

Predator presence suppresses immune function in a larval amphibian

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ABSTRACT

Background: The presence of predators frequently reduces developmental rate in larval amphibians. If physiological and evolutionary trade-offs exist between immunity and predator responses, optimal immune response may not be selected for but the immunological consequences of predator exposure are unknown.

Hypotheses: Amphibian larvae exposed to predators during development will develop more slowly and have weaker immune responses than those reared in a predator-free environment.

Organism: The anuran, *Rana sylvatica*, reproduces in vernal ponds throughout North America with varying levels of predation. *Rana sylvatica* tadpoles respond to predators through behavioural and morphological defences resulting in reduced development time.

Site of experiments: The Edwin S. George Biological Reserve, Livingston County, Michigan, March 2007 to July 2007.

Methods: *Rana sylvatica* tadpoles were randomly assigned to replicate experimental mesocosms. Those with caged insect predators provided the chemical cues of predation. Controls had empty cages. After 6 weeks, tadpoles in each treatment group were given an immune challenge (an injection of the immune elicitor phytohaemagglutinin, a common field test of T-cell mediated immunity). I measured body mass, developmental stage, and immune response (at 24 and 48 h) between experimental and control groups using linear mixed models. I used structural equation modelling (path analysis) to distinguish the direct effects of predator exposure from indirect effects mediated by developmental stage.

Results: Tadpoles reared with predators developed more slowly and had weaker immune responses at both 24 and 48 h compared with controls. Mean immune responses increased with time in both experimental animals and controls. The direct effect of predator exposure contributed more to reduced immune response than did indirect effects mediated by developmental stage.

Keywords: immune responses, indirect effects of predators, inducible defences, larval amphibians, natural enemies, plasticity, *Rana sylvatica*.

INTRODUCTION

Pathogens are important natural enemies that can have strong impacts on host survival and reproduction. Because they negatively affect fitness, the ability to defend against pathogens should be under strong positive selection. Despite evidence for selection for stronger immune response in the presence of pathogens, there is still a great deal of variation in observed levels of pathogen defence between species, populations, and individuals within a population (Lindstrom *et al.*, 2004; Cornet *et al.*, 2009). If immune function is important for fitness, what maintains heterogeneity in susceptibility to pathogens? One possibility is that community interactions may play a role in modulating immune responses. In particular, interactions with predators may alter immune function, as predator defences often have associated energetic and fitness costs and benefits, which could limit the ability of organisms to simultaneously defend against pathogens and predators.

Predator-induced defences have costs in a variety of taxa (reviewed in Harvell, 1990; Clark and Harvell, 1992; Benard, 2004). The amphibian model is particularly useful for studying the non-lethal effects of predators on immune function because amphibians respond to chemical cues of feeding predators. By using caged predators, researchers can expose amphibians to the chemical signal of predation without the confounding effects of thinning or injury (Petranka *et al.*, 1987). Exposure to predators generally results in the induction of behavioural and morphological defences and life-history shifts. In the presence of predators, larvae forage less and develop defensive tail morphology. Although these defences increase survivorship, they often reduce rates of growth and development (Benard, 2004; Relyea, 2007). In a review of 41 studies of amphibian larvae exposed to caged predators, Relyea (2007) found that in 40% of the studies development time increased significantly in the presence of predators, while only 5% reported that predators caused shorter development times. In addition to causing significant life-history shifts, the energetic costs of predation defences may also limit the ability of amphibians to optimally invest in immune function.

However, there are few empirical tests of the effect of predation defences on the immune system, either direct effects or effects mediated by predator-induced developmental delays. Tests in invertebrates indicate that predator-induced defences do reduce immune function (Rigby and Jokela, 2000; Mikolajewski *et al.*, 2008; Ramirez and Snyder, 2009). In amphibians, predator-induced changes in development rate may be an additional important factor in mediating parasite resistance because immune function is highly dependent on developmental stage in amphibians. Amphibians generally exhibit an increase in immune function throughout development, but experience a rapid decline of immune function during metamorphosis (Rollins-Smith, 1998, 2001; Carey *et al.*, 1999; Warne *et al.*, 2011). In light of the developmental and energetic consequences of predator defence, it is likely that exposure to predators affects immune function. However, the two studies of amphibians that tested for the effect of predators on parasite infection rates present conflicting results (Thiemann and Wassersug, 2000; Raffel *et al.*, 2010). To date, there have been no studies of the effect of predators specifically on immune response in amphibians, nor have any attempted to distinguish the energetic costs of predator-induced defences to the immune system from the effects of developmental delays that might decrease immune function.

