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Effects of nitrate and the pathogenic water mold *Saprolegnia* on survival of amphibian larvae

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ABSTRACT: We tested for a synergism between nitrate and *Saprolegnia*, a pathogenic water mold, using larvae of 3 amphibian species: *Ambystoma gracile* (northwestern salamander), *Hyla regilla* (Pacific treefrog) and *Rana aurora* (red-legged frog). Each species was tested separately, using a 3 × 2 fully factorial experiment with 3 nitrate treatments (none, low and high) and 2 *Saprolegnia* treatments (*Saprolegnia* and control). Survival of *H. regilla* was not affected significantly by either experimental factor. In contrast, survival of *R. aurora* was affected by a less-than-additive interaction between *Saprolegnia* and nitrate. Survival of *R. aurora* was significantly lower in the *Saprolegnia* compared to the control treatment when nitrate was not added, but there was no significant difference in survival between *Saprolegnia* and control treatments in the low and high nitrate treatments, consistent with increased nitrate preventing *Saprolegnia* from causing mortality of *R. aurora*. Survival of *A. gracile* followed a similar pattern, but the difference between *Saprolegnia* and control treatments when nitrate was not added was not significant, nor was the nitrate × *Saprolegnia* interaction. Our study suggests that *Saprolegnia* can cause mortality in amphibian larvae, that there are interspecific differences in susceptibility and that the effects of *Saprolegnia* on amphibians are context-dependent.

KEY WORDS: Pathogen · *Saprolegnia* · Amphibian

INTRODUCTION

Infectious diseases can bring about population declines and local host extinctions, contributing significantly to the global 'biodiversity crisis'. Emerging infections involving novel pathogens or bringing established pathogens in contact with naïve host populations often have particularly drastic effects on their hosts (Daszak et al. 2000, Cleaveland et al. 2002, Harvell et al. 2002, Altizer et al. 2003). In addition, pathogens capable of infecting multiple species may cause extinctions, as more resistant host species act as reservoirs of infection, preventing the demise of the pathogen along with its susceptible hosts (e.g. Gog et al. 2002, McCallum & Dobson 2002). Furthermore, the effects of pathogens on hosts are often context-dependent (e.g. Grossholz 1992, Kiesecker & Skelly 2001, Mitchell et al. 2002). For example, both biotic and abiotic stressors may act synergistically with pathogens to increase the adverse effects on hosts. Thus, if both stressors and pathogens are present, the effects on hosts may be greater than if either factor acted alone (Guth et al. 1977, Brown & Pascoe 1989, Khan 1990).

In several locations, amphibians may exemplify systems in which stressors and pathogens interact, thereby influencing population dynamics. The documentation of amphibian population declines (Alford & Richards 1999, Houlahan et al. 2000, Stuart et al. 2004) describes a number of factors as contributing to these declines, including abiotic and biotic stressors and pathogens (Alford & Richards 1999, Collins & Storfer 2003, Semlitsch 2003, Blaustein et al. 2004). Clearly, causes for amphibian population declines appear to be context-dependent and complex, often with more than one factor involved (Blaustein & Kiesecker 2002). It is also becoming clear that some amphibian populations have been severely impacted by 1 or more pathogens (Daszak et al. 2003). It is likely that the effects of these pathogens are influenced by co-factors (Taylor et al. 1999, Blaustein & Kiesecker 2002, Kiesecker 2002, Christin et al. 2003, Gendron et al. 2003)

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Saprolegnia, a water mold, is one important pathogen found in many amphibian populations (e.g. Strijbosch 1979, Banks & Beebee 1988, Blaustein et al. 1994). Furthermore, *Saprolegnia*-associated mortality appears to increase in the presence of abiotic stressors (Strijbosch 1979, Banks & Beebee 1988, Kiesecker & Blaustein 1995, Kiesecker et al. 2001a).

