400 assayed damselflies and then dividing them into four groups (hereafter 'activity levels', see Fig. 2) corresponding to significant differences in mean activity rates (Fig. 1, Fig. S2 in Siepielski et al. 2020). The difference between the mean activity level across all groups (e.g., before 'selection' occurred) and the mean in each group (e.g., equivalent to activity levels of the 'survivors' after selection occurred) represents the effect of selection, with each group reflecting a different intensity of selection. These groups represent population-level variation in activity levels commonly observed in odonates (Start 2018) and should represent differential survival across natural fish densities (Ousterhout et al. 2018).

To determine the effects of variation in damselfly density, we used 5.5L plastic tray mesocosms (0.25 m diameter, bottom area = 0.05 m^2) filled with filtered lake water, macrophytes (*Ceratophyllum* spp.), and a single inoculation of *Daphnia* prey (~ 50 individuals/L). *Daphnia* abundances were not monitored over the course of the experiment. Mesocosms were housed in a greenhouse under natural lighting and temperature conditions, with a fan circulating air. After completing the activity rate assays, damselflies were established at densities of 1, 2, 4, or 10 per mesocosm, which are equivalent densities of 20–198 damselflies/m^2, and similar to natural larval densities in this area (Ousterhout et al. 2019). Each of the four activity levels was then crossed with density in a factorial design with five replicates each ($n = 80$ total mesocosms). Larvae were collected after 21 days, which is sufficient time to allow for competitive effects to affect damselfly growth rates (Siepielski et al. 2020) and for differences in total PO values to manifest (De Block and Stoks 2008), then were stored at − 80 °C to later conduct PO assays.

Prediction 2: density dependent immune function along predator and prey density gradients

This second experiment was designed to examine how density-dependent responses of damselflies to intraspecific competition varied along gradients of fish predators and damselfly prey resources. Damselfly activity rates decrease with increasing fish predator density (McPeek 2004; Strobbe et al. 2011; Ousterhout et al. 2018; Siepielski et al. 2020), and the strength of density-dependent competition decreases with increasing fish density (Siepielski et al. 2020). These patterns, combined with variation in natural prey densities among lakes (Ousterhout et al. 2019) imply that damselflies not only experience spatial variation in resource availability, but also spatial variation in the strength of competition for these resources. Because immune function is tied to host resource acquisition (Siva-Jothy and Thompson 2002; Budischak and Cressler 2018; Budischak et al. 2018), spatial variation in damselfly prey resource availability (as dictated by natural prey densities) and acquisition (as dictated by damselfly activity levels) should explain variation in total PO activity among damselfly populations.

Thus, to examine how the effects of competition (as generated through negative density dependence in damselfly growth rates, McPeek 1990, 1998; Siepielski et al. 2010, 2011, 2020) affected damselfly immune function (total PO activity) among lakes varying in fish and prey densities, we established 20 submerged cages in the littoral zone of six lakes ($n = 120$ total cages). A full description of these methods and the experimental design can be found in Siepielski et al. (2020). Below, we include the most salient details. These lakes varied in both fish (range: 1.08–16.49 fish/m^2, based on the mean of three replicate seine hauls per lake) and prey densities (range: 11.85–121.68 prey/L, based on the mean of six replicate samples with a 6 L box sampler [100-μm mesh] placed over macrophytes where damselflies forage). Complete details for methods used to generate these estimates can be found in Ousterhout et al. (2019). Cages were constructed with PVC pipe (2.1 cm diameter) and enclosed in mesh netting (0.6×1.2 mm mesh), allowing prey to colonize the cages but keeping non-experimental larvae out. Each cage was stocked with macrophytes (*Justica americana*) to provide a foraging substrate for damselflies. Density treatments were 1, 2, 4, or 10 larvae per cage. Larvae were removed after 21 days and stored at − 80 °C to later conduct PO assays.

