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Transfer of a Pathogen from Fish to Amphibians

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Abstract: *Ecological studies of exotic species focus primarily on how invaders directly affect particular resident species. In contrast, little is known about the indirect effects of introduced species on native communities, including how pathogens may be spread by introduced species. We provide evidence suggesting that introduced fish may serve as a vector for a pathogenic oomycete, *Saprolegnia ferax*, that has been associated with embryonic mortality of amphibians in the Cascade Mountains of Oregon, U.S.A. In laboratory experiments, mortality induced by *S. ferax* was greater in western toad (*Bufo boreas*) embryos exposed directly to hatchery-reared rainbow trout (*Oncorhynchus mykiss*) experimentally infected with *S. ferax* and hatchery-reared trout not experimentally infected than in control embryos. Embryos also developed significant *S. ferax* infections when raised on soil that was exposed to trout experimentally infected with *S. ferax*. Furthermore, toad embryos exposed to *S. ferax* isolated from sites where *Saprolegnia* outbreaks are common experienced higher mortality than embryos exposed to *S. ferax* isolated from sites where *Saprolegnia* outbreaks have not occurred. Given the widespread practice of introducing hatchery-reared fishes, we suggest that fish used in stocking programs could be an important vector for diseases responsible for amphibian losses.*

Transferencia de un Patógeno de Peces a Anfibios

Resumen: *Los estudios ecológicos de especies exóticas están enfocados principalmente en el efecto de los invasores sobre ciertas especies residentes. En contraste, se sabe poco de los efectos de especies introducidas sobre comunidades nativas, incluyendo la dispersión de patógenos por las especies introducidas. Proporcionamos evidencia que sugiere que peces introducidos pueden ser el vector de un oomiceto patógeno, *Saprolegnia ferax*, que se ha asociado con la mortalidad embrionaria de anfibios en las montañas Cascade de Oregon, E.U.A. En experimentos de laboratorio, la mortalidad inducida por *S. ferax* fue mayor en embriones de sapo (*Bufo boreas*) expuestos directamente a truchas arco iris (*Oncorhynchus mykiss*) criadas en granjas y experimentalmente infectadas con *S. ferax* y con truchas no infectadas experimentalmente que en los embriones control. Los embriones también desarrollaron infecciones significativas con *S. ferax* cuando fueron criados en sitios expuestos a truchas infectadas experimentalmente con *S. ferax*. Más aún, los embriones expuestos a *S. ferax* aislado de sitios con brotes comunes de *Saprolegnia*, presentaron mayor mortalidad en comparación con embriones expuestos a *S. ferax* aislado de sitios en los que no han ocurrido brotes de *Saprolegnia*. Dada la introducción generalizada de peces criados en granjas, sugerimos que los peces utilizados en esos programas pudieran ser vectores importantes de enfermedades responsables de la pérdida de anfibios.*

Introduction

The introduction and spread of exotic species poses a threat to ecological communities and global biodiversity (Elton 1958; Drake et al. 1989; Lodge 1993). Introduced

exotic species have been linked to the displacement of native species (e.g., Zaret & Paine 1973; Petren et al. 1993; Gamradt et al. 1997; Kupferberg 1997; Kiesecker & Blaustein 1997a), modification of trophic structure (Wormington & Leach 1992; Holland 1993; Nicholls & Hopkins 1993), and alteration of ecosystem function (Vitousek 1989). The introduction of exotic species into freshwater systems may be of particular interest because

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introduced organisms have been associated with 68% of the 40 North American fish extinctions that have occurred in the 1900s (Miller et al. 1989).

The mechanisms that enable exotic species to thrive at the expense of natives often are unclear. Competition with or predation by introduced species is frequently proposed to explain losses of native species after the introduction of an exotic species. Rarely are these mechanisms isolated and tested. Moreover, studies that have attempted to examine the mechanisms that underlie the success of exotic species focus primarily on how invaders directly affect particular resident species (Simberloff 1981). In contrast, little is known about the indirect effects of introduced species on native communities (e.g., Kiesecker & Blaustein 1998), such as the effect of pathogens carried by introduced species. Evidence is increasing that indirect interactions are important in natural communities (Schoener 1993; Menge 1995), although the effects are not always obvious. Thus, attempts to assess the effects of introduced species may be underestimated if investigations fail to consider their indirect effects. The term *introduced species* is used broadly here and includes species moved outside of their normal range and those reared artificially and transferred to natural sites within their normal range.

