Susceptibility of newly-metamorphosed frogs to a pathogenic water mould (*Saprolegnia* sp.)

John M. Romansic, Elise M. Higashi, Kristin A. Diez & Andrew R. Blaustein

Department of Zoology, Oregon St. University, Corvallis, OR, USA

Recent losses of worldwide biodiversity include population declines and extinctions in many amphibian populations. Many factors, including pathogens, are contributing to amphibian population declines. One pathogen, a water mould of the genus *Saprolegnia*, causes mortality in early life stages of amphibians and may contribute to the declines of specific amphibian populations. Most of our knowledge of how *Saprolegnia* affects amphibians comes from studies of embryos. The effects of *Saprolegnia* on post-metamorphic amphibians are poorly known. Therefore, in the laboratory, we investigated the susceptibility of newly-metamorphosed juvenile amphibians to *Saprolegnia* in four frog species: *Bufo boreas* (western toad), *Pseudacris regilla* (Pacific treefrog), *Rana aurora* (red-legged frog) and *R. cascadae* (Cascades frog). We found that juvenile *R. cascadae* exposed to *Saprolegnia* had greater rates of mortality than unexposed controls. In the other species, survival was also lower in the *Saprolegnia* treatments compared with controls but these differences were not statistically significant. Combined effects of *Saprolegnia* in both embryonic and juvenile stages may make the populations of *R. cascadae* especially vulnerable.

Key words: amphibian population declines, *Bufo boreas*, life history stage, metamorph, *Pseudacris regilla*, *Rana aurora*, *R. cascadae*

INTRODUCTION

As part of worldwide losses in biodiversity, many amphibian populations are declining and disappearing (Houlahan et al., 2000; Stuart et al., 2004). Numerous factors appear to be contributing to these declines. They include habitat destruction (Dodd & Smith, 2003), introduced predators (Knapp & Matthews, 2000; Kats & Ferrer, 2003; Vredenburg, 2004), global climate change (Pounds & Crump, 1994; Pounds et al., 1999), pollution (Beebee et al., 1990) and ultraviolet-B (UV-B) radiation (Blaustein et al., 1998).

Pathogens are causing mortality in various amphibian life stages (e.g. Blaustein et al., 1994) and are implicated in the declines of several amphibian populations (Berger et al., 1998; Daszak et al., 2003; Lips et al., 2006; Pounds et al., 2006). Pathogens of amphibians are diverse. They include parasitic worms (e.g. Platt et al., 1993; Aisien et al., 2003; Blaustein & Johnson, 2003), oomycetes (e.g. Blaustein et al., 1994), fungi (e.g. Berger et al., 1998), viruses (e.g. Jancovich et al., 1997), bacteria (e.g. Worthylake & Hovingh, 1989) and myxozoans (e.g. Delvinquier et al., 1992).

The oomycete *Saprolegnia* is a genus of water mould that is commonly present in soil and freshwater (Johnson et al., 2002). *Saprolegnia* species are capable of both saprotrophism and parasitism (Johnson et al., 2002). *Saprolegnia* infects a variety of organisms, including mosquitoes (MacGregor, 1921), turtles (Tiffney, 1936), fish (e.g. Schaefer et al., 1981) and amphibians (e.g. Blaustein et al., 1994), and is associated with mass mortality in fish populations (Schaefer et al., 1981; Johansson et al., 1982).

Saprolegnia infects amphibian embryos (e.g. Blaustein et al., 1994; Kiesecker et al., 2001b), larvae (e.g. Schnetzler, 1888; Walls & Jaeger, 1987), and adults (Ford et al., 2004). Banks & Beebee (1988) found that *Saprolegnia*-associated mortality of *Bufo calamita* (natterjack toad) in England was associated with low temperature and acid conditions. In the Oregon Cascade range, a synergism between UV-B radiation and *Saprolegnia ferax* contributes to massive mortality of *B. boreas* and *Rana cascadae* embryos (Blaustein et al., 1994; Kiesecker & Blaustein, 1995). Thus, there is the potential for *Saprolegnia* to greatly affect amphibian populations. For *B. boreas* and *R. cascadae*, laying in communal egg masses increases embryo mortality, apparently because of the high density of susceptible hosts when eggs are laid in close proximity to one another (Kiesecker & Blaustein, 1997). For *B. boreas, S. ferax*-associated embryo mortality becomes greater when El Niño Southern Oscillation events cause a decrease in water levels and exposure to UV-B radiation increases (Kiesecker et al., 2001a). Koch's postulates (Pelczar & Reid, 1965) have been fulfilled for *B. boreas* embryos, illustrating that *Saprolegnia* is indeed one pathogen causing mortality in amphibians in the Pacific Northwest (U.S.A.) (Kiesecker et al., 2001b).