Saprolegnia (family Saprolegniaceae) is both saprobic and parasitic, obtaining nutrition from decaying organic matter or living hosts (Seymour 1970). *Saprolegnia* infects a wide variety of organisms, including insects, turtles, fishes, and amphibians (MacGregor 1921, Seymour 1970). In amphibians, embryos and larvae can become infected (Bragg & Bragg 1958, Walls & Jaeger 1987, Blaustein et al. 1994). *Saprolegnia*-infected embryos of fishes and amphibians become covered with visible white hyphal filaments and usually do not hatch (Blaustein et al. 1994). Infection can spread via contact from growing hyphae (in the case of immobile hosts such as amphibian egg masses) or through colonization by free-swimming zoospores (Wood & Willoughby 1986). Transmission can occur between species, for example, between fishes and amphibians (Kiesecker et al. 2001b). Fishes and amphibians may also be infected by *Saprolegnia* via contact with infected soil (Kiesecker et al. 2001b). Host species show strong interspecific variation in their susceptibility to infection (Richards & Pickering 1978, Wood & Willoughby 1986, Kiesecker & Blaustein 1995, 1997). Factors such as water temperature, pH, pollution, exposure to UV-B radiation, injury from biting, silt, and host behavior may modify the effects of *Saprolegnia* on its hosts (MacGregor 1921, Strijbosch 1979, Walls & Jaeger 1987, Banks & Beebee 1988, Carballo & Muñoz 1991, Bly et al. 1993, Pickering 1994, Carballo et al. 1995, Kiesecker & Blaustein 1995, 1997, Lefcort et al. 1997). *Saprolegnia* may influence community structure by altering competition between hosts (Kiesecker & Blaustein 1999). *Saprolegnia* has a world-wide distribution (Wood & Willoughby 1986, Blaustein et al. 1994, Kiesecker & Blaustein 1997). This ubiquitous distribution is influenced by the widespread introduction of hatchery-raised fishes that carry and transmit *Saprolegnia* to other species (Blaustein et al. 1994, Kiesecker et al. 2001b).

The purpose of this study was to examine amphibian larvae for susceptibility to mortality from *Saprolegnia* and to test for a possible synergism between *Saprolegnia* and nitrate, an important stressor on amphibian populations. Nitrate contamination is of global importance. In 2000, 87×10^6 metric tons of nitrogen were used as fertilizer in agriculture (Tilman et al. 2001). Nitrogen fertilization and sewage from humans and livestock are major sources of nitrate (Steinheimer et al. 1998, Tilman et al. 2001). In the 1990s in the Willamette Basin of Oregon about 63 000 t nitrate were applied (Rinella & Janet 1998). Ninety-eight percent of stream samples in the Willamette Basin contained detectable nitrate concentrations (0.054 to 22 mg l^{-1}) (Wentz et al. 1998). Anthropogenic nitrate enters aquatic ecosystems via runoff, groundwater, and sewage discharge (e.g. Giblin & Gaines 1990, Steinheimer et al. 1998, van Lanen & Dijksma 1999, Zhilang et al. 2003). There is a direct correlation between nitrate concentration and the proportion of the drainage area in agriculture. Nitrate concentrations vary seasonally, with highest concentrations coinciding with the beginning of rainfall induced run-off in the autumn and early winter (Wentz et al. 1998).

Anthropogenic nitrate contributes to eutrophication and can cause nitrate concentrations to reach toxic levels (OECD 1982, Rouse et al. 1999). Nitrate is toxic to a variety of organisms, including humans (Comly 1945, Lee 1970, Muir et al. 1991, Camargo & Ward 1995). There are well-documented effects on the susceptibility of amphibian species to nitrogenous fertilizers (e.g. Hecnar 1995, Marco et al. 1999, Rouse et al. 1999, Hatch et al. 2001). Effects of nitrate on amphibians include mortality, impaired feeding ability, reduced growth, slowed development, altered behavior, and methemoglobinemia (Huey & Beitinger 1980, Baker & Waights 1993, Watt & Oldham 1995, Marco et al. 1999, Hatch & Blaustein 2000, Hatch & Blaustein 2003). Nitrate can also interact with UV-B radiation to decrease growth or survival (Hatch & Blaustein 2003). Furthermore, nutrient enrichment from nitrogen-based fertilizers may alter community dynamics by increasing the abundance of herbivores, such as snails that are hosts for parasites linked to amphibian deformities (Johnson et al. 2002, Chase 2003a,b, Johnson & Chase 2004).

