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Water molds of the genera *Saprolegnia* and *Leptolegnia* are pathogenic to the North American frogs *Rana catesbeiana* and *Pseudacris crucifer*, respectively

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ABSTRACT: Water molds are commonly associated with amphibian mortality. Since water molds often act as saprophytes, it is important to test their effects on amphibians to determine whether they can also act as pathogens. In controlled experiments, the eggs of 2 amphibian species, the American bullfrog *Rana catesbeiana* and the spring peeper *Pseudacris crucifer*, suffered higher mortality when they were exposed to zoospores of water molds of the genera *Saprolegnia* and *Leptolegnia*, respectively. Water molds are important pathogens in many amphibian systems, yet their ecological impact on amphibians remains mostly unknown.

KEY WORDS: Water molds · *Rana catesbeiana* · *Pseudacris crucifer* · Amphibian disease · *Saprolegnia* · *Leptolegnia*

INTRODUCTION

Water molds of the Family Saprolegniaceae are associated with amphibian egg mortality worldwide (Blaustein et al. 1994). Although many species of the family are saprophytes (Johnson et al. 2002), some will opportunistically act as pathogens, particularly on weakened hosts (Kiesecker & Blaustein 1995, Lefcort et al. 1997). In the past several decades, amphibian species and populations have declined dramatically (Houlahan et al. 2000, Stuart et al. 2004) and the cause of many of these declines are due to the emergence of infectious diseases (Berger et al. 1998, Kiesecker et al. 2001a, Collins & Storfer 2003, Lips et al. 2006). Although many types of microbes have been associated with dead and moribund amphibians encountered in the field, controlled experiments that test the ability of these microbes to infect otherwise healthy individuals are necessary to determine if they are the cause of death and should be of concern to conservation biologists (Koch 1932, Berger et al. 1998, Romansic et al. 2006, 2007, Karraker & Ruthig 2009).

Water molds of the Phylum Oomycota (Kingdom: Stramenopila; Alexopolous et al. 2000), Infrakingdom Heterokonta (Baldauf et al. 2000) or Kingdom Chromista (Petrisko et al. 2008) are ubiquitous in freshwater systems. Water molds of the Family Saprolegniaceae are known pathogens of invertebrates (Martin 1981, Oidtmann et al. 1999, 2004) and vertebrates (Bangyeekhun et al. 2003, Robinson et al. 2003). There are 2 modes by which they can infect amphibian eggs. The first is by biflagellate zoospores that are produced by specialized hyphal tips called zoosporangia. Zoospores are chemotactic and will swim to suitable substrates where they attach and grow into hyphal colonies (Johnson et al. 2002). Hyphae can grow out from infected eggs and infect neighboring eggs (pers. obs.). Eggs laid in large masses, such as *Rana catesbeiana* eggs, may be more likely to be infected by hyphae growing from their infected neighbors (Green 1999).

Water mold infections have been associated with egg mortality on species of amphibians in many places around the world, including: *Crinia signifera* in Aus-

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tralia (Williamson & Bull 1994), *Rana arvalis*, *R. temporaria* and *Bufo bufo* in Europe (Robinson et al. 2003, Sagvik et al. 2008a,b), *R. sphenocephala* in southeastern North America (Ruthig 2008), *B. americanus* and *R. sylvatica* in northeastern North America (Gomez-Mestre et al. 2006) and *R. cascadae* and *B. boreas* in northwestern North America (Blaustein et al. 1994, Kiesecker & Blaustein 1995). To date, the eggs of *Hyla regilla* (Kiesecker & Blaustein 1995), *R. clamitans* (Karraker & Ruthig 2009) and *Ambystoma maculatum* (Gomez-Mestre et al. 2006, Karraker & Ruthig 2009) have been found to be resistant to infection when exposed to water molds in controlled experiments.