There are only limited data on the effects of *Saprolegnia* on post-embryonic amphibians. In the Oregon Cascades, there is experimental evidence that *Saprolegnia* influences competitive interactions between *R. cascadae* and *Pseudacris regilla* (Pacific treefrog). In an experiment performed in outdoor ponds, *Saprolegnia* prevented *R. cascadae* larvae from reducing survival, growth and development of *P. regilla* larvae, apparently because of decreased density of *R. cascadae* larvae due to *Saprolegnia*-induced mortality of their embryos (Kiesecker & Blaustein, 1999). Bragg & Bragg (1958) and Bragg (1962) reported *Saprolegnia* on dead amphibian

Correspondence: John M. Romansic, Department of Zoology, 3029 Cordley Hall, Oregon St. University, Corvallis, OR 97331, USA. *E-mail:* romansij@science.oregonstate.edu

larvae after mass mortality events. Walls & Jaeger (1987) noticed that mortality of *Ambystoma maculatum* (spotted salamander) larvae caused by exposure to *A. talpoideum* (mole salamander) larvae was associated with *Saprolegnia* infection of bite wounds from aggressive *A. talpoideum*. Lefcort et al. (1997) observed that mortality of *A. tigrinum* (tiger salamander) caused by exposure to silt was associated with *S. parasitica* infection. Romansic et al. (2006) showed that *Saprolegnia* can kill *R. aurora* (red-legged frog) larvae, and *Saprolegnia* infection was associated with red leg syndrome and mortality in captive *Xenopus laevis* (African clawed frog) adults that had undergone oocyte-harvesting surgery (Ford et al., 2004).

One step in examining the potential population effects of *Saprolegnia* is to test their susceptibility to the pathogen at various life stages. This seems especially important in light of models suggesting that mortality involving post-embryonic individuals may contribute significantly to population declines (Vonesh & de la Cruz, 2002; Biek et al., 2002).

The purpose of this study was to test experimentally the susceptibility of newly-metamorphosed amphibians (metamorphs) to mortality from *Saprolegnia*. We used four anuran species: *B. boreas, P. regilla, R. cascadae,* and *R. aurora*. *B. boreas, P. regilla* and *R. cascadae* are susceptible to mortality from *Saprolegnia* at the embryonic stage (Kiesecker & Blaustein, 1995; Kiesecker et al., 2001b). There have been no tests for the possible effect of *Saprolegnia* on *R. aurora* embryos. Romansic et al. (2006) found that larvae of *R. aurora* are susceptible to mortality from this pathogen, but found no evidence that larvae of *P. regilla* were. There have been no studies examining the effects of *Saprolegnia* on post-metamorphic frogs.

MATERIALS AND METHODS

We tested a set of three frog species (*B. boreas*, *P. regilla* and *R. cascadae*) collected from the Oregon Cascade range because *Saprolegnia* causes massive mortality in embryos of *B. boreas* and *R. cascadae* in this region (Blaustein et al., 1994; Kiesecker & Blaustein, 1995). We also tested *R. aurora* to gain a broader understanding of the effects of *Saprolegnia* on frogs. Testing frogs from the Oregon Cascades and *R. aurora* simultaneously was impossible due to differences in timing of oviposition and larval period between frog populations in the Oregon Cascades and the populations of *R. aurora* known to the authors. Therefore, *B. boreas*, *P. regilla* and *R. cascadae* were tested simultaneously in experiment 1 and *R. aurora* alone was tested in a separate experiment (experiment 2).