Quantification of total PO activity

To quantify innate immune function, we measured total PO activity using a modified protocol (Iserbyt et al. 2012; Mlynarek et al. 2015). Larvae were placed into microcentrifuge tubes with 300 µl of cacodylate buffer (0.01 M $C_2H_6AsNaO_2$–0.005 M $CaCl_2$), crushed in the cooled buffer, after which they were centrifuged at 15000 rpm for 10 min at 4 C. After centrifugation, 100 µl of the supernatant was placed into a well of a 96-well plate containing 35 µl of 50 mM PBS buffer, after which 5 µl of α-chymotrypsin (Sigma Aldritch #C4129) was added. After reacting for 5 min at room temperature, 60 µl of L-DOPA (10 mM/L of dihydroxyphenyl-L-alanine (Sigma Aldritch #D9628) in cacodylate buffer) was added as substrate for the reaction. Total PO values were measured in duplicate, and the mean of the readings was used for analyses.

The PO reaction was measured in a spectrophotometer (SprectraMax 190 Microplate Reader, Molecular Devices) for 30 min at 30 C and read at 485 nm. A reading was taken every 20 s, and the plate shaken between each reading. Total PO activity values were measured as the slope of the reaction curve. As in previous studies (e.g., Iserbyt et al. 2012; Mlynarek et al. 2015), to control for variation in body size, we measured the protein content of each damselfly using a modified Bradford protocol (Bradford 1976). Using the supernatant from the PO assay, we prepared the protein assay on a 96-well plate as follows: 40 µl of dye (Bio-Rad #5,000,006), 155 µl of Milli-Q water, 5 µl of supernatant, and 40 µl of Bradford solution. The plate was read at 595 nm at 30 C after 6 min of continuous shaking. Protein content was measured once at the endpoint and compared with a standard curve with Bovine serum albumin (Bio-Rad #500–0005). Protein was measured in duplicate, and the mean of the readings used for statistical analyses.

Statistical analysis

Ideally, we would have tracked individuals separately so that we could determine the relationship between individual activity rates and total PO values. However, except for the single individual density treatments it was impossible to mark individual larvae over the duration of the experiments (damselflies grow by molting, so any marks would be lost). To examine the association between individual-level activity and total PO we analyzed the correlation between activity levels and total PO values from the single density treatments in the mesocosm experiment.

In both experiments we had mortality from unknown causes (cannibalism was possible, but mortality occurred across all densities). Any change in damselfly density may have affected resource acquisition. Thus, for the models presented below we initially constructed two sets of models: one utilizing the initial experimentally applied densities, and one utilizing the final densities to account for mortality. There were, however, no qualitative differences between the models fit with initial and final densities. Because of this, and because it was impossible to determine when individuals died, we used initial densities as in Siepielski et al. (2020). Importantly, despite these occasional losses, the density treatments still resulted in declines in damselfly growth rates (e.g., Siepielski et al. 2020). During the field experiment our lakes also experienced intense storms and all cages from the 1 and 4 larvae density treatments were lost from Lake Fayetteville. In addition, we were unable to use data from several protein assays due to unsatisfactory standard curves ($r < 0.90$). In total, we used data from 58 mesocosms for experiment 1 and 78 cages from experiment 2.

Prediction 1: effects of predator consumption, trait, and selection on immune function

Here, we wanted to evaluate the direct effects of predator consumption (depressing damselfly densities), non-consumptive (indirect) trait-mediated effects on damselfly activity rates, and selection on damselfly total PO activity. To do so, we built a mixed-effects model of the form: individual total PO activity as the response variable with activity level, damselfly density, and their interaction as fixed effects, and mesocosm as a random effect. Individual protein content was included as a covariate to control for potential differences in size (Mikolajewski et al. 2008). Although activity rate was treated as a categorical term, it does represent differences in average damselfly activity rates (see Siepielski et al. 2020). For this model, a significant interaction term would indicate that the effect of damselfly density on total PO level depended on the activity level, thus revealing an effect of predator-driven selection on immune function.

