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Predation and disease: understanding the effects of predators at several trophic levels on pathogen transmission

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SUMMARY

1. Predators can directly and indirectly influence host–parasite interactions by consuming infected individuals, by removing infectious parasite stages and by changing host traits (e.g. behaviour). Because such effects can affect infection positively or negatively, understanding the net effects of predation on pathogen transmission under natural conditions is important.

2. We conducted a mesocosm experiment to examine the effects of predators on interactions between tadpole hosts (*Pseudacris regilla*) and trematode parasites (*Ribeiroia ondatrae*). We manipulated the presence of (non-lethal, i.e., caged) predators of tadpoles (dragonfly larvae) and (potentially lethal) parasite predators (damselfly larvae) to evaluate their individual and combined effects on host infection. We expected that dragonflies would reduce tadpole activity and thereby increase parasite infection through a reduction in antiparasite behaviour. Because damselflies can consume parasites in the laboratory, we predicted that damselflies would lower infection by consuming parasites before they infected tadpoles. Our goal was to evaluate the net consequences of these predator-mediated effects for host/prey infection.

3. The presence of caged dragonflies reduced tadpole activity, resulting in a ~50% increase in average infection load compared to treatments without predators. In contrast to our prediction that damselflies would reduce infection, damselflies elicited behavioural and morphological changes in hosts similar to dragonflies, with a comparable increase in parasite transmission. Thus, predator-mediated effects were evident predominantly through changes in host/prey behaviour, rather than through changes in the abundance of parasites. The lack of a direct effect of predators on infection (i.e. via consumption of parasites) could be the result of the presence of alternative prey (zooplankton) or a mismatch in timing between visual predators feeding during the day and parasites released from the first intermediate host and infecting amphibians at night.

4. The presence of predators also stimulated morphological defences in their tadpole prey, including increased tail and body depth. Interestingly, we found that parasite infection also induced morphological changes in tadpole tail and body depth, similar to changes produced by (non-lethal) cues from predators. Parasites caused malformations in tadpoles, but there were no effects on tadpole growth or development from either parasites or predators.

5. This research has key implications for linking predation and infectious disease in aquatic ecosystems. Our results emphasise the importance of indirect effects of predators on infection and highlight possible trade-offs in mitigating the concurrent risks of predation and disease. Parasites can also alter host morphology through trait-mediated effects similar to predators, supporting a broader inclusion of parasites in the study of the ecology of natural enemies.

Keywords: behaviour, malformation, parasite, phenotypic plasticity, *Ribeiroia*, trait-mediated indirect effects, trematode

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Introduction

Predation is a fundamental process that contributes to the structure and function of ecological communities (Lima, 1998; Preisser, Bolnick & Benard, 2005; Peckarsky *et al.*, 2008). Indeed, predators have a diversity of direct and indirect effects within food webs. Predators directly influence prey by reducing their population size and inducing changes in prey traits (e.g. phenotypic plasticity) (Lima, 1998; Peacor & Werner, 2001; Werner & Peacor, 2003; Preisser *et al.*, 2005; Peckarsky *et al.*, 2008). Because pairs of predators and prey are embedded within complex systems composed of many species, these direct effects can have cascading effects on other species within the community (Bruno & Cardinale, 2008). Such cascading or indirect effects can be mediated by changes in the density of prey species (i.e. density-mediated indirect effect, DMIE) and/or changes in the traits of the prey species (i.e. trait-mediated indirect effect, TMIE) (Abrams, 2007). For example, a reduction in prey density by predators can lead to a DMIE on prey resource, such as reduced grazing pressure (Abrams, 2007). However, a similar indirect effect can occur if predators reduce the foraging activity of their prey (i.e. a TMIE) (Werner & Peacor, 2003). Because density- and trait-mediated indirect effects are common within communities, there is a need to assess the relative importance of both mechanisms.

Growing evidence suggests that predation can also affect parasitism (Packer *et al.*, 2003; Ostfeld & Holt, 2004; Borer *et al.*, 2009). Predators can reduce the abundance of both susceptible and infected hosts through consumption, thereby reducing transmission (Packer *et al.*, 2003; Ostfeld & Holt, 2004). In addition, predators can consume parasites directly, including free-living infective stages of helminths and ectoparasites, increasing parasite mortality and reducing transmission (Johnson & Thieltges, 2010; Johnson *et al.*, 2010). Foraging activity can also result in predators becoming infected through trophic transmission (Lafferty, 1999; Hall *et al.*, 2007). Alternatively, predation can increase pathogen transmission indirectly by altering host behaviour (Thiemann & Wassersug, 2000; Decaestecker, De Meester & Ebert, 2002; Szuroczki & Richardson, 2012). For example, predatory fish can cause *Daphnia* to spend more time near the sediment, leading to increased risk of infection by fungal parasites within the sediment (Decaestecker *et al.*, 2002). Finally, inducible antipredator defences, including changes in prey morphology, may lower host immune responses due to energy allocation trade-offs, thereby increasing parasite infection success (Rigby & Jokela, 2000; Navarro *et al.*, 2004; Stoks *et al.*, 2006). As a result of these varied and sometimes conflicting influences, understanding the net effect of predators on host–parasite interactions remains a challenge in disease ecology.