Amphibians were collected as larvae. *B. boreas* were collected on 22 and 27 August 2002 from Todd Lake, Deschutes County, Oregon, USA (elevation about 1864 m). *P. regilla* were collected on 23 August from a subalpine meadow about 0.6 km NW of Todd Lake, Deschutes County (elevation about 1982 m) and *R. cascadae* were collected from this meadow on 23 August and 18 September 2002. *R. aurora* were collected from one pond (elevation about 12 m) at Baker Beach, Lane County, Oregon on 31 May 2003 and a second pond (elevation about 6 m) at Baker Beach in June 2003. Larvae were brought to the laboratory and maintained in aquaria filled with tapwater conditioned with NovAqua and Amquel water conditioners (hereafter, dechlorinated tapwater). *B. boreas* tanks were aerated. Larvae were fed a ground mixture of alfalfa pellets and Tetramin fish flakes. Metamorphs were transferred to tanks containing dechlorinated tapwater and tilted (part of the bottom of the tank was above water and part was below water), with the exception that *B. boreas* metamorphs were put in a plastic shoebox with paper towels moistened with dechlorinated tapwater placed on the bottom. Prior to use in experimentation, metamorphs were fed crickets, except for *P. regilla* metamorphs, which were fed a combination of *Drosophila* and crickets.

Saprolegnia was isolated from a water sample that was collected on 10 September 2002 at the shore of Lost Lake in the Oregon Cascade range (Linn County; elevation 1220 m), a site where *Saprolegnia* has contributed to massive mortality of *B. boreas* embryos (Blaustein et al., 1994, Kiesecker & Blaustein, 1995; Kiesecker et al., 2001a). The isolate was grown in pure culture in Petri dishes using YpG agar media (Fuller & Jaworski,1987). To obtain *Saprolegnia* for use in experiments, sterile hemp seeds were added directly to Petri dishes containing *Saprolegnia* cultures to allow seeds to become inoculated with *Saprolegnia*. Seeds were then removed and added to standardized Petri dishes (diameter 85 mm, height 12 mm) filled approximately half full with ultrapure water. Thirty seeds were added to each dish. After seeds were added to water, dishes were incubated for seven days at approximately 20–23 ºC and then transferred to a refrigerator (~4 ºC) for two days prior to use. *Saprolegnia* hyphae grew between seeds in dishes, producing clumps of seeds connected by a mycelium of *Saprolegnia* containing hyphae and zoosporangia. These clumps of seeds were used to apply *Saprolegnia* treatments in laboratory experiments.

Experimental units consisted of plastic cups (diameter 9.5 cm, height 7.5 cm) with covers of 1.5-mm fibreglass mesh. Unbleached paper towels were placed at the bottom of each cup and flooded initially with 15 ml of ultrapure water. In experiment 1 (*B. boreas*, *P. regilla* and *R. cascadae* tested simultaneously), we manipulated treatment and frog species in a 2 × 3 fully factorial design. Units received, at random, either a *Saprolegnia* treatment (a clump of 30 hemp seeds overgrown with *Saprolegnia* hyphae and zoosporangia) or a control treatment (30 sterile hemp seeds) and five metamorph individuals of either *B. boreas*, *P. regilla* or *R. cascadae*. There were five replicates of each species–treatment combination, for a total of 30 units and 150 metamorphs. Experiment 2 used the same methods, except that it used *R. aurora* metamorphs only (five metamorphs per unit). Here, there were five replicates of each treatment (*Saprolegnia* and control), for a total of 10 units and 50 metamorphs. Metamorphs were visually inspected for hyphal growths consistent with *Saprolegnia* infection (Ford et al., 2004) immediately prior to addition to units. No hyphal growths were found during these inspections. Addition of all metamorphs to

Table 1. Mean length (±1 standard error) of preserved specimens.

Species	Mean length (mm)	*n*
Experiment 1		
Bufo boreas	14.0±0.1	50
Pseudacris regilla	12.5±0.1	39
Rana cascadae	13.6±0.2	47
Experiment 2		
Rana aurora	15.8±0.2	27

units was random with respect to treatment. Each experiment lasted for two weeks. Experiment 1 began on 27 October 2002, and experiment 2 on 6 August 2003.

The initial number of *Saprolegnia* zoospores and zoospore cysts (a combined total) applied in the *Saprolegnia* treatment was estimated at the start of each experiment using leftover clumps of *Saprolegnia*-laden hemp seeds from the same stock used in the experiment but not added to cups. These clumps were lifted out of their water, placed in a sterile container and washed with ultrapure water. The number of zoospores and cysts in a sample of the resulting solution was counted using a hemacytometer and these counts provide an estimate of the initial number of zoospores and cysts per cup in the *Saprolegnia* treatment, assuming that the number of zoospores and cysts present from sources besides the *Saprolegnia*-laden hemp seeds was zero. Estimates of initial number of *Saprolegnia* zoospores and cysts were 1.1×10^8 (standard error = 2.4×10^7, n=2) and 2.4×10^7 (standard error = 3.3×10^6, n =2) in experiments 1 and 2, respectively.