MATERIALS AND METHODS

Embryos of the northwestern salamander *Ambystoma gracile*, Pacific treefrog *Hyla regilla* and red-legged frog *Rana aurora* (hereafter, *Ambystoma*, *Hyla*, and *Rana*) were collected in 2002. *Ambystoma* and *Rana* were collected on 10 February and 17 March, respectively, at Coast Pond (approximately 20 km south of Walport, Lincoln County, Oregon, USA). *Hyla* were collected on 10 March from a pond at Baker Beach (approximately 10.5 km north of Florence, Lane County, Oregon, elevation about 12 m). Whole clutches of *Ambystoma* and *Hyla* were collected, while portions of *Rana* clutches were collected.

Amphibians were maintained and hatched in the laboratory at 13 to 17.5°C in tanks filled with dechlorinated tapwater treated with Novaqua® and Amquel® water conditioners. These conditioners do not remove nitrate or

nitrite. Each species was kept separately. *Hyla* and *Rana* were reared in tanks containing about 30 l of water and *Ambystoma* were reared in tanks containing about 8 of water. Approximate densities of larvae were 6.3, 2.1 to 3.1, and 12.5 individuals l^{-1} for *Hyla*, *Rana*, and *Ambystoma*, respectively. Frog larvae were fed a ground mixture of alfalfa pellets and Tetramin fish flakes. *Ambystoma* were given *Artemia* (brine shrimp). A natural photoperiod was provided during the maintenance and experimental periods in the form of artifical light combined with natural daily sunlight through unshaded windows.

Each species was tested in a separate experiment. Each experiment used a 3 × 2 fully factorial design to manipulate nitrate and *Saprolegnia*. There were 3 nitrate treatments (no nitrate, low nitrate, and high nitrate) and 2 *Saprolegnia* treatments (*Saprolegnia* and control). Treatments were assigned to units randomly, and larvae were added to units haphazardly with respect to treatment. For each species, there were 5 replicates of each treatment combination. Thus, there were 30 units per species, for a total of 90 units.

Experimental units consisted of plastic boxes (dimensions: 31 × 18 × 8 cm) containing 2 l of water from one of 3 stock solutions (no nitrate, low nitrate, and high nitrate stock solutions). We added 0.29 ml l^{-1} of Novaqua® and 0.29 ml l^{-1} of Amquel® to each stock solution. We added sodium nitrate to the low nitrate and high nitrate stock solutions to achieve nominal nitrate concentrations of 0, 5, and 20 mg l^{-1} in the no nitrate, low nitrate, and high nitrate treatments, respectively.

Ten larvae were added per unit for *Hyla* and *Ambystoma*, while 7 larvae were added per unit for *Rana*. Larvae were added to units, and treatments were applied on 14 April 2002. *Hyla* and *Rana* ranged in age from about 2 to 5 and 7 to 9 wk post-hatching, respectively. Gosner developmental stages (Gosner 1960) were 25 to 28 in *Hyla* and 25 to 30 in *Rana*. The oldest *Ambystoma* larvae used in experiments were 25 d post-hatch.

Larvae were chosen for experimentation haphazardly. For *Ambystoma*, 1 larva was chosen haphazardly from each of 10 clutches, and individuals that appeared to be small and recently hatched were excluded. *Hyla* were chosen from 2 tanks, 1 containing larvae from 12 clutches and 1 containing larvae from 13 clutches. *Rana* were chosen from 4 tanks and from 4 to 8 clutches.