Aquatic communities in general, and larval amphibians in particular, serve as important model systems for investigating the influence of predators on host–pathogen interactions (Benard, 2004; Koprivnikar *et al.*, 2012). For instance, predator cues are well known to reduce tadpole activity, which lowers their detectability to visual predators (Caldwell, Thorp & Jervey, 1980; Lawler, 1989). However, by also limiting their antiparasite behaviour, including evasive manoeuvres to dislodge attacking parasites, a decrease in tadpole activity can increase infections by trematode parasites (Thiemann & Wassersug, 2000; Szuroczki & Richardson, 2012). Belden and Wojdak (2011) further illustrated the potential for multiple, simultaneous effects of predators on parasitic infection through reductions in host activity, reductions in host density or transmission to predators. Predation directly on free-living infective stages can also influence the transmission of amphibian pathogens; for instance, zooplankters consume zoospores of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, while larval damselflies prey upon cercariae of the virulent trematode, *Ribeiroia ondatrae* (Buck, Truong & Blaustein, 2011; Orlofske *et al.*, 2012). Each of these previous studies focusses on the effects of an individual predator on either hosts or parasites separately; however, some generalist predators could simultaneously influence both parasites and hosts, directly through consumption and indirectly through changes in traits (e.g. behaviour, morphology). Given the diversity of interactions that could occur in nature, this emphasises the need to isolate the mechanisms through which predators influence disease (Orlofske *et al.*, 2012).

Here, we extend current knowledge of the effects of predation on parasite infection by testing the relative importance of two competing effects: the ability of predators to reduce infection directly by consuming parasites before they encounter hosts and the capacity of predators to increase infection indirectly by reducing host antiparasite behaviour (i.e. a trait-mediated indirect effect). We used a semi-natural mesocosm experiment to represent aquatic communities, consisting of larvae of the Pacific chorus frog (*Pseudacris regilla*) as hosts, dragonfly larvae (*Anax* sp.) as predators of hosts and damselfly larvae (*Enallagma* sp. and *Lestes* sp.) as purported predators of the free-living infective stages of a trematode parasite (*R. ondatrae*). We included

zooplankton in the mesocosms to provide a more realistic prey community for damselflies. We hypothesised that cues from caged (therefore non-lethal) dragonflies would reduce tadpole activity in general, including antiparasite behaviour and elicit energetically costly morphological changes, thereby increasing host exposure or reducing energy available for immune defence and lead to higher infection by *R. ondatrae* (Fig. 1). Concurrently, we hypothesised that damselflies would consume the infective stages of *R. ondatrae*, leading to a lower rate of infection (Fig. 1). Finally, we assessed the joint effects of both predators, to reveal disease outcomes when predators simultaneously affect host–parasite contact or change the abundance of parasites. By extending our community to include more than one type of predator, we sought to integrate both the direct and indirect effects of potential interactions among species feeding at a range of trophic levels (predation on parasites with a complex life cycle versus predation on herbivorous hosts) on disease dynamics to better understand how parasite transmission occurs in nature (Preston *et al.*, 2013a).

Methods

Study system

Ribeiroia ondatrae is a trematode with a complex life cycle, sequentially infecting first intermediate host snails (*Helisoma trivolvis*), second intermediate host amphibians and, finally, amphibian-eating birds as definitive hosts (Johnson *et al.*, 2004). Transmission of *R. ondatrae* from snail to amphibians occurs through direct infection by free-living aquatic stages known as cercariae, which then form encysted metacercariae in the amphibian (Johnson *et al.*, 2004). Mortality and pathology in amphibians are based on the intensity of infection (i.e. total number of parasites in the host) highlighting the need for understanding the role of the broader aquatic community in transmission dynamics (Johnson *et al.*, 1999). The Pacific chorus frog, *Pseudacris regilla*, serves as our focal amphibian host because it is among the species with the highest reported frequencies of severe malformations (extra, misshapen or reduced limbs) caused by the encystment of the parasite in the developing limb buds. Malformation frequencies approach 90% among newly