In this study, I tested whether predation risk (exposure to predator cues) affects immune response in wood frog tadpoles (*Rana sylvatica*). Dragonfly larvae (genus: *Anax*) were used to generate predator cues in this study, as they are a common predator of *R. sylvatica* and because the effects of their chemical feeding cues on behaviour, development time, and

morphology are well studied in wood frog tadpoles (Relyea, 2004). I reared *R. sylvatica* tadpoles either in experimental mesocosms with caged, fed predators to simulate predation risk, or with empty cages (controls), and measured their immune responsiveness and development rate. This experimental design allowed me to examine the effects of predator exposure on developmental rate and immune response, as well as to distinguish the direct effects of predator exposure on immune response from the indirect effects mediated by developmental delays.

METHODS AND MATERIALS

I conducted this experiment at the University of Michigan's Edwin S. George Reserve in Livingston County, Michigan during an 8-week period in the spring and summer of 2007. The experiment utilized a common predator-prey system found in many North American ponds and wetlands: *Rana sylvatica* tadpoles and dragonfly larvae (genus: *Anax*). Six newly oviposited *R. sylvatica* egg masses were collected from ponds on the Edwin S. George reserve and maintained in the laboratory in individual containers at 10°C until hatching. After hatching, individuals were randomly assigned to one of five 200-L outdoor wading pools containing well water and fed rabbit chow *ad libitum* for 10 days. Fourteen experimental tanks (750-L cattle watering tanks) were prepared 2 weeks before the start of the experiment, according to a well-established protocol (Relyea, 2004; Seiter, 2009). A total of 560 tadpoles from a mixture of all egg masses were selected, of which 40 were added to each experimental tank. *Rana sylvatica* tadpoles respond to the odour of dragonfly larvae rather than the scent of injured conspecifics, thus predation risk was simulated by placing three caged dragonfly larvae (genus: *Anax*) in each of the seven predator treatment tanks (Petranka and Hayes, 1998). Dragonfly larvae were fed approximately 0.2 g of tadpole, three times per week, a rate previously demonstrated to induce changes in behaviour and morphology in *R. sylvatica* larvae (Relyea, 2004). Control tanks were outfitted with empty cages and these were removed from the water during feeding to equalize disturbance between control and predator treatments (Relyea, 2004).

Immune challenge

After 6 weeks in the experimental tanks, 12 tadpoles per tank were selected at random for the immunoassay. Immune function was measured by administering a standard challenge, an injection of phytohaemagglutinin (Sigma Aldrich, St. Louis, MO). Phytohaemagglutinin (PHA) causes T-lymphocytes to proliferate rapidly both *in vivo* and *in vitro* (Smits *et al.*, 1999; Martin *et al.*, 2006). When used as an *in vivo* assay, it causes a measurable swelling at the injection site; greater swelling indicates a stronger T-lymphocyte response and therefore stronger immune function. The PHA assay is a commonly used field method for measuring immune response in birds, fish, and reptiles (Binns *et al.*, 1990; Smits *et al.*, 1999; Ardia and Clotfelter, 2006; Martin *et al.*, 2006; Calsbeek *et al.*, 2008; Boughton *et al.*, 2011). Its usefulness has also been recently demonstrated in amphibians (Gervasi and Foufopoulos, 2008).

Tadpoles were removed from tanks less than 5 min before administration of the assay to minimize handling stress. To facilitate handling, tadpoles were anaesthetized using 0.3 mg·mL⁻¹ MS-222. Before injection, tadpoles were weighed, and tail thickness was measured using fine-gauge digital calipers (Mitutoyo, Precision Graphic Instruments, Seattle, WA). The immunoassay was prepared by dissolving 2 mg of PHA in 1 mL of