Saprolegnia was isolated from a water sample taken next to a *Rana aurora* embryo mass in Coast Pond on 10 February 2002. Isolation of *Saprolegnia* was achieved using sterile hemp seeds and YpG (yeast-glucose) agar media (Fuller & Jaworski 1987). *Saprolegnia* was prepared for experiments by placing a hemp seed laden with *Saprolegnia* into a Petri dish (diameter = 85 mm, height = 12 mm) filled approximately half full with ultrapure water and containing 7 sterile hemp seeds. Dishes were incubated at ~13 to 15°C for 6 d. These hemp seeds, laden with *Saprolegnia*, were used in *Saprolegnia* treatments. Three seeds were added to each unit in the *Saprolegnia* treatment. Immediately prior to the experiment, seeds were connected by *Saprolegnia* hyphae. Seeds were disconnected and added to units haphazardly. Units in the control treatment received 3 sterile hemp seeds. Addition of sterile seeds was haphazard with respect to nitrate treatment.

Leftover seeds laden with *Saprolegnia* were placed in a refrigerator (~4 to 5°C) at the start of the experiments. Non-spherical zoosporangia were counted 4 d later using a dissecting microscope on 5 seeds with heavy growth, selected haphazardly. Number of zoospores per seed (mean ± SE) was 16.6 ± 4.0. Spherical zoosporangia may have been present (Seymour 1970); however, they would have been indistinguishable from oogonia under the dissecting microscope. Since the number of spherical zoosporangia was not quantified, our counts of zoosporangia may be underestimates.

Within 5.5 h of addition of larvae to units and application of *Saprolegnia* treatments, units from 1 of each treatment combination were selected haphazardly for each species and a water sample was taken to obtain measurements of initial water quality. Conductivity, total alkalinity, and calcium hardness were measured in subsets of water samples. We measured pH for every water sample.

To minimize handling stress of animals in the experiments, initial measurements of their total length and mass were not taken. Instead, on 15 April, unused larvae from the same stocks were selected (in the same manner that larvae were selected for experimentation) and measured to obtain estimates of total length and mass of the larvae in the experiments (Table 1).

During the experiment, anurans were fed ad libitum a ground mixture of alfalfa pellets and Tetramin fish

Table 1. *Hyla regilla*, *Rana aurora* and *Ambystoma gracile*. Measurements of total length (mean ± 1SE) and mass (mean ± 1 SE) from laboratory stocks of amphibian larvae

Species	Total length (mm)	N	Mass (g)
H. regilla	12.7 ± 0.3	32 (8 batches of 4 larvae each)	0.03[a]
R. aurora	23.5 ± 0.7	23 (23 larvae)	0.14 ± 0.02
A. gracile	16.6 ± 0.4	30 (10 batches of 3 larvae each)	0.03[a]

[a]SE could not be calculated because larvae were massed in batches

flakes. On 20 April, *Ambystoma* were given *Artemia* (brine shrimp). The experiment was checked at least once per day. Live larvae were monitored visually for hyphal structures consistent with descriptions of *Saprolegnia* growth on amphibian larvae (Bragg & Bragg 1958, Bragg 1962). Dead larvae were removed and examined for hyphal structures with a dissecting microscope. Each experiment lasted for 7 d. Surviving larvae were anesthetized with MS-222 and sacrificed. During the experiment, laboratory temperature was maintained at approximately 13 to 15°C.

Survival was analyzed separately for each species using ANOVA. All data met parametric assumptions on the original scale, and conversion of the data to proportion of individuals surviving, followed by arcsin square-root transformation, had negligible effects on normality and inequality of variances. Therefore, data on the original percent survival scale were used for analyses. Tukey tests ($\alpha = 0.05$) were used for pairwise comparisons between treatment combinations.

RESULTS

In *Hyla*, no significant effects of nitrate or *Saprolegnia* on survival were found (Fig 1, Table 2). In contrast, for *Rana*, significant effects of *Saprolegnia* and the interaction between nitrate and *Saprolegnia* were detected, although the nitrate term was not significant (Table 2). Survival of *Rana* is presented in Fig. 2. For *Rana*, in the no nitrate treatment, survival was significantly lower in the *Saprolegnia* treatment compared to the control treatment ($0.005 < p < 0.01$). However, in the low nitrate and