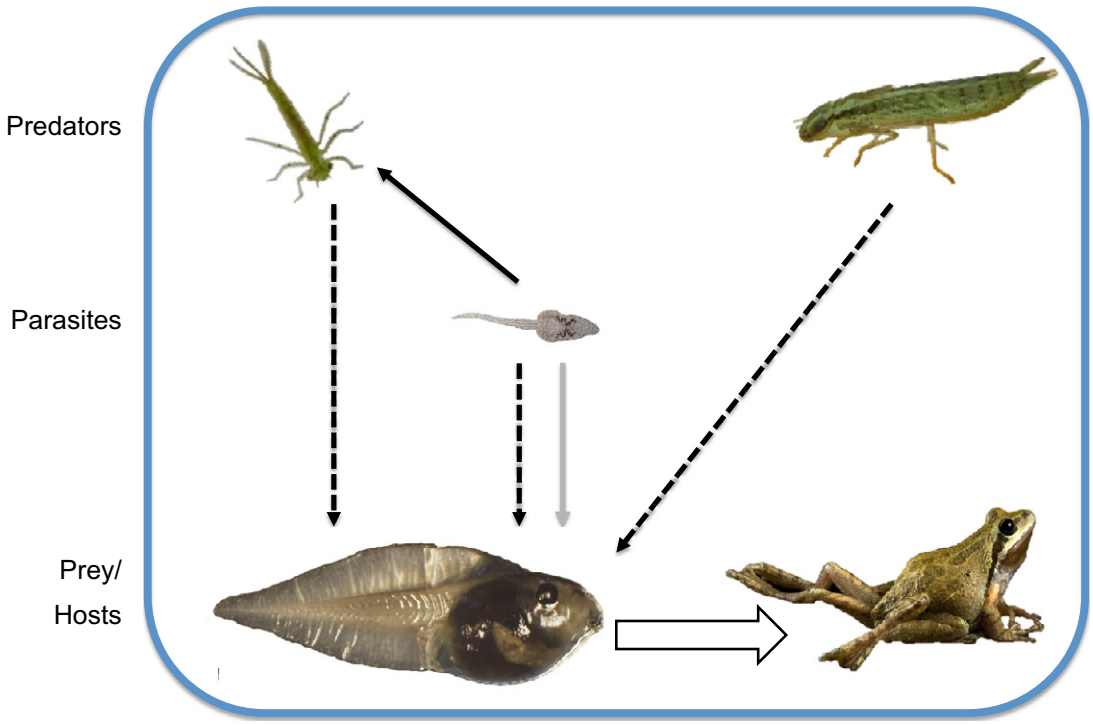

Fig. 1 Conceptual diagram illustrating the hypothesised direct and indirect effects of predators on parasite transmission and host pathology. Damselfly larvae may directly consume (solid black line) *Ribeiroia ondatrae* cercariae leading to a lower density of parasites of tadpoles (solid grey line). Both damselflies and dragonfly nymphs may induce behavioural and morphological changes in tadpoles (dashed lines). Parasites may also alter host behaviour and morphology (dashed line). Increased pathology (white arrow) may result from increased infection due to indirect effects of predators (note: dragonflies were prevented from direct consumption of tadpoles in our experiment and therefore that direct interaction was not included in this diagram).

metamorphosed (juvenile) frogs in some wetlands (Johnson *et al.*, 1999, 2001, 2002, 2013). In addition, this species expresses inducible defences (e.g. reduced activity, deeper tails) in response to aquatic predators (Benard, 2006).

Biologically relevant assemblages of aquatic predators were created using damselflies and dragonflies, which are highly abundant and frequently coexist in wetlands supporting the intermediate hosts of *R. ondatrae* (Orlofske *et al.*, 2012; Preston *et al.*, 2013b). Additionally, neither dragonflies nor damselflies are hosts of *R. ondatrae* (Orlofske *et al.*, 2012). Damselfly larvae (*Enallagma* sp. and *Lestes* sp.) were selected as predators of *R. ondatrae* cercariae, because previous small-scale laboratory studies have shown that they actively consume cercariae even in the presence of alternative prey and can reduce transmission (Schotthoefer, Labak & Beasley, 2007; Orlofske *et al.*, 2012). By manipulating the presence of free-ranging damselflies that consume parasites, we were able to assess density-mediated indirect effects on host infection. Dragonfly larvae (*Anax* sp.) are predators of tadpoles. Tadpoles reduce their activity, and their tail and body morphology is modified in the presence of chemical cues from dragonfly larvae (Relyea, 2004). By caging the dragonflies in our experiment, we were able to evaluate the influence of predator-induced changes in prey traits on host–parasite interactions (i.e. trait-mediated indirect effects). Furthermore, because the trophic level of the cercariae can be interpreted as equivalent to their previous feeding stage, this represents a distinct trophic interaction from that occurring between tadpoles and dragonflies (Preston *et al.*, 2013a). Although dragonflies also eat damselflies, previous research has indicated that caged dragonflies do not influence damselfly foraging activity, which is the primary mechanism by which we expected damselflies to influence parasite infection (Stoks *et al.*, 1999; Stoks, 2001).

Establishment and maintenance of mesocosms

On 14 June 2011, 32 mesocosms were established using 68-L tubs (Rubbermaid) within a greenhouse following standard methods (Johnson *et al.*, 2012; see Appendix S1). Mesocosms were filled with 5.7 kg of sand and 50 L of tap water. After 1 week, 4 g of crushed rabbit food, 0.015 L of pond sediment and 0.4 L of filtered pond water collected from local wetlands were added. Finally, a 0.095-L aliquot of concentrated zooplankton from local wetlands was added. A 20 cm length of polypropylene rope was added to simulate aquatic vegetation and provide added substratum for tadpoles and damselflies (Michel & Burke, 2011). Water temperature was monitored continuously in a subset of mesocosms throughout the experiment ($n = 14$; Hobo underwater datalogger, Onset Computer Corporation, Bourne, MA; Appendix S1).

The experiment started on 02 July 2011 with the addition of 10 tadpoles that had been collected as eggs and maintained in the laboratory until being added to each mesocosm (Appendix S2). Tadpoles were at stage 28 or 29 (Gosner, 1960) and had a mean mass 0.180 mg $\pm$ 0.005 standard error (SE). Treatment conditions were randomly assigned to mesocosms based on a $2 \times 2 \times 2$ factorial design, with the presence/absence of *Ribeiroia ondatrae* cercariae, presence/absence of (free-ranging) damselflies and presence/absence of (caged) dragonflies. Each treatment was replicated four times.

Mesocosms assigned to the damselfly treatment were inoculated with 10 larvae randomly selected from a mixture including *Enallagma* sp. and *Lestes* sp. collected from local wetlands (mean body length = 14.0 mm $\pm$ 1 SE; Appendix S2). Densities of larval damselflies were approximately 37 m^{-2} (0.2 damselflies L^{-1}), which is within the range of published estimates for natural populations and consistent with previous mesocosm experiments (Anholt, 1990; McPeek, 1990; Stoks & McPeek, 2003). Furthermore, this number of damselflies allowed us to assess and maintain densities throughout the experiment.

Dragonflies were maintained in cages throughout the experiment to examine trait-mediated effects on the host–parasite interaction. For mesocosms assigned to a caged predator treatment, a single *Anax* sp. dragonfly larva collected from local wetlands (mean body length = 41.2 mm $\pm$ 1.2 SE) was placed in a 0.24-L plastic cup covered with a piece of plastic window screen to prevent escape (Appendix S2). Dragonflies were fed a similar-sized conspecific tadpole as the experimental tadpoles before being added to the mesocosms and were fed one tadpole every 3 days (tadpole mass range 0.180–0.600 mg). If a dragonfly did not eat the tadpole within 12 h, it was replaced with an extra dragonfly that had eaten in the laboratory (see Appendix S2). Mesocosms that did not receive a dragonfly predator contained an empty 0.24-L plastic cup cage that was taken out and replaced when dragonflies were fed to control for any effect of disturbance. Dragonflies and damselflies in the mesocosms were monitored daily and replaced if they were found dead or emerged (damselflies only) from the mesocosms, as indicated by the presence of exuviae along the edges of the mesocosm.