During this century, unprecedented numbers of fish species and other aquatic organisms have been transferred from one geographic location to another (e.g., Taylor et al. 1984; Minckley & Deacon 1991; Kiesecker & Blaustein 1998). In fact, the stocking of hatchery-reared fishes for sport fishing still occurs in several national parks and wilderness areas (e.g., Bahls 1992; Fraley 1996). There are numerous examples of native species declining after the arrival of an exotic fish species (Taylor et al. 1984; Bradford 1989; Bradford et al. 1993; Gamradt & Kats 1996; Tyler et al. 1998). Frequently, competition or predation is proposed to explain the decline of native species associated with introduced fishes. In addition to the direct effects that introduced fishes can have on native species, they may also be the source of introduced pathogens (e.g., Kennedy et al. 1991; Ganzhorn et al. 1992). Few studies, however, have attempted to assess the role that fish introductions can play in the dispersal of pathogens.

In the Pacific Northwest of the United States, massive amphibian embryo mortality is associated with the presence of the oomycete pathogen *Saprolegnia ferax* (Blaustein et al. 1994a; Kiesecker & Blaustein 1995, 1997b, 1999). Outbreaks have been observed at several sites, whereas at other sites embryo mortality remains relatively low (Blaustein et al. 1996; Kiesecker & Blaustein 1997b). This variation may be attributed to a number of factors. For example, the difference may be the result of variation in abiotic conditions between sites (e.g., UV-B exposure; Kiesecker & Blaustein 1995) or may result from variation in the strains of *S. ferax* present. We hypothesized that embryos exposed to isolates of *S. ferax*

from sites where *S. ferax* outbreaks have occurred would experience higher mortality than embryos exposed to isolates of *S. ferax* from sites where outbreaks have not occurred.

Saprolegniasis is a common disease of fish, particularly those reared in hatcheries (Richards & Pickering 1978). Many of the fish species introduced into Pacific Northwest lakes (*Salmo* sp., *Salvelinus* sp., and *Oncorhynchus* sp.; see Johnson et al. 1985) are common carriers of *Saprolegnia*, including *S. ferax* (Seymour 1970; Richards & Pickering 1978; Wood & Willoughby 1986). Although a large body of research has focused on attempting to understand the factors that influence *Saprolegnia* outbreaks in wild and captive stocks, relatively little work has assessed how or even if *Saprolegnia* can be transmitted to wild fishes or other vertebrate species. During stocking, infected fish might directly transmit *S. ferax* to developing amphibians; alternatively, *S. ferax* may be transferred to the lake substrate, where it may become established. Therefore, we hypothesized that amphibian embryos exposed to infected fish or soil have higher mortality than embryos not exposed to either. We tested these hypotheses in a series of laboratory experiments using embryos of the western toad, *Bufo boreas*, a species whose embryos are infected by *Saprolegnia* in Oregon.

Methods

Collection and Infection Protocol

Using standardized protocol (Laskin & Lechevalier 1978), we cultured *S. ferax* in the laboratory on 20-mL cornmeal agar in standard petri dishes. Boiled hemp seeds were added to cultures, and cultures were incubated for approximately 168 hours. To inoculate tanks with *S. ferax*, we introduced hemp seeds from the inoculated cultures. Seeds were added to tanks to produce zoospore levels similar to those observed under field conditions (approximately 4000 zoospores/L; Kiesecker & Blaustein 1995). We quantified zoospore densities with a hemacytometer. Water was removed from each tank, and 1 mL was transferred to a hemacytometer. The number of zoospores in seven 0.04-mm² grids was scored, and the mean number of cells in three subsamples was used to estimate the relative number of zoospores per liter. Boiled hemp seeds were placed into control tanks receiving no *S. ferax*. Prior to use, all tanks were rinsed with a 2-ppm solution of malachite green, an antioomycete agent (Kiesecker & Blaustein 1995), and then repeatedly rinsed with dechlorinated tapwater to remove malachite green. Isolates of *Saprolegnia* used to infect fish were collected from a site where high amphibian embryo mortality has been reported (Parrish Lake, Linn County, Oregon).