phosphate-buffered saline solution. Each individual was then injected subdermally at the base of the tail with 15 μL of the PHA-saline solution using a 0.3-mL, 32-gauge insulin syringe (Becton-Dickerson, Franklin Lakes, NJ). Skin thickness measurements of each individual were taken before injection, and at 24 and 48 h post injection. Control injections of saline were logistically difficult due to the small body sizes of the tadpoles. During the immune challenge, animals were housed individually in the laboratory in a climate-controlled room, at 21–23°C on a 16:8 light–dark cycle, in accordance with approved institutional animal care protocols (University Committee on Use and Care of Animals Protocol #07765). Immune response was assayed by subtracting the pre-injection tail thickness from the tail thickness at 24 and 48 h. After the 48-h measurement, animals were euthanized in a 1 g·L⁻¹ solution of MS-222 and preserved in ethanol. Preserved animals were scored for developmental stage using the Gosner staging index (Gosner, 1960).

Statistical analysis

All statistical analyses were performed in R (v.2.11.0), using the lmer, lme4, and sem libraries. I performed two sets of statistical analysis. I first used linear mixed models to test for an effect of predator exposure on mass, Gosner stage, and response to PHA. I then used structural equation modelling to quantify the direct and indirect associations between these response variables. I used separate linear mixed models to test for an effect of treatment on Gosner stage, mass, and PHA response. Because mass and Gosner stage were assessed only once during the experiment, I tested only for a treatment effect and not a time effect on these variables. I analysed the effect of predator treatment on Gosner stage with a two-level, linear mixed effects model, where each individual's measured Gosner stage was nested within tank (replicate), and predator exposure was included as a fixed effect. I used a similar two-level model to test for an effect of predator exposure on mass, but I found no significant effect of predator exposure on mass, and therefore excluded it from subsequent analyses. To test for an effect of predator exposure on PHA response, I used a three-level linear mixed model, in which immune function observations at 24 and 48 h were nested within each animal, and then animals were nested within tanks. Time and predator treatment were included as fixed effects; tank and animal were included as random effects. The analyses, done in R using the library lme4, use a maximum likelihood framework. Thus I performed χ^2 -tests to compare the full model (which included tank, treatment, and time) with models where each of these terms was omitted.

I used structural equation modelling to quantify the relative contributions of the direct effect of predator exposure on immune response versus the indirect effect of predator exposure mediated by developmental stage. The PHA response was analysed using separate structural equation models for observations at 24 and 48 h. Treatment was included as an exogenous variable, while Gosner stage and PHA-induced swelling were treated as endogenous variables. Because mass was not significant in the linear mixed model, I fitted two sets of models, one which included mass and one which omitted it, and compared model fit using the Bayesian information criterion. The Bayesian information criterion indicated that models excluding mass fit better at both 24 and 48 h. Because the linear mixed models did not detect an effect of tank, I did not include tank effects in the structural equation models and used individual PHA response values in the models. The standardized path coefficients estimated from the model are regression coefficients (beta weights). For indirect effects, individual path coefficients are multiplied along the path to obtain the total

path contribution. I use the terms ‘direct’ and ‘indirect’ to refer to the structure of the variables in the model, rather than the complexity of the mechanisms that produce the observed effects. For example, the ‘direct’ effects of predator exposure may be mediated by other, unmeasured variables, such as induction of a stress response, or through energetically costly defensive behaviour and morphology.

RESULTS

Predator-exposed animals developed significantly more slowly (achieved an earlier Gosner stage) than control animals ($F_{12,90} = 60.5$, $P < 0.001$), consistent with previous studies (Relyea, 2007). I tested for an effect of tank on Gosner stage, by comparing a two-level model in which animal was nested within tank with a single-level model that was not structured by tank. The log likelihood of the two models was the same (log likelihood = 2697.164, likelihood ratio = $9.095e^{-13}$, $P = 1.000$), indicating that there is no effect of tank on Gosner stage, and the Akaike information criterion indicates that the model which excludes tank is a slightly better fit. Linear mixed models also show that the strength of the PHA swelling response was significantly reduced in the predator treatment group (Fig. 1, Table 1). Response to PHA in both groups also increased significantly from 24 to 48 h after the challenge (Fig. 1, Table 1). However, there was no significant interaction between treatment and time.