Parasite exposure

For all experimental procedures, *R. ondatrae* were obtained from a population of field-collected snails identified as carrying the parasite and subsequently maintained in the laboratory (see Appendix S2). To collect newly emerged cercariae from snails, each snail was placed into an individual 50-mL centrifuge tube from 1800 to 2200 h. Cercariae were counted using a glass pipette under a dissecting microscope and transferred to 50-mL centrifuge tubes before being added to the mesocosms. Cercariae were added to mesocosms at midnight, when they would be released in nature (Johnson *et al.*, 2004). Mesocosms in no-parasite treatments received 35 mL of snail-conditioned water as a sham exposure. Immediately before adding the parasites, predator cups were verified to be free of uneaten tadpoles that could potentially become infected and reduce transmission to the experimental tadpoles. A total of 400 cercariae were added to each mesocosm, with further additions of 50 cercariae on 2 and 3 July and 100 cercariae on 6, 9 and 11 July. This incremental exposure was sufficient to produce differences between the predator treatments while minimising the lethal effects on tadpoles of *R. ondatrae* that would have occurred had a single, higher exposure been used.

Amphibian behaviour

Tadpole activity (the number of active tadpoles, defined as any movement of the tadpole through the water) was quantified using scan sampling (Michel & Burke, 2011; Szuroczki & Richardson, 2012). Because of the small size and limited structural complexity of mesocosms, all tadpoles were visible during observations. Each mesocosm was observed 10 times during daily measurement periods (1200–1300 h) from 07 to 13 July 2011. The response variable in subsequent analyses was the average number of tadpoles active in each mesocosm from the 10 measurements during each observation period. The observer was blind to treatment; cages were deployed in every mesocosm and opaque so that the observer could not distinguish empty cages from those with dragonflies. Additionally, the small, cryptic damselflies were not readily apparent during scan sampling.

Amphibian morphology and parasite infection

The experiment concluded on 14 July 2011 to assess treatment effects on tadpole traits (i.e. phenotypic plasticity) prior to metamorphosis. All tadpoles were euthanized in MS-222 (Tricaine methanesulphate, Western Chemical Inc., Ferndale, WA, U.S.A.) buffered with sodium bicarbonate, weighed to the nearest 0.001 mg and preserved in 10% buffered formalin. Tadpoles were staged according to Gosner (1960). To assess phenotypic plasticity, each tadpole was photographed on the right side with a glass slide under the tail in identical positions using a dissecting microscope (Olympus SZX10) and digital camera (Olympus Corporation, Center Valley, PA, U.S.A.) with a 1-cm scale bar. Tadpole morphology was characterised based on seven linear measurements (total length = TOL, tail length = TL, tail depth = TD, tail muscle depth = MD, body length = BL, body depth = BD, and mouth width = MW) using Image Processing and Analysis in Java software (ImageJ, National Institutes of Health, Bethesda, MD, U.S.A.; Relyea, 2001; Appendix S3). These measurements were selected on the basis of previous research indicating that they are antipredator responses (Relyea, 2001).

To evaluate pathology, every tadpole was examined under a dissecting scope for the presence, and type of, any malformations. To quantify *R. ondatrae* transmission success, five of the 10 tadpoles in each mesocosm were randomly selected to quantify parasite infection intensity (Appendix S4). In addition, a random subset of one to two tadpoles per mesocosm that were not in parasite treatments were dissected to verify that they were not infected.

Zooplankton abundance

To assess the role of damselflies in influencing the abundance of their presumed (non-cercarial) prey in the mesocosms, zooplankton density was quantified at the end of the experiment. Cercariae have a short (<24 h) lifespan in the water column and were therefore not quantified with the zooplankton. Samples of the water column were collected with a tube sampler (30 cm in length × 5 cm in diameter, two combined samples per mesocosm) and passed through a 45-μm sieve. Samples were preserved in 70% ethanol and later identified and counted under a dissecting microscope.

Statistical analyses

Ribeiroia ondatrae infection intensity was normally distributed and was examined using a linear mixed effects (LME) model, with mesocosm as a random effect with predator treatments and interactions as main effects (R package nlme; R Development Core Team, 2008); however, no tadpoles from unexposed treatments had been

infected so our analyses included only mesocosms exposed to *R. ondatrae.* To examine pathology, generalised linear mixed models with the Laplace approximation method, binomially distributed error and logit-link function were used to test for the effects of predators on malformation presence or absence (malformed or normal) with mesocosms included as a random effect (R package lme4; Zuur *et al.*, 2009). Because we never observed malformations in mesocosms without parasites, we only analysed malformations from mesocosms exposed to *R. ondatrae*.

A repeated-measures analysis of variance (rm-ANOVA) was used to test the effects of different communities on tadpole behaviour over the course of the experiment (JMP Pro 9). Similar to the infection analysis, the role of different species on tadpole mass and developmental stage was evaluated using LME with mesocosm identity as a random effect.