Throughout each experiment, dechlorinated tapwater was added as necessary to maintain a thin film of water over towels, thus allowing hydration of amphibians and preventing *Saprolegnia* from desiccating. Experiments were checked at least once per day. Frogs were not fed during the experiments. Dead individuals were removed and preserved in 70% ethanol. At the end of each experiment, percent survival was recorded for each cup and surviving individuals were anaesthetized with MS-222 and preserved in 70% ethanol.

After the experiments, preserved specimens were measured for length (Table 1). Snout–vent length was measured, unless there was some tail present, in which case the snout–base-of-tail length was measured. Specimens that were damaged or distorted due to desiccation or decomposition prior to preservation or handling after preservation were not measured. Approximate range of ages at the start of experimentation was 2–6 weeks post-metamorphosis for *B. boreas* and *P. regilla* and 2–8 weeks post-metamorphosis for *R. aurora* and *R. cascadae*. We defined metamorphosis as having about 50% or more of the tail resorbed.

Preserved specimens were examined for oomycotic growth consistent with *Saprolegnia* infection (Ford et al., 2004) using a dissecting microscope (25× magnification). For each metamorph, a sample of or the entirety of any hyphae, shedding skin, unusual-looking skin, unusual-looking structures or unidentified debris present on the exterior of the specimen was removed using a pair of forceps and examined using a compound microscope (100–400× magnification). Metamorphs with coenocytic hyphae were scored as having oomycotic growth consistent with *Saprolegnia* infection.

Each experiment was analysed separately. In experiment 1, percent survival data and prevalence of oomycotic growth data were heteroscedastic, violating the parametric assumption of homoscedasticity. In the survival data there was no variation in the *B. boreas*, control treatment combination, while in the prevalence of oomycotic growth data there was no variation in the control treatment. Therefore, all analyses were nonparametric. For experiment 1, survival and prevalence of oomycotic growth were analysed separately, using multiple-comparisons procedures. For each dependent variable, an analogue to the Student–Newman–Keuls test (Zar, 1999) was used to test for pairwise differences between treatments for each of the three frog species in the experiment (*B. boreas*, *P. regilla* and *R. cascadae*). For experiment 2, we tested for a difference in survival between the two treatments in *R. aurora* using a rank-sum test. No oomycotic growth was observed on *R. aurora*.

RESULTS

Mean percent survival of *B. boreas*, *P. regilla* and *R. cascadae* in experiment 1 is displayed in Figure 1. Survival was not significantly different between *Saprolegnia* and control treatments in *B. boreas* ($q_{0.05,\infty,3}$= 2.85, $0.10<P<0.20$) and *P. regilla* ($q_{0.05,\infty,2}$= 2.6588, $0.05<P<0.10$). In *R. cascadae*, survival was significantly lower in the *Saprolegnia* treatment compared to the control treatment ($q_{0.05,\infty,3}$= 5.0292, $0.005<P<0.01$). Mean percent and proportion of specimens showing oomycotic growth is displayed in Table 2. In *R. cascadae*, *B. boreas* and *P. regilla*, prevalence of oomycotic growth was higher in the *Saprolegnia* compared to the control treatment, but none of these differences was statistically

Fig. 1. Percent survival of metamorphs of *Bufo boreas*, *Pseudacris regilla* and *Rana cascadae* in experiment 1 and *Rana aurora* in experiment 2. Error bars are + 1 standard error. * Denotes the statistically significant difference between treatments for *Rana cascadae*.