We obtained rainbow trout (*Oncorhynchus mykiss*) on 22 April 1995 from a fish hatchery and transported

them to a laboratory at Oregon State University. One-third were placed into disinfected (*Saprolegnia*-free) 38-L tanks and left undisturbed; they are hereafter referred to as "unmanipulated hatchery fish." The remaining two-thirds of the fish were placed into 38-L tanks containing 2-ppm solution of malachite green for 6 hours. Fish were then rinsed with dechlorinated tap water and randomly assigned to one of two regimes. "Infected fish" were placed into 38-L aquaria and exposed to *S. ferax* for 2 days, and "uninfected control fish" were placed in 38-L aquaria that had been disinfected to remove *S. ferax*. In all regimes (unmanipulated hatchery fish, uninfected fish, infected fish), four fish were placed in each aquarium.

We collected freshly oviposited *B. boreas* embryos from Lost Lake (Linn County, Oregon). Embryos were transported to a laboratory at Oregon State University, where they were initially rinsed in a dilute (2-ppm) solution of malachite green to eliminate *Saprolegnia* on them (Kiesecker & Blaustein 1995). These embryos were then immediately placed into experimental chambers. All tests were conducted with early-stage embryos (stages 1–3; Gosner 1960). All experiments were conducted simultaneously in the same laboratory at approximately 16° C on a 12:12 light:dark cycle, and all treatments within an experiment were assigned randomly according to a randomized block design.

Transfer of *Saprolegnia* from Fish to Developing Embryos

Tests to examine whether fish transmit *Saprolegnia* infections to developing amphibian embryos took place in 20 38-L aquaria. Each aquarium was divided with a fiberglass screen partition (pore size 750 μ m) that prevented access of fish to the embryos but allowed the movement of *S. ferax* zoospores. One end of each aquarium held the experimental fish, and the other end held the developing embryos whose survival was observed. We forced air through an air-stone to provide oxygen for experimental animals. We wrapped the top of each tank with clear plastic to prevent contamination with *Saprolegnia*. Prior to use, all experimental tanks were rinsed with a 2-ppm solution of malachite green to remove any *Saprolegnia* and then rinsed repeatedly with dechlorinated tapwater to remove malachite green.

Testing began when one *O. mykiss* was placed into 15 out of 20 of the 38-L tanks and acclimated for 8 days. After the acclimation period, 100 *B. boreas* embryos were placed in the tank. We added 10 embryos from 10 different clutches into each tank. All fish were of similar size among replicates and treatments ($x = 8.47 \text{ cm} \pm 0.31 \text{ SE}$). Fish were fed Purina trout chow.

The experiment consisted of four treatments, each replicated five times. Focal embryos were exposed to either infected *O. mykiss*, uninfected *O. mykiss*, unmanipulated hatchery *O. mykiss*, or nothing. During the embryonic development of *B. boreas*, we monitored tanks

daily and recorded the proportion of embryos surviving to hatching per tank. We terminated the experiment when all embryos had either hatched or died.