To analyse differences in phenotypic plasticity across parasite and predator treatments, we first needed to remove differences that were due to the allometric relationship between morphological traits and tadpole size (Hoverman & Relyea, 2012). To address size variation, analysis of covariance (ANCOVA) was used with $\log_{10}$-transformed mass as the covariate (Hoverman, Auld & Relyea, 2005). A critical assumption of the ANCOVA is a common slope of the regression across treatments (i.e. similar allometric relationships). Three traits met this assumption (tail depth, tail muscle depth and body depth) and were used for further analysis. The mass-adjusted treatment mean and residuals from the within-treatment regression were used to calculate each individual's size-adjusted trait value. The mean response for each experimental unit within each sample was calculated and used as our morphological response variable. Because these measurements represent multiple responses from the same individual, a multivariate analysis of variance (MANOVA) was used, followed by univariate analyses (ANOVA) for each trait that was significant in the previous analysis.

To investigate whether observed increases in infection were more strongly associated with predator-mediated changes in host behaviour or host phenotypic traits, we analysed average infection per mesocosm ($\log_{10}$x+1 transformed) as the response and both the proportion of tadpoles active (averaged over all time points) and phenotypic traits as predictors using generalised linear models (GLM). If behavioural changes were the primary mechanism for higher infection, we expected a better fit to the infection data evaluated by AIC_c compared with the models including the morphological variables.

To compare zooplankton abundance across treatments, an LME was used on log-transformed total zooplankton density with mesocosm as a random effect. Non-significant interactions were sequentially removed from the final models. Tadpoles from one mesocosm within the 'no-predator or parasite' treatment developed unusually slowly and were very small, probably due to an overgrowth of cyanobacteria and were removed from all analyses.

Results

Amphibian malformation and parasite infection

The presence of either dragonflies or damselflies caused an increase in amphibian infections by *Ribeiroia ondatrae* relative to treatments without predators (LME; Dragonfly: $t_{12} = 2.5$, $P = 0.029$; Damselfly: $t_{12} = 2.4$, $P = 0.035$; Dragonfly × Damselfly: $t_{12} = -1.8$, $P = 0.102$; Fig. 2). Hosts maintained with either or both predators supported, on average, 52 to 54% more parasites than those with no predator present. While there was no significant interaction between the predators, this lack of statistical significance could be a function of the degree of replication. Only two tadpoles died during the experiment and neither was exposed to parasites, verifying our sublethal exposure levels and reducing any influence of density-dependent changes in transmission. As expected, malformations were only detected in the region of the developing hind limb buds and only in mesocosms exposed to *R. ondatrae*. Types of malformation included twisted or bent limbs, extra digits and extra limb buds.

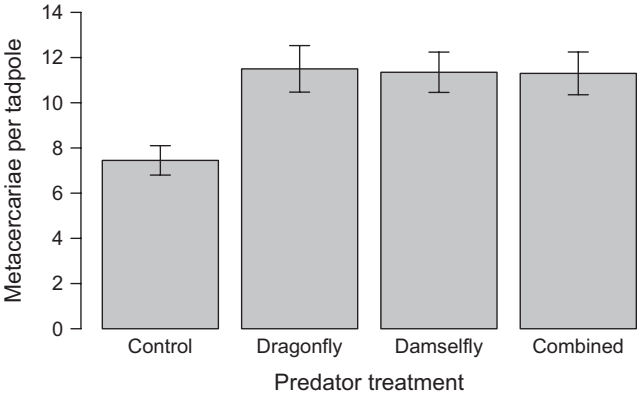

Fig. 2 Mean (±SE) infection of Pacific chorus frog (*Pseudacris regilla*) tadpoles from mesocosms ($n = 4$ mesocosms/treatment) exposed to *Ribeiroia ondatrae* in the absence (control) or presence of caged dragonflies, unrestrained damselflies, or both predator species combined.

The proportion of malformations varied from 10 to 28% but did not differ significantly as a function of predator treatment (GLMM; $Z > -1.23$, $P > 0.22$).

Amphibian behaviour

Predator treatment, time and the time-by-dragonfly interaction all affected tadpole behaviour during the experiment (see Appendix S5; Table S1). The presence of caged dragonflies reduced tadpole activity, and this reduction was most pronounced (~20% decrease relative to no-predator treatment) during the first 3 days of observations (ANOVA; dragonfly x time, $F_{6,19} = 12$, $P < 0.0001$; Fig. 3a). Damselflies also reduced tadpole activity, but this occurred over the entire experimental period (ANOVA; $F_{1,24} = 7.8$, $P = 0.010$; Fig. 3b). Furthermore, tadpole activity was reduced by only 13% in response to the damselflies. Tadpoles exposed to *R. ondatrae* did not show any differences in activity compared with unexposed tadpoles (ANOVA; $F_{1,24} = 1.5$, $P = 0.230$). In general, tadpole activity increased over time (ANOVA; $F_{6,19} = 25.6$, $P < 0.0001$). As predicted, tadpole activity (averaged over the experiment) was negatively associated with mean infection intensity (GLM; $t_{15} = -2.44$, $P = 0.028$, $AIC_c = -27.9$; Fig. 3c).