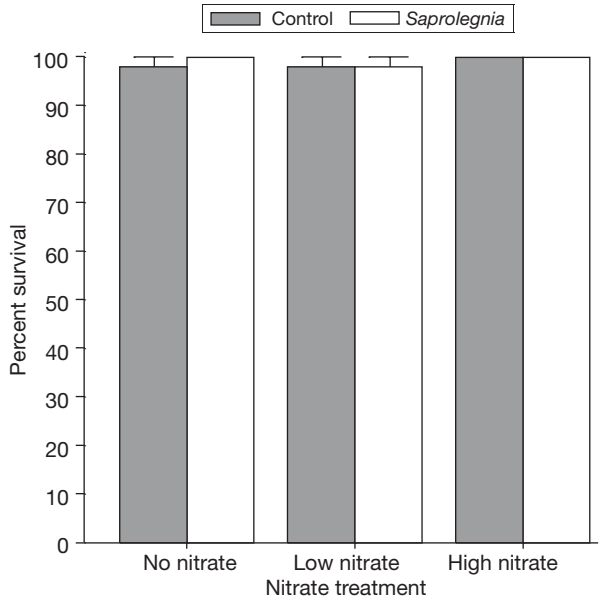

Fig. 1. *Hyla regilla.* Survival of larvae. Error bars are +1 SE

Table 2. *Hyla regilla*, *Rana aurora* and *Ambystoma gracile.* ANOVA results for the survival of larvae of the 3 amphibian species

Source of variation	MS	df	*F*	p
H. regilla				
Nitrate	10.000	2	1.000	0.383
Saprolegnia	3.333	1	0.333	0.569
Nitrate × *Saprolegnia*	3.333	2	0.333	0.720
Error	10.000	24		
R. aurora				
Nitrate	20.408	2	0.128	0.881
Saprolegnia	1149.660	1	7.192	0.013
Nitrate × *Saprolegnia*	700.680	2	4.383	0.024
Error	159.864	24		
A. gracile				
Nitrate	13.333	2	2.667	0.090
Saprolegnia	13.333	1	2.667	0.116
Nitrate × *Saprolegnia*	13.333	2	2.667	0.090
Error	5.000	24		

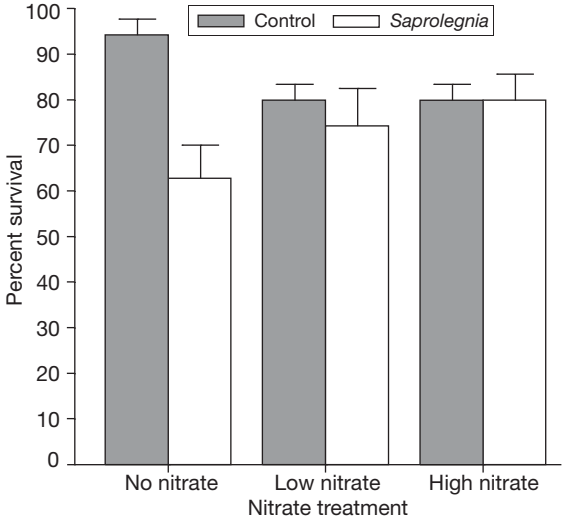

Fig. 2. *Rana aurora.* Survival of larvae. Error bars are +1 SE

in the high nitrate treatments, survival was not significantly different between *Saprolegnia* and control treatments. Nitrate alone did not significantly affect survival (none of the differences between treatment groups were significant). However, survival was always lower when nitrate was added, compared to the no nitrate, control treatment group (no nitrate or pathogen added). One *Rana* individual with severe tail damage was observed being preyed upon by 2 conspecifics. It was removed and scored as dead. Scoring this individual as dead or eliminating it from the analysis did not alter the qualitative interpretations regarding *Rana* survival.

In *Ambystoma*, survival when nitrate was not added was lower in *Saprolegnia* compared to control treatments (Fig. 3), but all larvae, in both *Saprolegnia* and control treatments, survived when nitrate was added.