Transfer of *Saprolegnia* from Fish to Soil

Tests to examine whether fish transmit *Saprolegnia* to soil were identical to those described for transfer from fish to amphibian embryos, except that sterile soil was used in place of the developing embryos. Each of the 20 38-L aquaria held a $10 \times 10 \times 10 \text{ cm}$ soil container with 350 g of heat-sterilized soil (12 hours at 287.7° C). Soil was obtained from a commercial supplier and mixed thoroughly before use. Soil containers had tops perforated with 0.25-cm holes that would allow colonization of *Saprolegnia*. Fish were introduced into the tanks for 1 week, after which time the soil containers were removed, placed in a sealed plastic bag, and dried at room temperature (approximately 20° C) for 16 days. Tanks were rinsed with a 2-ppm solution of malachite green to remove *Saprolegnia* and then rinsed repeatedly with dechlorinated tapwater to remove malachite green. Disinfected tanks were filled with dechlorinated tapwater, and the soil containers were placed randomly back into the tanks for 8 days. We then added 10 *B. boreas* embryos from each of 10 different clutches onto the tops of the soil containers. We monitored tanks daily and recorded the proportion surviving to hatching per tank. We terminated the experiment when all embryos had either hatched or died. All fish were of similar size among replicates and treatments ($x = 9.43 \text{ cm} \pm 0.37 \text{ SE}$).

Variability in Virulence of *S. ferax*

To examine variability in *S. ferax* virulence, we isolated *S. ferax* from amphibian embryos from four different populations. The first population, was from Lost Lake, (Linn County, Oregon) in the Cascade mountains; Lost Lake has a large breeding population of *B. boreas* and Pacific treefrog (*Hyla regilla*), and fish stocking is common there (Johnson et al. 1985). In fact, 5000 fingerling rainbow trout are introduced into Lost Lake each year (Wayne Hunt, personal communication). Embryonic mortality for *B. boreas* at this site has ranged from 60% to 100% in 6 out of 7 years from 1990 to 1997 (Blaustein et al. 1994a; Kiesecker & Blaustein 1997b, unpublished data). The second population, from Three Creeks (Deschutes County, Oregon) is also located in the Oregon Cascade Mountains and had large breeding populations of *B. boreas*, *H. regilla*, and Cascades frog (*Rana cascadae*). Fish stocking is also common at this site (Johnson et al. 1985), with 4000 legal-sized rainbow trout introduced into Three Creeks Lake each year (Steve Marx, personal communication). Embryonic mortality for *B. boreas* at this site has ranged from 60% to 90% in all 4 years from 1993 to 1996 (Kiesecker & Blaustein 1997a).

The third population, from Corvallis Watershed Pond (Benton County, Oregon) of the Oregon Coast Range, had breeding populations of red-legged frog (*Rana aurora*) and Northwestern salamander (*Ambystoma gracile*). The fourth population, from Waldport Pond (Lincoln County, Oregon) was located along the coast and had a population of *R. aurora* and *H. regilla*. We have not observed embryo mortality exceeding 10% (unpublished data) for any of the amphibians at the third and fourth site, and neither of these sites has stocked fish.

We removed embryos from each site and isolated the fungal pathogens by removing a portion of the jelly matrix from an infected embryo and culturing it in the laboratory on cornmeal agar. Isolates from all four populations were positively identified as *S. ferax*. Pure cultures of *S. ferax* were maintained in the laboratory on 20-mL cornmeal agar in standard petri dishes.

The experiment consisted of five treatments, each replicated five times. Embryos were either exposed to *S. ferax* from one of the four populations or to nothing. To inoculate tanks with *S. ferax*, we introduced hemp seeds infected with *S. ferax*, described above. Control containers received clean boiled hemp seeds. *Bufo boreas* embryos used in this experiment were handled in a manner identical to that described above. After disinfection, 100 embryos (10 embryos from each of 10 separate clutches) were placed in plastic containers (32 × 18 × 8 cm) filled with 3 L of dechlorinated tap water. During the embryonic development of *B. boreas*, we monitored tanks daily and recorded the proportion surviving to hatching per tank. We terminated the experiment when all embryos had either hatched or died.

Post-Experimental Analysis of Infection

To identify oomycete pathogens associated with mortality of embryos in our experiments, we isolated the *Saprolegnia* from three dead embryos from each tank and cultured them on cornmeal agar. Individual isolates were prepared for each tank and were kept separate. We also isolated *Saprolegnia* from "unmanipulated hatchery fish." The mucus coatings of five fish were scraped with a sterile swab and transferred to a cornmeal agar plate. Oomycete isolates were sent to the University of Washington, Seattle, for identification. Samples were examined at 400×, and the reproductive structures (oogonia) were used to identify the oomycetes to the species level. When oogonia were not present, we used the structure of secondary zoospore cysts to make the identification (Mueller 1994).