Amphibian growth and morphology

Both dragonflies and parasites influenced the relative morphology of tadpoles (MANOVA; dragonflies: $F_{3,21} = 42.4$, $P < 0.0001$, Parasite: $F_{3,21} = 4.2$, $P = 0.039$; interactions: $F_{3,21} < 2.6$, $P > 0.08$; Fig. 4a–d). The effects of damselflies on tadpole morphology were significant only when examined for individual traits. Tadpoles reared in the presence of either predator or parasites formed deeper tails (ANOVA; dragonflies: $F_{1,23} = 109.8$, $P < 0.0001$, damselflies: $F_{1,23} = 5.4$, $P = 0.029$, parasite: $F_{1,23} = 7.5$, $P = 0.012$; Fig. 4e), but the presence of predators only also contributed to deeper tail muscles (ANOVA; dragonflies: $F_{1,23} = 53.5$, $P < 0.0001$, damselflies: $F_{1,23} = 4.7$, $P = 0.041$). Both the presence of parasites and dragonflies were associated with deeper tadpole bodies (ANOVA; dragonfly: $F_{1,23} = 16.0$, $P = 0.0005$, parasite: $F_{1,23} = 7.0$, $P = 0.014$). However, the change in body depth due to parasites was greater without dragonflies present (ANOVA; dragonfly x parasite: $F_{1,23} = 4.4$, $P = 0.047$; Fig. 4f). Mean parasite infection intensity was marginally negatively related to morphological changes (GLM; mean tail depth: $t_{15} = 2.11$, $P = 0.054$, $AIC_c = -26.6$, mean tail muscle depth: $t_{15} = 1.84$, $P = 0.086$, $AIC_c = -25.6$, mean body depth: $t_{15} = 2.09$, $P = 0.056$, $AIC_c = -26.5$). Although the model including behaviour was the best-fitting (see above), models incorporating morphological traits were almost equally well supported (ΔAIC_c 1.3–2.2).

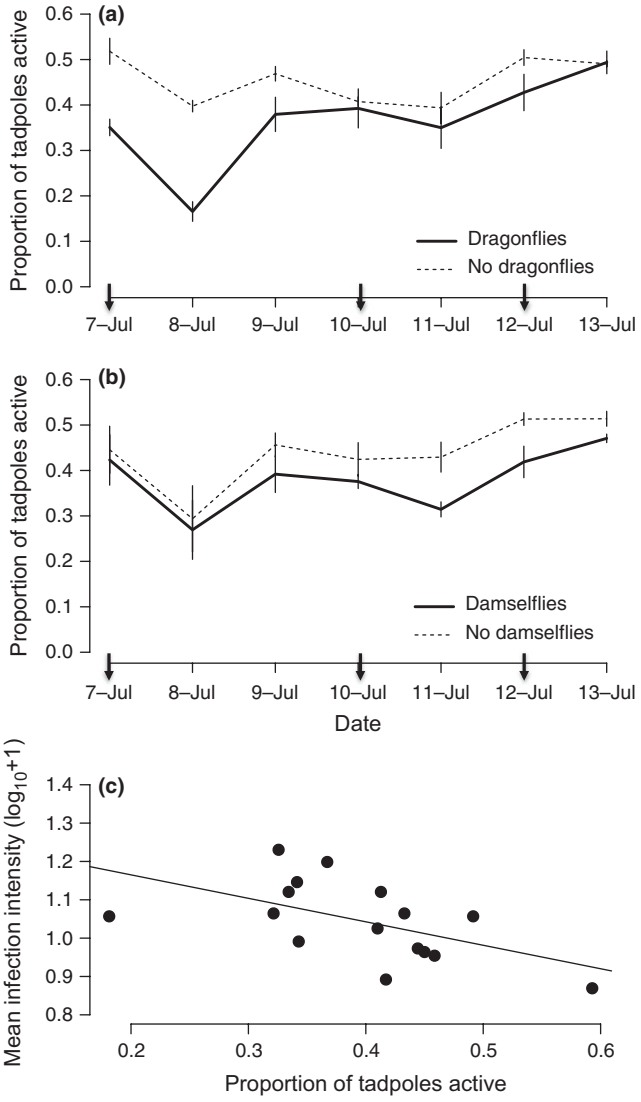

Fig. 3 Proportion (±SE) of active Pacific chorus frog (*Pseudacris regilla*) tadpoles ($n = 10$) based on daily snap-shot observations of individual aquatic mesocosms ($n = 4$ per treatment), a) in the presence or absence of caged dragonfly nymphs fed conspecific tadpoles or b) in the presence or absence of 10 unrestrained damselfly larvae. Predators were present prior before and on the days on which behaviour was monitored, while parasites were added at midnight as indicated by the black arrows. c). Regression showing the relationship between the mean proportion of tadpoles active over the entire course of observations and the mean infection level in each mesocosm ($n = 16$, $F_{1,14} = 5.97$, $R^2 = 0.29$, $P = 0.028$).

There were no significant effects of predators, parasites or their interactions on tadpole mass (LME; $t_{28} = 1.59$, $P > 0.123$) or developmental stage (LME; $t_{28} = -1.37$, $P > 0.183$). Across all treatments, tadpole