I used path analysis (structural equation models) to compare the relative magnitude of the direct effects of predator treatment with their indirect effects mediated via Gosner stage

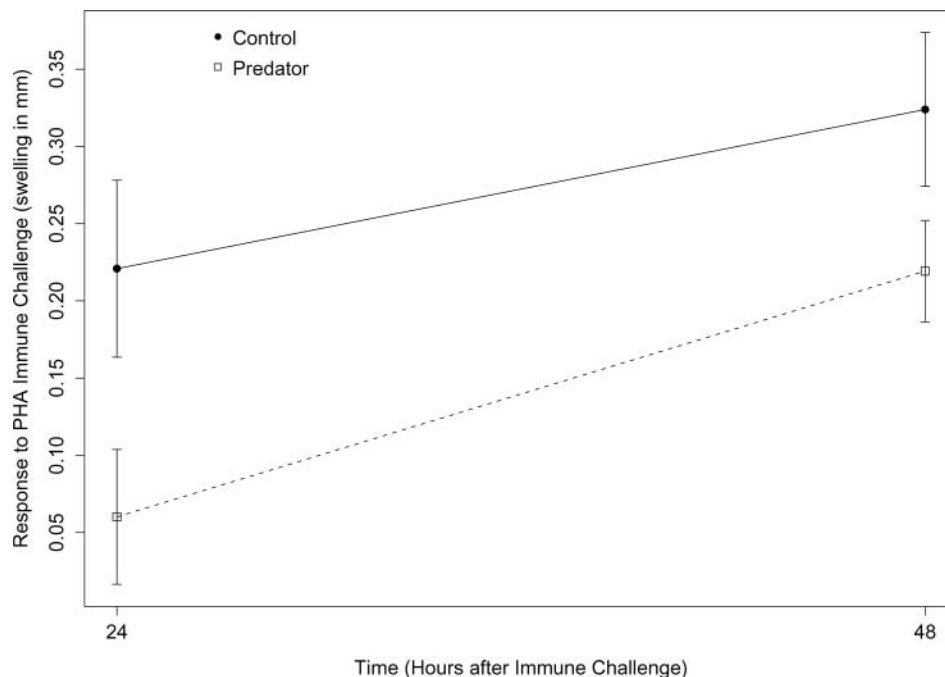


Fig. 1. Predator-exposed animals had weaker responses to the PHA immune challenge than control animals. Swelling response increased from 24 to 48 h in both groups. Error bars represent standard errors.

Table 1. Comparison of full model with models omitting tank, time, and treatment

	d.f.	AIC	BIC	Log likelihood	χ^2	χ^2 d.f.	<i>P</i>
Full model (fixed effects: time, treatment; random effects: animal, tank)	8	-87.79	-62.89	51.89			
Full model minus predator treatment	7	-80.14	-58.36	47.07	9.644	1	0.002
Full model minus time	7	-47.85	-26.06	30.92	41.94	1	<0.001
Full model minus tank	5	-89.79	-69.00	51.89	0	1	1.000

Note: I used linear mixed models to test for an effect of predator treatment, time, and tank on immune response. I performed χ^2 tests to compare the full model (which includes time and treatment as fixed effects and tank as a random effect) with models where each of these terms was omitted. Including predator treatment and time significantly improved the fit of the model. However, the Akaike information criterion indicates that excluding tank from the model actually provided a better fit, indicating that there was no effect of tank.

in each model. As expected, predator exposure reduces Gosner stage (-0.67 , $P < 0.001$; Fig. 2). However, at both 24 and 48 h, the magnitude of the direct effect of predator exposure on PHA response was much greater than the indirect effect of predator treatment on PHA response mediated by Gosner stage. At 24 h, the direct effect of treatment on PHA response (-0.28 , $P = 0.037$; Fig. 2a) was significant and an order of magnitude larger than the indirect effect (treatment to Gosner stage path: $-0.67 \times$ Gosner stage to PHA path: $0.052 = -0.035$, $P = 0.701$; Fig. 2a). At 48 h the magnitude of the direct effect of predator treatment (-0.15 , $P = 0.18$) on PHA response was not significant. However, the direct effect was still nearly twice as large as the indirect effect of predator treatment (treatment to Gosner stage path: $-0.67 \times$ Gosner stage to PHA path: $0.15 = -0.105$, $P = 0.261$; Fig. 2b). I repeated these analyses using the mean values for each tank to determine if tank effects influenced the results of the path analysis. Despite reduced statistical power, the results were qualitatively the same for analyses using tank means.