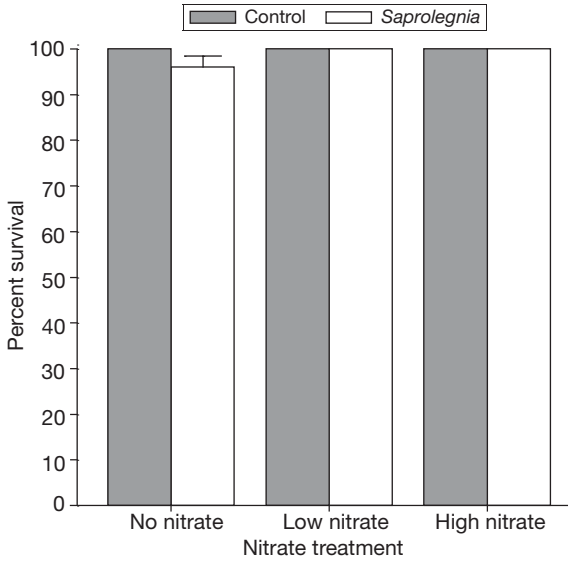

Fig. 3. *Ambystoma gracile.* Survival of larvae. Error bars are +1 SE

Table 3. *Hyla regilla*, *Rana aurora* and *Ambystoma gracile.* Examination of larvae for hyphal growth. We checked for hyphal growth on living larvae on 10 occasions for *Hyla* and *Ambystoma* and 12 times for *Rana.* At the end of the experiment, hyphal growths were not found on any of the live larvae. NA: no mortality; ND: microscopy data not recorded for the one individual that died in this treatment combination. No hyphae were noted during visual inspection of this individual

Treatment	Dead individuals with hyphae	Total no. of instances live individuals were counted as having hyphal growth
H. regilla		
No nitrate addition/control	ND	0
No nitrate addition/*Saprolegnia*	0/1 (0%)	0
Low nitrate addition/control	NA	0
Low nitrate addition/*Saprolegnia*	NA	0
High nitrate addition/control	0/1 (0%)	0
High nitrate addition/*Saprolegnia*	NA	0
R. aurora		
No nitrate addition/control	0/2 (0%)[a]	0
No nitrate addition/*Saprolegnia*	6/13 (46%)[b]	2[d]
Low nitrate addition/control	2/7 (25%)	0
Low nitrate addition/*Saprolegnia*	3/7 (43%)[c]	4
High nitrate addition/control	1/7 (14%)	1
High nitrate addition/*Saprolegnia*	0/5 (0%)[c]	2
A. gracile		
No nitrate addition/control	NA	0
No nitrate addition/*Saprolegnia*	2/2 (100%)	0
Low nitrate addition/control	NA	0
Low nitrate addition/*Saprolegnia*	NA	0
High nitrate addition/control	NA	0
High nitrate addition/*Saprolegnia*	NA	0

[a]Including 1 individual that was removed prior to death. Hyphae were not noted on this individual
[b]Not including 1 carcass that may have had hyphal growth
[c]Not including 2 carcasses that were not discovered
[d]Not including 1 count in which an individual may have had hyphal growth on its tail

Only 2 larvae died; both were in the no nitrate, *Saprolegnia* treatment. ANOVA revealed no significant effects of the treatments or their interaction; however, there were non-significant trends toward a main effect of nitrate treatment and a nitrate × *Saprolegnia* interaction (Table 2). Thus, there was a non-significant trend of the same less-than-additive interaction between the 2 factors that we detected in the experiment using *Rana*.

In *Rana*, 4 carcasses were observed being eaten by conspecifics, and signs of scavenging (i.e. holes in the skin or missing structures obviously due to scavenging) were observed on 25 of the 31 remaining carcasses. For an additional 2 carcasses, it could not be determined whether cannibalism was involved in causing the missing structures (this may have resulted from parasitism, e.g. from *Saprolegnia*, or decomposition without the involvement of cannibalism). Two larvae (1 in the no nitrate, control treatment combination and 1 in the low nitrate, *Saprolegnia* treatment) were observed being chewed upon by 1 or more conspecifics while still alive.