We also evaluated if total PO levels were condition-dependent (e.g., as reflected by differences in growth rates). To do so, we tested for a correlation between mesocosm mean total PO activity and mesocosm mean growth rate (changes in body size through time) from Siepielski et al. (2020) across all treatments and mesocosms.

Prediction 2: density dependent immune function along predator and prey density gradients

We examined if the effects of competition (as generated through differences in damselfly cage densities; Siepielski et al. 2020), affected damselfly immune function (total PO activity) among lakes varying in fish and prey densities. Specifically, we were interested in whether there were significant interactions between total PO activity and damselfly, fish, and prey densities among lakes; fish and prey densities were not correlated among lakes ($n = 6$, $r = 0.148$, $p = 0.780$). To examine this, we built a mixed-effects model of the form: individual total PO activity as the response variable, with damselfly cage density, fish density, prey density, and interactions between damselfly density and fish or prey density as fixed effects, with cage nested within lake as random effects (Table 1). Marginal and conditional r^2 for this model were obtained as described in Nakagawa and Schielzeth (2013). As above, individual protein content was included as a covariate to control for potential differences in size. To examine if total PO levels were condition-dependent, we again tested for a correlation between cage mean total PO activity and cage mean growth rates from Siepielski et al. (2020) across all treatments and cages. All analyses were performed in R ver 4.0.0 (R Core Team 2020).

Results

Effects of predator consumption, activity, and selection on immune function

There was considerable variation in both individual activity rates and total PO activity among damselflies, although there was no correlation between these factors (Fig. 2e–g). For the single density treatments, where we had individual-level data, there was also no correlation between activity rates and total PO activity ($n=11$, $r=-0.004$, $p=0.99$, Fig. S1). For the mixed-effect model, there was no effect of damselfly density (a predator consumptive effect, $F=0.52$, $p=0.47$), average activity level (a trait mediated effect, $F=0.04$, $p=0.99$), or selection (the interaction between density and mean activity level, $F=0.07$, $p=0.98$, Fig. 2a–d). Likewise, there was no significant correlation between total PO activity and mean growth rates ($n=58$, $r=0.14$, $p=0.29$, Fig. S2a).

Immune function along environmental gradients

Total PO activity varied significantly among lakes (mixed effect model, Lake effect, $F=50.10$, $p<0.0001$; Fig. 3a–f). Larvae from Lake Wilson had the lowest total PO