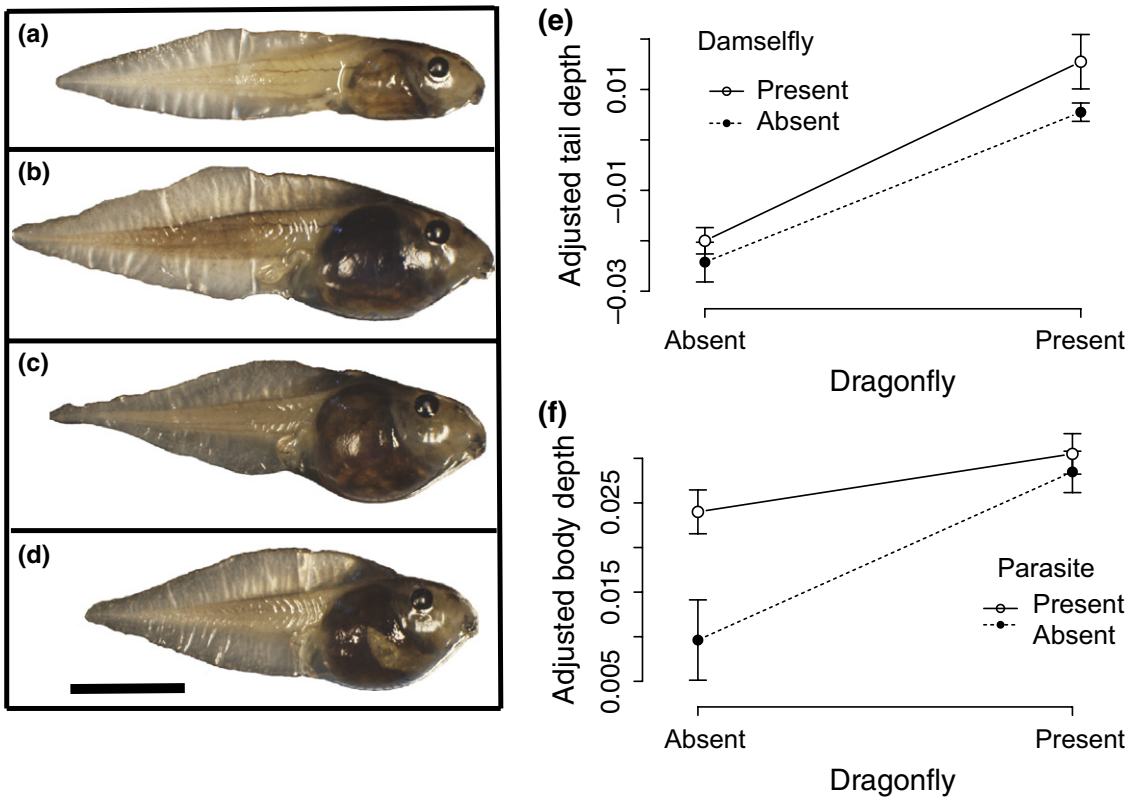

Fig. 4 Images of representative Pacific chorus frog (*Pseudacris regilla*) tadpoles from mesocosms with factorial combinations of caged dragonflies, unrestrained damselflies and the trematode parasite *Ribeiroia ondatrae*: a) no predators or parasites present; b) dragonflies only present; c) parasite only present; d) all species (dragonflies, damselflies, and parasites) present. Scale bar = 1 cm. Interaction plots (means ± SE) of tadpole morphological data adjusted for tadpole mass and averaged across mesocosms: e) tail muscle depth by dragonfly and damselfly absence or presence; f) body depth by dragonfly and parasite absence or presence.

wet mass increased to 0.824 mg ± 0.011 SE and developmental stage increased to 36.1 ± 0.1 SE (Gosner, 1960).

Zooplankton abundance

Daphnia middendorffiana comprised over 97% of the zooplankton community in our mesocosms, with copepods composing the rest of the community. Zooplankton abundance across the pooled samples was 338 ± 70 per mesocosm and did not differ significantly across predator or parasite treatments (LME; $t_{27} > 0.83$, $P > 0.41$).

Discussion

Using a mesocosm experiment designed to represent natural aquatic communities, we were able to isolate and quantify pathways through which predation influences host–parasite interactions. Overall, we observed a consistent increase in *R. ondatrae* infection in amphibian hosts in the presence of dragonflies, damselflies and both predators combined. This result was probably driven by predator-mediated reductions in host activity, which led to greater colonisation and penetration success by parasite free-living stages (Thiemann & Wassersug, 2000; Szuroczki & Richardson, 2012). While most research on the interface between predation and disease has focussed on the role of predators in altering host density, and therefore transmission, these findings add weight to the indirect role of predators in altering disease risk through trait-mediated effects.

Consistent with our predictions and previous laboratory-based research (Thiemann & Wassersug, 2000; Szuroczki & Richardson, 2012), the presence of caged dragonflies reduced host activity, increased *R. ondatrae* transmission and induced the development of antipredator defences. Indeed, tadpoles exposed to predators had a 50% higher infection rate than tadpoles exposed in the absence of predators. We suspect that predator-driven reductions in host activity were the primary mechanism for this increase in infection (Thiemann &

Wassersug, 2000). Over the course of our experiment, we observed a 13–20% decrease in activity in the presence of predators that correlated significantly with the observed increases in infection (Fig. 3c). Because tadpoles engage in antiparasite behaviour, including avoidance and rapid, evasive manoeuvres to dislodge infective stages (Taylor, Oseen & Wassersug, 2004), predator-induced reductions in activity limit an important defence mechanism in hosts (Thiemann & Wassersug, 2000; Szuroczki & Richardson, 2012). Laboratory studies using anaesthesia to simulate the reduced activity associated with predators have shown an increase in infection compared with active tadpoles (Koprivnikar, Forbes & Baker, 2006; Daly & Johnson, 2011), and previous laboratory studies reported an increase in *Echinostoma trivolvis* infection in amphibians in the presence of predatory fish (Thiemann & Wassersug, 2000; Szuroczki & Richardson, 2012). Somewhat surprisingly, however, damselflies elicited a similar reduction in tadpole activity, which might reflect tadpoles interpreting a damselfly cue as a predator cue, damselflies physically disrupting the tadpoles, or changing the community (e.g. reducing foraging by zooplankton). Overall, enhanced parasite transmission may be a common outcome of increased predation risk on hosts, such that hosts/prey face a trade-off between the immediate, lethal risk of predation and the long term, sublethal danger of increased infection accumulated over time (see also Decaestecker *et al.*, 2002).