No signs of hyphal growth were observed in *Hyla* (Table 3). *Rana* carcasses had hyphal growths more often in *Saprolegnia* compared to control treatments in the no nitrate and low nitrate treatments, but the reverse was true in the high nitrate treatment. In contrast, hyphae were noted in live *Rana* more frequently in *Saprolegnia* than in control treatments in each of the nitrate treatments. At the end of the experiment, hyphal growths were not observed on any live *Rana*. It is possible that in *Rana*, shredded tadpole structures (e.g. muscle and connective tissue) due to scavenging by conspecifics may have been misidentified as hyphae in some cases. Both of the *Ambystoma* that died, which were in the no nitrate, *Saprolegnia* treatment combination, were covered with hyphae, but hyphae were not noted on any live *Ambystoma*.

Conductivity ranged from 153 to 195 µS cm^{-1} (n = 6, not all treatment combinations included), total alkalinity ranged from 33 to 41 mg $CaCO_3$ l^{-1} (n = 5, not all treatment combinations included), and calcium hardness ranged from 34 to 40 mg $CaCO_3$ l^{-1} (n = 5, not all treatment combinations included). pH varied from 6.5 to 7.2 (n = 18).

DISCUSSION

Our results suggest that *Saprolegnia* caused mortality of *Rana*, but only in the no nitrate treatment. This is consistent with increased nitrate preventing *Saprolegnia* from causing mortality of *Rana.* In *Rana*, the combined effects of nitrate and *Saprolegnia* were less than additive, rather than synergistic. Although determining the mechanism behind such an interaction was not a goal of this study, some possible mechanisms deserve note. Nitrate may decrease zoospore production or kill zoospores in *Saprolegnia*. Nitrate may also have induced a physiological response in *Rana* that increased their resistance to *Saprolegnia*.

Less-than-additive stressor–pathogen interactions have been reported previously in different organisms, including amphibians. For example, Parris & Baud (2004) demonstrated a negative interaction between copper and the amphibian pathogen *Batrachochytrium dendrobatidis.* Both copper and the pathogen increased the length of the larval period in gray treefrogs *Hyla chrysoscelis* larvae, but the magnitude of the effect of the pathogen on larval period was lower when the copper concentration was high than it was in regimes in which there was a lower copper concentration. Parris & Baud (2004) hypothesized that copper may have decreased the growth of the fungus on *H. chrysoscelis*. Poleo et al. (2004) found that aluminum and zinc decreased the number of parasites in Atlantic salmon *Salmo salar*. Parasite diversity and intensity in 2 snail species (*Physella columbiana* and *Lymnaea palustris*) were lower in lakes polluted by heavy metals than in reference lakes, which may have influenced competitive interactions between the 2 snail species (Lefcort et al. 2002). Riggs & Esch (1987) and Riggs et al. (1987) studied the tapeworm *Bothriocephalus acheilognathi* in *Gambusia affinis* (mosquitofish), *Notropis lutrensis* (red shiners), and *Pimophales promelus* (fathead minnows) in a cooling pond receiving thermally and selenium-enriched fly ash from a coal-fired power plant in North Carolina, USA. Three sites were characterized as polluted, interface or unpolluted, based upon proximity to the fly ash input and selenium concentration. A complex pattern of mean number of worms per fish, mean number of gravid worms per fish, growth and biomass of worms, ratio of gravid proglottids per gravid worm, and number of eggs shed per gravid proglottid emerged (Riggs & Esch 1987, Riggs et al. 1987). In several cases, the pattern was consistent with increased pollution causing decreased worm population size or decreased performance of individual worms (Riggs & Esch 1987, Riggs et al. 1987).

Other stressors may also affect host–pathogen interactions. For example, high temperatures may also reduce the effects of infectious disease or even eliminate infection. Woodhams et al. (2003) found evidence consistent with high temperatures eliminating *Batrachochytrium dendrobatidis* infection in juvenile *Litoria chloris* (red-eyed treefrogs). However, the influence of temperature on amphibian diseases may not be straightforward. Aspects of diseases such as mortality from infection, time to death after exposure, and production of pathogenic propagules may show conflicting patterns with respect to temperature (Berger et al. 2004, Rojas et al. 2005).