DISCUSSION

My results indicate that exposure to predators significantly delays development and reduces immune function in *R. sylvatica* tadpoles. These experiments and analyses also show that the effect of predator exposure on immune function is largely mediated by factors other than developmental stage. My results are consistent with previous work that predator exposure commonly (but not always) results in slower development in amphibian larvae (Lardner, 2000; Benard, 2004; Relyea, 2007). Although the mechanism by which predator cues reduce developmental rate is not clear, this is further evidence for the energetic trade-off between development and predation defences (Relyea, 2002; Relyea and Auld, 2004; Teplitsky *et al.*, 2005).

Predator exposure reduced immune response in *R. sylvatica* tadpoles, a finding consistent with studies in invertebrates, but differing from some previous work in amphibians. Studies in insects and snails demonstrate that caged or simulated predators reduced immune response (Rigby and Jokela, 2000; De Block *et al.*, 2008; Ramirez and Snyder, 2009). In a previous test of the effects of caged predators on pathogen resistance in amphibians (Thiemann and Wassersug, 2000),

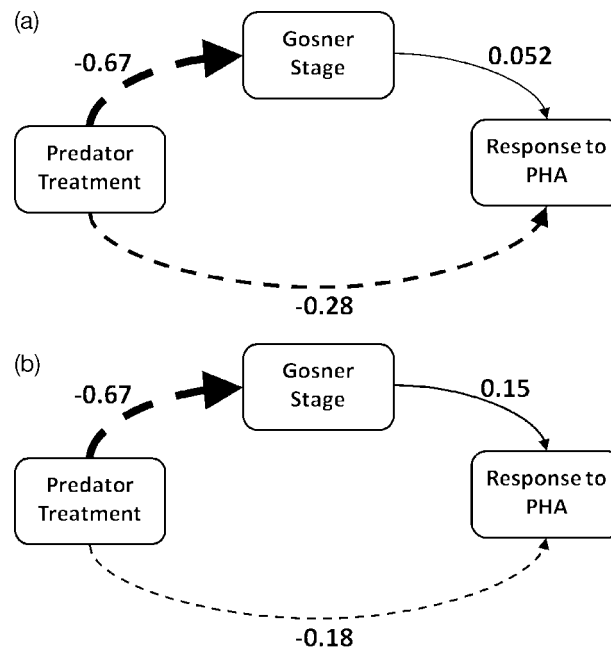


Fig. 2. At both 24 and 48 h the direct effect of predator exposure on PHA response was much greater than the indirect effect of predator treatment on PHA response (mediated by Gosner stage). (a) At 24 h the direct effect of treatment on PHA response (-0.28) was greater than the indirect effect (treatment to Gosner stage path: $-0.67 \times$ Gosner stage to PHA path: $0.052 = -0.035$, $P = 0.701$). (b) At 48 h the direct effect of predator treatment (-0.18) was larger than the indirect effect (treatment to Gosner stage path: $-0.67 \times$ Gosner stage to PHA path: $0.15 = -0.105$). Direct effects of treatment contributed more to decreased immune response at 24 h (direct effects were an order of magnitude greater) than at 48 h (direct effects were only twice as large).

Rana sylvatica and *Rana clamitans* tadpoles both had reduced rates of development and higher parasite burdens in the presence of predators, consistent with the results of the present study. The authors attributed the difference in infection rate to behavioural avoidance of predators: tadpoles evade predators by resting in pond substrate, thus exposing them to benthic helminth parasites (Thiemann and Wassersug, 2000). However, my results indicate that reduced immune function, as well as increased exposure, may account for the higher infection rates observed in that study. A second study in American toad tadpoles (*Bufo americanus*) found that predators had no effect on the infection rates of helminth parasites (Holland *et al.*, 2007). However, Holland and colleagues did find a strong effect of developmental stage on susceptibility, as helminths can infect tadpoles only early in development (Holland *et al.*, 2007). *Bufo americanus* is atypical in that it accelerates its development when exposed to predators, thus predators reduced the time spent in susceptible developmental stages (Raffel *et al.*, 2010). More work is needed to separate the role of stage-dependent susceptibility and increased contact with parasites from the direct effects of predator exposure on the immune system. Further experiments are also required to determine whether the patterns observed in this study are generalizable to other species of hosts, parasites, and predators.