Statistical Analysis

For all three experiments, we tested for differences in hatching success between treatments using an analysis of variance (ANOVA). We used Tukey's (HSD) tests to

compare treatment means where significant differences ($p < 0.05$) were found with the ANOVA. We arcsine transformed the data on survivorship before the analysis, and after transformation the data met the parametric assumptions of normality and homogeneity of variance.

Results

Embryos exposed to infected *O. mykiss* or unmanipulated hatchery *O. mykiss* had less hatching success than embryos exposed to uninfected *O. mykiss* or nothing (Table 1 & Fig. 1a). There was no difference in hatching success between embryos exposed to uninfected *O. mykiss* and embryos exposed to nothing ($p = 0.947$). The hatching success of embryos from the treatments of infected fish ($p < 0.001$) and unmanipulated hatchery fish ($p \leq 0.035$) were different from both control and uninfected treatments, respectively. There was also a difference in hatching success between embryos exposed to infected *O. mykiss* and embryos exposed to unmanipulated hatchery *O. mykiss* ($p = 0.05$).

Similarly, embryos raised on soil exposed to infected *O. mykiss* had less hatching success than embryos raised on soil exposed to uninfected *O. mykiss*, soil exposed to unmanipulated hatchery *O. mykiss*, and soil exposed to nothing (Table 1 & Fig. 1b). There were, however, no differences between uninfected fish, unmanipulated hatchery fish, and the control treatment ($p > 0.190$).

There were also clear differences in the hatching success of embryos exposed to different isolates of *S. ferax*. Embryos exposed to *S. ferax* isolated from both sites where *Saprolegnia* outbreaks had been observed experienced 12.6–13.7% less hatching success than embryos exposed to either *S. ferax* from sites where embryo mortality has remained relatively low or to the control with no *S. ferax* (Table 1 & Fig. 2).

Dead embryos from both the infected fish and unmanipulated hatchery fish treatments became covered with a crown of white hyphal filaments characteristic of *Sap-*

Table 1. Analysis of variance on percent survival for *Bufo boreas* exposed to infected or uninfected fish (fish exposure experiment), infected or uninfected soil (soil exposure experiment), or different strains of *S. ferax* (virulence experiment).

Source of variation	df	F	p
Embryo exposure experiment			
treatment	3	16.18	0.001
error	16		
Soil exposure experiment			
treatment	3	7.01	0.003
error	16		
Virulence experiment			
treatment	4	7.83	0.002
error	20		