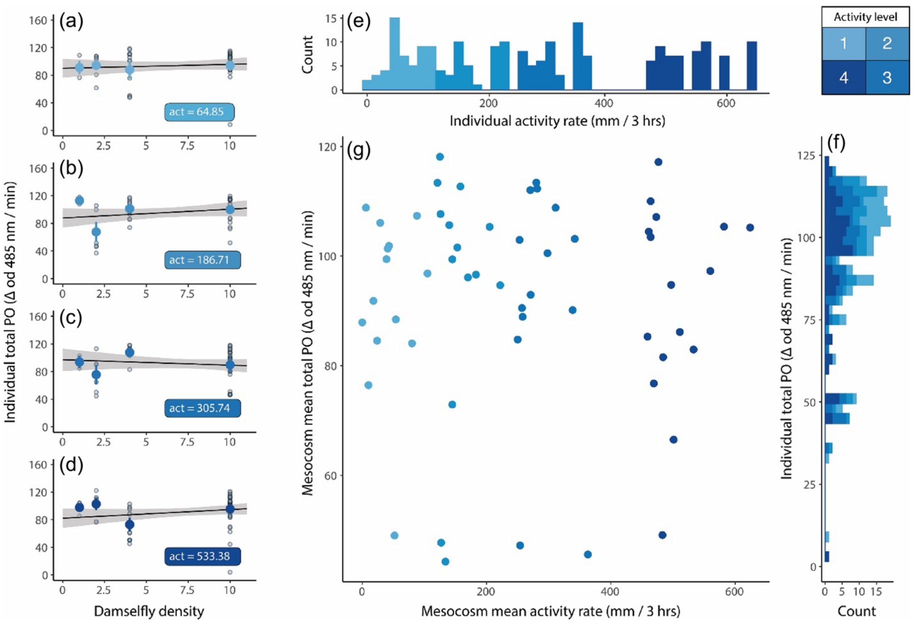

Fig. 2 There was no effect of damselfly density or activity level on damselfly total PO activity. Shown are the linear regressions (grey bands denote 95% CI) between total PO activity and larval density for each of the four activity levels, ordered from least active to most active (**a–d**). The large dark points denote density treatment means and one s.e.; smaller light shaded points denote total PO activity from individual damselflies. Inset values denote mean activity rate (mm moved/3 h) for each activity level. Also shown are histograms of (**e**) individual damselfly activity rates and (f) individual total PO activity levels from the mesocosms. Points in (**g**) represent mesocosm mean total PO activity in relation to mesocosm mean activity level ($r=0.0006$, df$=56$, $p=0.99$). Colors denote the activity level treatments

activity (mean = 37.40, s.e. = 3.95), while it was almost three-times higher on average for the larvae in the other five lakes, as those from Bobb Kidd Lake had the highest total PO activity (mean = 106.62, s.e. = 3.49). Tukey post-hoc tests revealed that Lake Wilson larvae had significantly lower total PO activity than larvae from the other lakes ($p < 0.001$ for all tests), but total PO activity did not differ for larvae from the other five lakes ($p > 0.58$ for all tests). Though total PO activity was not correlated with average activity rates among lakes (Fig. S3), it was weakly positively correlated with mean growth rates ($n = 78$, $r = 0.23$, $p = 0.04$, Fig. S2b).

Although total PO activity varied among lakes, we found no evidence for an effect of damselfly density on total PO activity (Table 1, Fig. 3a–f), nor an effect of fish density (Table 1, Fig. 3g). However, we did find that total PO activity increased with greater prey density among lakes (Table 1, Fig. 3h). Graphical inspection revealed that the relationship between total PO and prey density was non-linear. Thus, we built an asymptotic regression model of total PO on prey density to quantify this association using the nls function (R Core Team 2020). This model was of the form total PO = $a(1 - e^{(-c^{*}\mathrm{prey/L})})$, where a is the estimated upper total PO limit and c is a parameter describing the proportional increase in total PO with prey/L. This model showed that total PO activity rapidly increased once prey density exceeded about 17/L and then stabilized at an upper limit of about 110 (Δod 485 nm/min) (Fig. 3h).

Discussion

Predators frequently generate cascading effects on competitive interactions that affect host resource acquisition (Gurevitch et al. 2000; Chase et al. 2002; Siepielski et al. 2020). Because the ability to mount an immune response is also resource limited and condition-dependent, we posited that trait- and density-mediated effects of predators on competitive interactions suppressing damselfly resource acquisition would in turn influence total PO. Yet, our results fell contrary to this overall hypothesis. We found no support for the prediction that total PO would vary due to non-consumptive trait-mediated effects on activity rates, or through effects of selection on activity that affects resource acquisition. Similarly, although we expected to observe declines in total PO with increases in damselfly density, we found no relationship between density and total PO. Despite the lack of trait- or density-mediated effects, we did find that total PO increased with prey density among lakes. Taken together, the replicated nature of these results among two independent sets of experiments imply that predation and competition do not constrain a key aspect of immune function.