While behaviour appeared to play a prominent role in driving infection, the presence of predators also may have affected host resource allocation and the effectiveness of their defences against invading parasites. In the presence of predators, for instance, tadpoles developed deeper bodies and tails, both of which are antipredator responses. These defences could have come at the cost of reduced investment in host immune responses (Rigby & Jokela, 2000; Navarro *et al.*, 2004; Stoks *et al.*, 2006; Middlemis Maher, Werner & Denver, 2013). Although we found marginally significant relationships between the mean infection intensity and the degree of morphological change in the tadpoles, more work is needed to isolate the contributions of morphology and behaviour to infection risk because these traits were tightly correlated in our experiment ($r = -0.51$ to -0.83). However, in a similar mesocosm study that measured host immune responses to non-lethal predation cues, Raffel *et al.* (2010) did not find a relationship between predators and tadpole immune responses. Consistent with other studies of non-lethal predator cues, we found no effects of non-lethal dragonfly cues or damselflies on tadpole growth rate or development, despite alterations to host morphology and behaviour (Thiemann & Wassersug, 2000; Benard, 2004). In our study, we observed the greatest reduction in tadpole activity early on in the experiment (first few days). However, once morphological responses were formed, activity increased during the experiment, which probably allowed the tadpoles to compensate for any reductions in growth that may have occurred early in the experiment. Moreover, any fitness consequences associated with antipredator responses are likely to depend on resource availability, the density of competitors and the timing of the effects (Werner & Peacor, 2003; Benard, 2004; Relyea, 2004).

In contrast to our predictions, the antipredator responses elicited by damselflies led to subsequent increases in parasite transmission to larval amphibians. Based on previous laboratory studies, showing that damselflies consume trematode free-living stages, we predicted lower infection in amphibian larvae raised with damselflies (Orlofske *et al.*, 2012). Unfortunately, the small size of cercariae prevented the straightforward observation of predation in the mesocosms, and the lack of a hard exoskeleton precluded conventional gut content analysis on the damselflies. So we could not assess the consumption of cercariae directly (Kaplan *et al.*, 2009). The observed differences between the previous laboratory study and our results may be due to some important features of our somewhat more realistic mesocosm experiment, including greater availability of alternative prey (e.g. zooplankton). One consideration is that damselflies could have been saturated with other prey (i.e. zooplankton), which limited their feeding on parasites. A second hypothesis involves a temporal disconnect between infection by *R. ondatrae* and foraging by damselflies. *Ribeiroia ondatrae* has a nocturnal circadian rhythm, emerging from the first intermediate host at night (Johnson *et al.*, 2004), which is also when we added parasites to mesocosms. As visual predators, however, damselflies may be ineffective at night (S.A. Orlofske, R. C. Jadin & P. T. J. Johnson, unpubl.), emphasising the importance of realistic experimental conditions. This also raises the intriguing possibility that nocturnal emergence helps to protect actively swimming, relatively large cercariae (~1000 μm), as in *R. ondatrae*, from predation. Predation may therefore function as a selective force on parasite body size, shedding time or both, to reduce detection by visual predators. We caution, however, that many ecological communities support a wide range of both predators and parasites with variable activity periods. Thus, additional research is needed to understand the net effects of predators, including

consumption of parasites, consumption of hosts and alterations of host phenotype, on parasite transmission.

Intriguingly, we also observed changes in tadpole morphology, including body depth, in the presence of parasite infection. These changes may reflect a general stress response involving corticosterone, which has been associated with morphological changes due to predators (Middlemis Maher *et al.*, 2013). However, Thiemann & Wassersug (2000) found no effect of *E. trivolvis* cercariae on tadpole morphology, suggesting that the response may be parasite species specific. Importantly, morphological changes induced by *R. ondatrae* may help the tadpoles to remove cercariae or be a direct response of the tadpole to these parasites (that frequently encyst in the area of the developing limb buds and along the base of the tail). In comparison, *E. trivolvis* infects the tadpole kidneys and may not elicit an external morphological change. The increase in body depth due to parasite exposure was similar to that induced by dragonflies, suggesting that there were no morphological trade-offs in the responses to different natural enemies (Raffel, Martin & Rohr, 2008). Future research should investigate the functional significance of both host behaviour and morphological responses to parasites in the presence of other natural enemies as one step towards integrating parasites into natural enemy ecology (Raffel *et al.*, 2008).

Overall, our research highlights the importance of examining interactions over several trophic levels and the underlying mechanisms (density and trait-mediated indirect effects) linking predation risk and parasite transmission to develop an ecological framework for understanding disease in natural systems. By isolating potential mechanisms of predation on infection, we found that predator-induced changes in host behaviour (i.e. reduced activity level) outweighed any consumptive effects on parasites ultimately increasing infection when predators were present. While previous studies have examined the effects of predators on host abundance (Packer *et al.*, 2003; Ostfeld & Holt, 2004; Borer *et al.*, 2009; Orlofske *et al.*, 2012) and the effects of predators on prey activity and physiology, only recently have these research areas been integrated (Rigby & Jokela, 2000; Decaestecker *et al.*, 2002; Raffel *et al.*, 2010). Furthermore, we provide evidence that parasite infection may influence host morphology to a similar extent as do predators and suggest that these effects should be more broadly included in the ecology of natural enemies. To integrate predation and disease dynamics fully, the direct and indirect effects of predators and parasites need to be considered simultaneously.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Additional mesocosm methods.

Appendix S2. Animal collection and maintenance.

Appendix S3. Landmarks for amphibian morphological assessment.

Appendix S4. Methods for amphibian necropsy.

Appendix S5. Statistical results of tadpole behaviour.

Table S1. Results of repeated-measures ANOVA on the effects of caged dragonfly predators, unrestrained damselflies and *Ribeiroia ondatrae* parasites on tadpole activity over time.

Figure S1. Digital image of preserved Pacific chorus frog (*Pseudacris regilla*) tadpole illustrating the positions of the morphological measurements taken to evaluate phenotypic plasticity including total length (red), tail length (orange), tail depth (yellow), tail muscle depth (green), body depth (light blue), body length (dark blue), and mouth size (purple).

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