Importantly, effects of stressor–pathogen interactions on parameters of individual hosts do not necessarily translate into corresponding effects at the level of the host population. Indeed, modeling of the influence of environmental stress on the impact of infectious disease caused by a parasite that is host specific and has a population closed to recruitment from outside the population, suggests that environmental stress that increases the susceptibility of individual hosts to the parasite is most likely to decrease the impact of the disease on the host population (Lafferty & Holt 2003). However, if stress that increases the susceptibility of individual hosts to parasites occurs in an infectious disease system in which the parasite population is open to outside recruitment, or is not host-specific and the other host(s) are not affected by the stress, then the stress will most likely cause an increase in the impact of the disease on the host population (Lafferty & Holt 2003). In this scheme, the ability to grow as a saprobe (as *Saprolegnia* can) should have the same qualitative effect on a host population as open recruitment or ability to use 1 or more alternate hosts unaffected by the stressor. Lafferty & Holt (2003) did not model situations in which the effects of stress and an infectious disease on individual hosts were less-than-additive. However, it seems logical that if nitrate prevents *Saprolegnia* from affecting survival of amphibian larvae, then it will also prevent *Saprolegnia* from having effects on larvae that influence population-level parameters. However, effects on other life stages besides larvae may factor in to determine the combined effects of nitrate and *Saprolegnia* on an amphibian population, and the effects on survival of other life stages may be different from the effect on survival of larvae.

Our study concentrated on testing the separate and combined effects of nitrate and *Saprolegnia* on survival of amphibian larvae. Thus, we did not focus on possible sublethal effects. Nitrate and *Saprolegnia* may interact to influence sublethal parameters in amphibian larvae.

The nominal nitrate concentrations we used were realistic for amphibian larvae in habitats receiving fertilizer runoff. In the Willamette Valley of Oregon, average nitrate concentrations of 17.8 and 21.9 mg N l^{-1}

were reported in water samples from some crop soils receiving recommended rates of nitrogen fertilization (Brandi-Dohrn et al. 1997, Marco et al. 1999). These average values are highly toxic to some amphibians (Marco et al. 1999). Flow-weighted mean nitrate concentrations ranging from zero to 14.8 mg l^{-1} have been recorded in streams draining the English Lake District, Cumbria, England (Thornton & Dise 1998). Average nitrate concentrations in 30 mg l^{-1} have been recorded in relatively large streams in the Great Lakes region of North America (US EPA 1998, Rouse et al. 1999). Rouse et al. (1999) suggest that such streams will typically have average nitrate concentrations lower than small ponds and ditches close to point sources of nitrate. Thus, amphibian larvae in agricultural landscapes may be exposed to nitrate levels well in excess of 30 mg l^{-1}. Exposure to these levels may occur for extended periods of time (Rouse et al. 1999).

Observations of *Rana* carcasses suggest that the *Saprolegnia* treatment caused infection in the low and no nitrate treatments, but not in the high nitrate treatment. Furthermore, our results suggest *Saprolegnia* can kill larvae of *R. aurora* in water lacking nitrate pollution. Because most of the studies of the effects of *Saprolegnia* on amphibians have been examined in embryos, our study of larvae is an important step in determining the effects of this pathogen on post-embryonic life history stages. The effects of *Saprolegnia* on amphibian populations may be far different, and possibly more severe, if the pathogen causes mortality in both embryos and larvae rather than in just 1 life stage. Recent models have shown differential effects on populations when mortality occurs in different life stages in amphibians (Biek et al. 2002, Vonesh & De la Cruz 2002a,b).

Under the conditions of this study, *Rana* was susceptible to mortality from *Saprolegnia* when nitrate was not added, while *Hyla* and *Ambystoma* were not susceptible to mortality from *Saprolegnia* in any of the nitrate treatments. These interspecific differences may indicate that *R. aurora* larvae are more susceptible to *Saprolegnia* than larvae of the other 2 species. However, the relative susceptibilities of these species may depend on the dose of the pathogen.

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