Table 1 Results from the mixed effects model of damselfly immune function (total PO activity) in relation to damselfly cage density, fish density, and prey density among lakes

Term	Estimate	s.e	t	*p*
Intercept	34.03	12.21	2.78	0.005
Prey density	0.47	0.15	3.06	0.002
Fish density	0.40	1.46	0.27	0.783
Cage density	0.70	0.85	0.83	0.409
Prey density × cage density	−0.01	0.02	−0.83	0.405
Fish density × cage density	0.04	0.13	0.36	0.716

This model had a marginal $r^2 = 22.26\%$ and conditional $r^2 = 78.46\%$

We found no relationship between total PO levels and differences in activity rates arising through either a non-consumptive trait-mediated effect or an effect of selection. This was surprising as i) our mesocosm experiment showed that differences in activity rates affect damselfly prey acquisition by lowering attack rates and increasing handling times, and ii) damselflies in the field experiment had reduced activity rates as an adaptive response to increasing fish predation (Siepielski et al. 2020). Mlynarek et al. (2015) also found no consistent difference in total PO levels between *Enallagma* species found in lakes with either fish or dragonflies as the top predator. These differences among species in lakes with different top predator are noteworthy, as fish select for reduced activity levels (Stoks and McPeek 2003; Strobbe et al. 2011; Siepielski et al. 2020) while dragonflies select for more active damselflies that may have higher prey attack rates (Strobbe et al. 2009). Thus, collectively, these population-level and comparative findings imply that activity-based adaptations to predators do not constrain this aspect of immune function.

Though we tested for an effect of predators on one key aspect of immune function, we acknowledge that immune function is multi-faceted. Prey responses to predators have been shown to have no effect on some components of immune function, but also increase and simultaneously decrease other components (Rigby and Jokela 2000; Vinterstare et al. 2019). Resource limitation can also differentially affect immune function components. For example, De Block and Stoks (2008) found that haemocyte levels returned to normal after a period of resource restriction, but total PO and proPO levels remained low into the adult stage for damselflies. However, the PO cascade is not only an important defense against many parasites and pathogens, it is also used in pigment synthesis, egg production, and wound-healing of damaged tissue (reviewed in González-Santoyo and Córdoba-Aguilar 2012). Damselflies engage in attempted cannibalism (Anholt 1994) and direct interference competition (McPeek et al. 2001), both of which result in wounding that would require PO-facilitated repair. Additionally, defense against parasites in insects often relies on melanization (Siva-Jothy et al. 2005; González-Santoyo and Córdoba-Aguilar 2012). For example, the most common ectoparasite in this system are *Arrenurus* water mites which parasitize odonates at the transition to the adult stage (Smith et al. 2010), and adult damselflies defend themselves from these mites by melanizing their feeding tubes via the PO cascade (Marmaras et al. 1996). PO responds to experimental parasite challenges much like other immune parameters (Stoks et al. 2006; Gershman 2008; Srygley and Jaronski 2011) and PO levels positively correlate with not only melanization (Zhang et al. 2008), but also with defense against parasites (Fedorka et al. 2013). Thus, our use of total PO is an appropriate, though limited, representation of overall immune function (Gershman 2008; Srygley and Jaronski 2011; Stahlschmidt et al. 2020). Future studies should nevertheless consider a multitude of immune defenses such as haemocyte counts, nitric oxide, and proPO (e.g., Siva-Jothy et al. 2005; Mlynarek et al. 2015). However, because the PO cascade is the primary defense against *Arrenurus* mites, our results imply that indirect effects of predators are decoupled from mite parasitism in this system.

A key aspect of our mesocosm experiment was that by having predator cues absent it uncoupled changes in activity rates from any other possible non-consumptive effects of predators on immune function. This is important to consider as other studies have found that the mere presence of predators affects immune function (Stoks et al. 2006; Mikolajewski et al. 2008; Duong and McCauley 2016). For example, Duong and McCauley (2016) found that the presence of dragonfly predators did not affect activity levels of their prey, but it did increase prey melanization levels via the PO cascade. However, this is also a strength of our experimental design because unlike previous studies it allowed us to specifically isolate the effects of activity relative to any other confounding effects of plastic responses to