Path analysis indicates that the effects of predator exposure on the immune system are not due to developmental stage in *R. sylvatica* tadpoles. Although the mechanism by which predators reduce immune function is unknown, energetic investment in predator defences may be an important limit to immune function. In addition to conferring fitness, immune defences have substantial energetic costs, and competing energetic demands such as growth and reproduction may limit the energy invested in the immune system. Selection may also produce plastic allocation of energy to the immune system; investment in parasite defence is often contingent on environmental variables and continuously adjusted to maximize fitness (Lochmiller and Deerenberg, 2000; Martin *et al.*, 2003). For example, many species of birds reduce investment in immune function during reproduction when energetic demands are high or when resources are limited (Ardia, 2005; Greenman *et al.*, 2005). Because energetic costs of predator defence are often implicated in reducing development time in amphibians, they may also limit immune function (Relyea, 2002; Relyea and Auld, 2004). There is some experimental evidence that defending against both predators and pathogens has developmental costs for amphibians. In an experiment using grey treefrog tadpoles (*Hyla crysoscelis*), individuals infected with the fungal pathogen *Batrachochytrium dendrobatidis* developed more slowly than uninfected individuals, but only in the presence of predators (Parris and Beaudoin, 2004). These results suggest that the costs of predator defences were amplified by the costs of defending against the fungus (Parris and Beaudoin, 2004). My results imply that a similar energetic trade-off may occur in *Rana sylvatica*, and that the costs of defending against predators may preclude optimal investment in immune function, and that such trade-offs may be a general phenomenon in amphibians.

However, energetic trade-offs may not be the sole cause of reduced immune response in *R. sylvatica* tadpoles. Predator cues have been shown to elicit a stress response in many taxa, resulting in elevated glucocorticoids (CORT) in birds, fish, and mammals (Boinski *et al.*, 1999; Barcellos *et al.*, 2007; Sheriff *et al.*, 2009). Elevated CORT has been shown to reduce immune function in a variety of taxa, including amphibians (Demas *et al.*, 2011). A recent study demonstrated that *R. sylvatica* tadpoles exposed to caged dragonfly larvae had elevated corticosterone (Middlemis-Maher, 2011). It is possible that the predator-exposed tadpoles in our study had higher concentrations of CORT, resulting in suppressed immune function. In fact, studies in *R. sylvatica* show that when CORT is experimentally reduced, tadpoles are unable to develop defensive morphology, suggesting a link between CORT and predation defences (Middlemis-Maher, 2011). It could be that the CORT concentrations required to induce predator defences may also be sufficient to suppress immune function. However, the energetic costs of inducible predator defences and elevated CORT may both play a role in reducing immune function in predator-exposed tadpoles, and may work jointly to reduce immune responses. More work is needed to understand the roles of CORT and predator defences, and their relationship to immune function in amphibians.

Both ultimate factors (such as evolutionary trade-offs) as well as proximate causes (stress responses and developmental delays) may contribute to reduced immune responses in individuals exposed to predators. Although the observed reductions in immune response may appear maladaptive, it is possible that overall fitness is maximized by plastically diverting resources to predator responses. Presumably there could be some optimal, if reduced degree of investment in immune responses that balances investment in immunity and other components of survival and reproduction. Testing such an adaptive hypothesis is beyond the scope of the current study, but presents an interesting hypothesis for future studies.

In addition to affecting individual fitness, predator presence at the population level may have important effects on disease or population dynamics. It is well known that parasites influence predation rates by making prey insensitive to predation risk more easily detected by predators. Infected hosts may also be more vulnerable to predation because they are simply in poor condition (Giles, 1983; Dobson, 1988; Hudson *et al.*, 1992). This study suggests that the converse may also be true; predation risk may facilitate infection by parasites. Amphibian–predator systems have been a useful model for studying community interactions because of the ability to separate the direct and indirect effects of predators (Relyea, 2007). They may prove an equally productive system for studying parasite and predator facilitation. In addition to providing a tractable system for studying the interactions of multiple types of natural enemies, understanding predator-mediated effects on immune function and disease dynamics is particularly important in the context of global declines in amphibians (Carey *et al.*, 1999). As disease has become a serious conservation issue for many amphibian species, understanding the causes of heterogeneity in immune function may have important conservation implications.

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