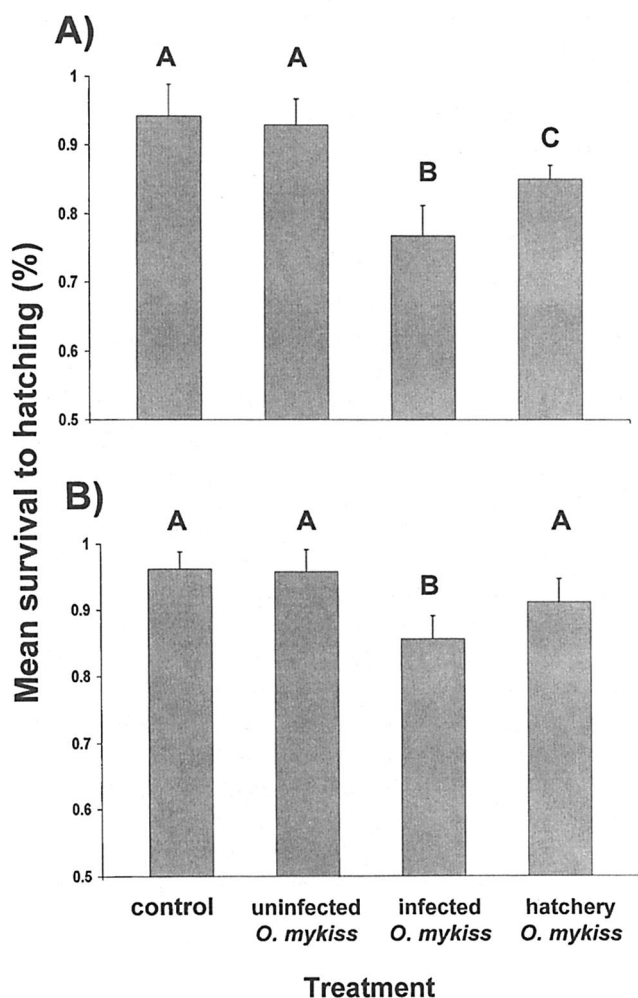


Figure 1. Mean (± 1 SE, $n = 5$) hatching success of *Bufo boreas* embryos exposed to potential *Saprolegnia* infection in experiments designed to examine (a) whether fish transmit *Saprolegnia* to developing amphibian embryos and (b) whether fish transmit *Saprolegnia* to soil.

rolegnia infection (Smith et al. 1985; Blaustein et al. 1994a; Kiesecker & Blaustein 1997a). *Saprolegnia ferax* was isolated and positively identified from all unmanipulated hatchery fish and infected embryos.

Discussion

We demonstrated the existence of a relationship between exposure of western toad embryos to *O. mykiss* or to soil that was exposed to *O. mykiss* and *S. ferax* infections that result in mortality. *Saprolegnia ferax* infections developed on embryos exposed directly to fish experimentally infected with *S. ferax* or to hatchery fish that were not manipulated. This suggests that either infected or seemingly healthy hatchery fish may transmit

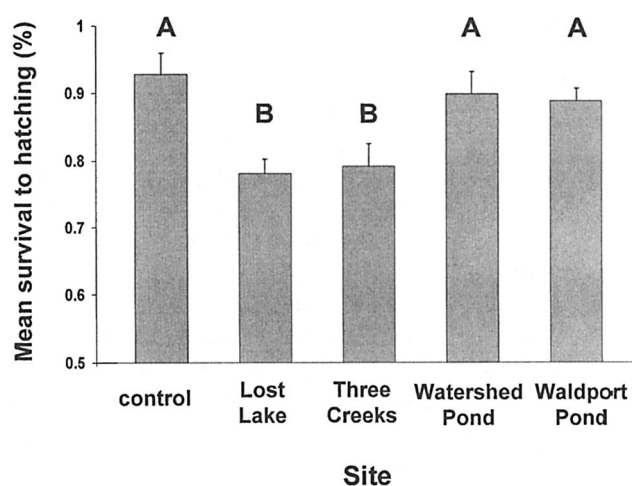


Figure 2. Mean (± 1 SE, $n = 5$) hatching success of *Bufo boreas* embryos exposed to isolates of *Saprolegnia ferax* from two sites with high embryo mortality (Lost Lake and Three Creeks) and two sites with low embryo mortality (Watershed Pond and Waldport Pond).

S. ferax directly to developing amphibian embryos. Embryos exposed to experimental soil, however, developed significant *S. ferax* infections only when the soil was exposed to fish experimentally infected with *S. ferax*. Thus, *S. ferax* may become established in soil only when it is exposed to heavily infected fish. Although our results do not rule out other potential sources of *Saprolegnia* introduction in the wild, they do identify one potential artificial source of *S. ferax* in the ponds we sampled.

Our experiments revealed significant differences in hatching success among treatments. These differences were relatively small however, resulting in only about a 15% decrease in survivorship between experimentally infected and control treatments. Our previous work demonstrates the complex interaction between exposure to environmental stress (UV-B radiation) and outbreaks of *S. ferax* (Kiesecker & Blaustein 1995). Thus, we did not anticipate high mortality in our laboratory experiments. Our main goal with these experiments was to determine whether hatchery fish could serve as a vector for *S. ferax*. Given that fish stocking did occur at the sites we sampled, it is important to understand that fish are a potential avenue for *S. ferax* introduction.