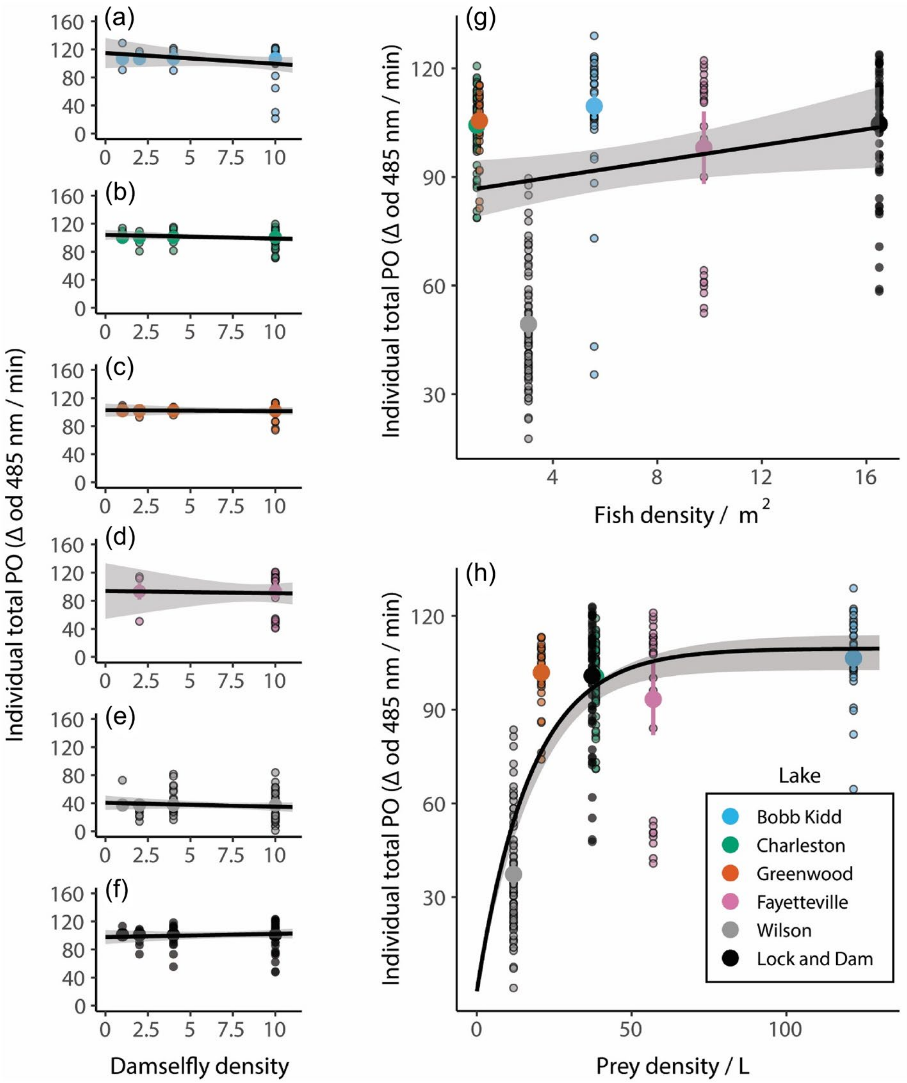

Fig. 3 There was no effect of damselfly density or fish density on damselfly total PO activity, but immune function increased with prey density among lakes. Panels in (**a**–**f**) are linear regressions of total PO activity on damselfly density from each of the six lakes. (**g**) Shows the linear regression of total PO activity in relation to fish density, and (**h**) shows the asymptotic regression of total PO activity in relation to prey density (a, estimated upper limit = 108.50, s.e. = 2.87, t = 37.77, $p < 0.001$; c, proportional change = 0.06, s.e. = 0.01, t = 12.58, $p < 0.001$, r^2 = 87.56). Grey bands in all panels denote 95% CI, larger dark points in (**a**–**f**) denote density treatment means and one s.e., while larger dark points in (**g**) and (**f**) denote lake means and one s.e. Small, light points in all plots denote total PO activity values from individual damselflies

predator cues. Notably, however, in the field experiment such cues were present, and higher fish densities should correspond with stronger cues that illicit predator threat responses (Siepielski et al. 2014, 2016; Tollrian et al. 2015). Yet there was no correlation between total PO activity and fish densities. Thus, despite inhabiting communities where the risk of predation is greater (Ousterhout et al. 2018), damselflies maintained consistently high total PO, implying that this aspect of immune function is not constrained by predators, and that the lack of finding an effect on total PO is likely not an artifact per se of not having predator cues present (e.g., Stoks et al. 2006).