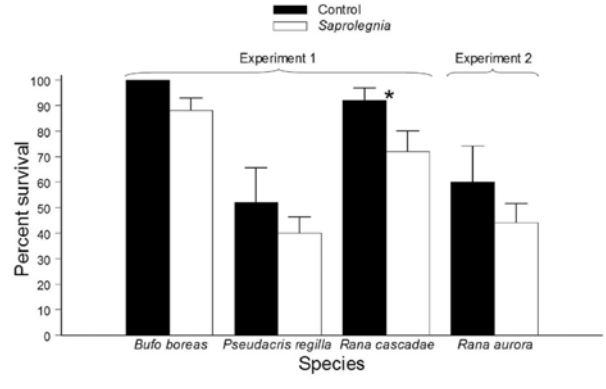

Table 2. Mean percent and proportion (in parentheses) of preserved specimens showing oomycotic growth consistent with *Saprolegnia* infection in control and *Saprolegnia* treatments. If variation in mean percent is present, ± 1 standard error is given. Mean percent data are for means across all relevant experimental units. Proportions are for all individuals in that category lumped together.

Species	Treatment	Died[a]	Survived[b]	Total
Experiment 1				
Bufo boreas	Control	NA	0.0 (0/25)	0.0 (0/25)
	Saprolegnia	100.0 (3/3)	0.0 (0/22)	12.0±4.9 (3/25)
Pseudacris regilla	Control	0.0 (0/12)	0.0 (0/13)	0.0 (0/25)
	Saprolegnia	16.7±10.5 (2/15)	0.0 (0/10)	8.0±4.9 (2/25)
Rana cascadae	Control	0.0 (0/2)	0.0 (0/23)	0.0 (0/25)
	Saprolegnia	66.7±28.9 (3/5)[c]	0.0 (0/19)	13.0±5.4 (3/24)[c]
Experiment 2				
Rana aurora	Control	0.0 (0/10)	0.0 (0/15)	0.0 (0/25)
	Saprolegnia	0.0 (0/14)	0.0 (0/11)	0.0 (0/25)

[a]Individuals that died during the experiment.
[b]Individuals that survived the experiment.
[c]Mean percent and proportion do not include one individual with possible oomycotic growth.

significant (P>0.50 for *R. cascadae*, P>0.05 for *B. boreas* and *P. regilla*). In these three species, oomycotic growth was not observed on any of the individuals in the control treatment.

Mean percent survival of *R. aurora* in experiment 2 is also shown in Figure 1. Survival was lower in the *Saprolegnia* treatment compared to the control treatment, but this difference was not statistically significant (P=0.2828). Oomycotic growth was not observed on any *R. aurora*.

DISCUSSION

Our results suggest that newly metamorphosed *R. cascadae* are susceptible to mortality from *Saprolegnia*. In each of the other three species, mortality was higher in the exposed compared to the control group, but none of these differences were statistically significant. However, longer exposures and/or higher doses of the pathogen may cause significant mortality in these three species. Also, differences in dosage (number of zoospores) and differences in age may have caused some of the differences in results across the four frog species. For example, despite the use of the same culturing methods in both experiments, estimated initial number of zoospores and cysts applied in the *Saprolegnia* treatment for experiment 2 (*R. aurora*) was only about one-fifth that of experiment 1 (*B. boreas*, *P. regilla* and *R. cascadae*). Furthermore, approximate age post-metamorphosis was 2–6 weeks in *B. boreas* and *P. regilla*, and 2–8 weeks in *R. cascadae* and *R. aurora*.

Prevalence of oomycotic growth consistent with *Saprolegnia* infection was higher in the *Saprolegnia* compared to the control treatment for *B. boreas*, *P. regilla* and *R. cascadae*, but none of these differences was statistically significant. Oomycotic growth was not observed in *R. aurora*. It is possible that some metamorphs had oomycotic growth that was not detected. Failure to detect cases of oomycotic growth could have prevented detection of a statistically significant effect of the *Saprolegnia* treatment on prevalence of oomycotic growth in *R. cascadae*, the species in which we found a statistically significant negative effect of the *Saprolegnia* treatment on survival.

Our study suggests that *Saprolegnia* kills newly-metamorphosed individuals of at least one anuran species (*R. cascadae*). Our study constitutes an important step in determining the effects of this pathogen on post-embryonic life-history stages in nature. Taken together, the results of this study and those of previous studies (Romansic et al., 2006; Walls & Jaeger, 1987; Lefcort et al., 1997) suggest that *Saprolegnia*-induced mortality of amphibians in nature is not restricted to embryos and may greatly influence mortality rates in larvae and newly-metamorphosed individuals. Efforts to evaluate whether or not a pathogen contributes to amphibian population declines should not be restricted to a single life-history stage.

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