The results of the experiment designed to assess variability in the virulence of *S. ferax* indicate that embryos exposed to different isolates of *S. ferax* experience different levels of mortality. Embryos exposed to isolates from sites where embryo mortality had been high had higher mortality than those exposed to isolates from sites where embryo mortality had been relatively low. Because embryos were exposed to the isolates under identical conditions, the differences in mortality may have been due to inherent characteristics of the isolates. The ob-

served differences in mortality may have been the result of several factors. First, sites historically may have contained strains of *S. ferax* that naturally differed in virulence. Alternatively, a novel, highly virulent strain of *S. ferax* may have been introduced to these sites. Given the small number of populations surveyed, it is unclear whether this is a general pattern, but fish stocking is common practice at sites where *Saprolegnia* outbreaks have been observed in recent years (Blaustein et al. 1994a; Kiesecker & Blaustein 1997b).

It is unknown whether *S. ferax* is a non-native Oomycete in our aquatic environments or if it has always been present. Unfortunately, historical records do not exist for most oomycetes. Regardless of the origin of *S. ferax* at our sites, fish stocking could influence the density of *S. ferax*, which in turn could increase infection rates. Given the practice of transporting and introducing hatchery-reared fishes for commercial and sport fishing, introduced fishes may be an effective mechanism for rapidly introducing novel pathogens to native species over wide geographic areas. The common practice of repeatedly restocking the same site could allow for dissemination of new strains of pathogens as they emerge.

Historically, in the western contiguous United States, as much as 45% of the 16,000 mountain lakes have been stocked with fish (Bahls 1992). In some areas of the western United States, fish stocking is still common practice. During 1998–1999, for example, lakes in the Cascade Mountains of Oregon were stocked with over 10 million rainbow trout (*O. mykiss*, native to western United States), over 480,000 brook trout (*Salvelinus fontinalis*, native to eastern North America), and over 100,000 brown trout (*Salmo trutta*, native to Europe and Asia) from fish hatcheries (John Leppink, personal communication). Introduced fishes have been implicated in the decline of a variety of native species, including fishes and amphibians (e.g., Bradford 1989; Minckley & Deacon 1991; Bradford et al. 1993; Gamradt & Kats 1996; Kiesecker & Blaustein 1998).^{*} The effect of introduced fishes on native species will likely persist with their continued introduction. If introduced pathogens become established, effects could persist even after fish stocking has been discontinued.

An apparent increase in the mortality rates of *B. boreas* has occurred since the late 1980s (e.g., Blaustein et al. 1994a; Kiesecker & Blaustein 1997b). Some of the increase may be due to the introduction of *S. ferax* from hatcheries where agents used in controlling this pathogen have changed, perhaps altering the effectiveness of control. For example, malachite green was often the primary agent used to control the growth of pathogenic water molds, including *Saprolegnia* (Schreck et al. 1993). Due to suspected teratogenicity, it was banned in 1991 (Schreck et al. 1993), so fish may be reared in hatcheries where *Saprolegnia* is not controlled as well as when malachite green was in use.

Like many amphibian species, the western toad has experienced severe declines throughout its historical range (Corn et al. 1989; Carey 1993). Several factors, including interaction with introduced predators, increased prevalence of disease, and exposure to ultraviolet radiation have all been suggested as potential causes of the decline (Corn et al. 1989; Blaustein et al. 1994b; Blaustein & Kiesecker 1997). Although it is unlikely that a single factor can explain all losses, disease outbreaks have been implicated in several mass-mortality events among western toads (Carey 1993; Blaustein et al. 1994a, Kiesecker & Blaustein 1997b). In addition, the introduction of exotic species such as predatory fish could influence the outcome of important host-pathogen interactions by either acting as a stressor of host organisms or transporting pathogens. These types of indirect effects of exotic species are rarely considered (Simberloff 1981). Western toads breed in habitats that would potentially expose them to direct interaction with exotic fishes. But fish predation is not likely to be important to survival of the western toad because *B. boreas* larvae are noxious to fish (e.g., Kiesecker et al. 1996). Thus, any effect that introduced fishes have on *B. boreas* is likely to be the result of indirect interactions. Assessing the effects of introduced fish on the western toad may provide a model system with which to measure the indirect effects of exotic species on native species.

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