The lack of any association between the direct, indirect, or combined effects of predators on total PO in *Enallagma* damselflies is surprising, as previous studies have demonstrated that predators can limit (Stoks et al. 2006) or enhance (Duong and McCauley 2016) odonate immune function. One possibility is that the response of total PO to the threat of predation varies among species. Both *Enallagma*, and *Lestes* damselflies studied in Stoks et al. (2006), exhibit similar decreases in production efficiency (i.e., converting consumed resources into biomass) in the presence of predator threats (McPeek et al. 2001; Stoks 2001; Stoks and McPeek 2003; McPeek 2004), yet the indirect (non-consumptive) effects of fish predators only limit total PO production in *Lestes* (Stoks et al. 2006), not *Enallagma* (this study). A second possibility is that less-active *Enallagma* larvae may have immune compensatory mechanisms which could offset reductions in resource acquisition. For example, *Enallagma* possess compensatory mechanisms for growth to offset predator-induced reductions in resource acquisition (e.g., Van Dievel et al. 2016). Though we did not test for this possibility, future studies should investigate if *Enallagma* have similarly adaptive immune compensatory mechanisms to offset the costs of reduced foraging in the presence of fish predators.

Contrary to our predictions, reduced damselfly activity could instead be expected to correspond with lower total PO levels, since lower activity is associated with reduced resource acquisition (Strobbe et al. 2011; Siepielski et al. 2020), and total PO levels are food-limited (Campero et al. 2008; De Block and Stoks 2008). However, resource acquisition is only the first step in resource utilization (McPeek 2004), which is also dictated by an individual's ability to digest and assimilate resources. Consequently, simply acquiring more prey resources through greater foraging rates and reduced competitive effects may not be the limiting step. Predators and conspecifics can also suppress digestive physiology just by being present (McPeek et al. 2001; McPeek 2004). Thus, the lack of an association between activity and total PO may indicate that immune function is more strongly coupled to other aspects of digestive physiology. Indeed, Tye et al. (2020) found no association between larval damselfly (*E. vesperum*) prey consumption rates and immune function, but did find a positive correlation between assimilation efficiency and immune function.

We found that total PO levels increased markedly with increases in prey density among lakes, supporting the widely-posited relationship between immune function and resource availability (Siva-Jothy and Thompson 2002; De Block and Stoks 2008; Kristan 2008; Forbes et al. 2016; Budischak et al. 2018; Hite and Cressler 2019). However, this association was non-linear as immune function rapidly increased with prey density, after which there was generally little variation among lakes. This pattern implies that some minimal amount of prey is necessary to generate a more robust immune function, but levels above that generate no further benefit. This apparent threshold could be adaptive, as the PO cascade produces toxic by-products that can harm the host (Dowling and Simmons 2009). Thus, by limiting investment in immune function, despite increasing levels of resource abundance, hosts can protect themselves from self-harm (e.g., melanization of host tissue, Sadd and Siva-Jothy 2006). We do note, however, that prey density was not experimentally manipulated (e.g., Forbes et al. 2016). Total PO levels could be responding to other factors correlated with prey densities that vary among lakes (i.e., prevalence of parasites, temperature, lake productivity), and in addition to spatial variation, damselfly total PO levels are also known to vary over time (Córdoba-Aguilar et al. 2011). The relationship between immune function and resource availability requires further study to determine to what degree individual total PO levels are resource-limited, and if there is some threshold, after which immune function does not increase.

That we found an effect of prey resources among lakes, but no corresponding effect of density-dependence in total PO is counterintuitive. Like many organisms, damselfly growth is resource-limited (Anholt 1990; McPeek 1990, 1998; Siepielski et al. 2010, 2020; Ousterhout et al. 2019), and aspects of immune function are resource-limited (this study, Campero et al. 2008; De Block and Stoks 2008; Forbes et al. 2016; Budischak et al. 2018), both of which are often density dependent. Thus, our results are contrary to long-held and widely supported views that immune function, at least for total PO, is density dependent (Wilson and Reeson 1998; Barnes and Siva-Jothy 2000; Wilson et al. 2002; Kong et al. 2018; Murray et al. 2020). Instead, our results support evidence for a lack of density-dependent immune function found in other studies (Svensson et al. 2001; Miller and Simpson 2010; Thomas et al. 2010; Piesk et al. 2013). In combination with these other studies, such results highlight the lack of a clear and direct relationship between these two facets (Elliot and Hart 2010).

That we only supplied *Daphnia* prey once at the onset of the mesocosm experiment could have limited our ability to detect an effect of damselfly density on PO. We assumed that higher damselfly densities would result in lower per capita prey (*Daphnia*) availability to damselflies. However, *Daphnia* densities are known to vary substantially over relatively short time periods (Bruijning et al. 2018). We did not measure *Daphnia* densities throughout the course of the experiment, so we cannot rule out this possibility. Presumably, though, any such effects of varying *Daphnia* densities among experimental replicates would have added noise, and despite this possibility we still detected an effect of damselfly density on growth rates in the mesocosm experiment (Fig. 1 in Siepielski et al. 2020). That, combined with the identical results in this study from both the mesocosm and field experiments lend support to our overall conclusion that density-dependent competition was not strong enough to limit this aspect of damselfly immune function.

Although total PO levels did not vary with damselfly density, it may be that growth is prioritized over, and decoupled from, immune function (Stoks et al. 2006; van der Most et al. 2010). This decoupling makes sense, as growth must be prioritized if organisms are to complete their life cycles (Brodin and Johansson 2004; De Block and Stoks 2004; Stoks et al. 2006), while parasitism by ectoparasitic mites rarely leads to the death of the host and represents a lesser cost. Thus, any effects of food-limitation would primarily affect growth as we and others previously found. Indeed, we found no association between total PO activity and growth rates in the mesocosm experiment, and only a weak positive association in the field experiment, where natural resource levels were sufficiently limiting in at least one lake to reduce total PO. However, it may also be that variation among individuals in resource acquisition and energetic investment toward immune function may obscure the ability to detect associations with particular traits at the population level (Reznick et al. 2000; Kortet et al. 2007; Tye et al. 2020) although this seems unlikely (e.g., Fig. S1).

The ability to simultaneously defend against predators, thwart off competitors, and generate immune responses are but one example of the conflicting demands species face when living in complex communities (Stearns 1992). Although the consequences of these conflicting demands can be promulgated by trait and density-mediated effects, few studies have considered them simultaneously. By investigating how predators exert direct consumptive, indirect trait-mediated, and combined effects though natural selection on a key behavioral trait that mediates resource acquisition underlying immune function, our results suggest that the tradeoffs arising between resource acquisition and predation do not always constrain the key aspect of immune function considered here. Although we only investigated a single aspect of immune function, and future studies should consider alternative components of immunity, our results suggest that predators and competitors may not constrain immune function if resources are sufficiently abundant. As a result, the potential cascading and constraining effects of predators on immune function, and by extension any effects on parasitism, are likely population specific (Kortet et al. 2007). Determining under what ecological conditions predators and competitors do and do not constrain immune function will provide critical insight into how species defend themselves against parasites in complex food webs.

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Authors' contribution AMS designed the study. All authors collected data, and both AZH and AMS performed modeling work and analyzed data. AZH wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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Declarations

Consent for publication All authors consent to the publication of this manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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