The impact of pathogenic water molds on amphibian populations has the potential to increase due to anthropogenic effects on the environment, including climate change, pollution and human transport of pathogens. The ability of water molds to grow and produce flagellated zoospores is dependent on temperature, pH and dissolved oxygen (Cotner 1930, Willoughby & Copland 1984, Ruthig 2006) and their ability to become parasitic is enhanced if their host is weakened (Kiesecker & Blaustein 1995). For example, salamander adults are more susceptible to *Saprolegnia parasitica* in water polluted by motor oil (Lefcort et al. 1997), and frog eggs are more susceptible to infection under exposure to ambient levels of UV-B radiation in shallow water (Kiesecker & Blaustein 1995, Kiesecker et al. 2001b). Temperature also affects the susceptibility of amphibian eggs to infection (Ruthig 2006, 2008, Sagvik et al. 2008b). Water molds are also important pathogens of overcrowded and injured fish in fisheries (El-Sharouny & Badran 1995) that may potentially carry pathogenic strains of water molds to naïve amphibian populations (Kiesecker et al. 2001b). Since many environmental factors can kill amphibians by themselves and water molds quickly colonize dead amphibians (pers. obs.), controlled experiments are critical to determine the ecological impact of water molds on their amphibian hosts (Karraker & Ruthig 2009).

I observed water molds growing on various organisms, including dead tadpoles, frogs, newts, insects and amphibian eggs at Riopel Pond at the Mountain Lake Biological Station in western Virginia. In the field, it is difficult to determine whether water molds are acting as pathogens or if they are colonizing organisms only after they die. To determine the ecological role of water molds on amphibian eggs I exposed the eggs of the American bullfrog *Rana catesbeiana* and the spring peeper *Pseudacris crucifer* to water molds that I isolated from colonies growing on the eggs of each species in the field. I compared the mortality of eggs exposed to water molds to eggs that were exposed to a sterile control. I also tested whether the density of *R. catesbeiana* eggs affected their likelihood of becoming infected. I predicted that eggs that were exposed to water molds would be more likely to die before they hatched and that high densities of eggs would make them more likely to become infected.

MATERIALS AND METHODS

Isolation and identification of water molds. In July 2002 I collected an infected *Rana catesbeiana* egg at Riopel Pond at the Mountain Lake Biological Station (MLBS; Giles County, Virginia, USA). The embryo within the egg had stopped developing and was consumed by white water mold hyphae. Once these hyphae were isolated (Ruthig 2008), this culture was considered an isolate of a single strain and was the parent line in all of the experiments using *R. catesbeiana* eggs as hosts.

I also collected a water mold isolate from a *Pseudacris crucifer* egg. Since *P. crucifer* eggs are laid individually and are difficult to find in the field, I collected a male and a female *P. crucifer* adult in May 2002 and placed them in a plastic bin with approximately 2 l of water collected from Riopel Pond. Overnight the female laid several hundred eggs that developed at room temperature in the laboratory. A small number of the eggs developed water mold infections. I collected and isolated a strain of water mold in the same manner as the isolate from the *Rana catesbeiana* egg.

Determining the species of water molds anatomically is impossible without the presence of sexual structures, which can be difficult to obtain (Willoughby 1985, 1994, Woo & Bruno 1999, Hulvey et al. 2007). Therefore, I sequenced the internal transcribed spacer (ITS) regions of the ribosomal DNA of the strains used in the experiments. ITS sequences are commonly used as characteristics for the taxonomy of Saprolegniaceae (Hulvey et al. 2007, Petrisko et al. 2008) and have been sequenced in many other types of organisms (Lee & Taylor 1992, Vilgalys & Sun 1994, Bridge et al. 1998, LeClerc et al. 2000, Gemeinholzer et al. 2006). Amplification reactions consisted of 1 mM of each primer, 1 mM $MgCl_2$, 3.2 mM buffer, 1.6 mM of each dNTP, 4 U of *Taq* polymerase, and 1 µl of extracted water mold DNA in a volume of 10 ml. The amplifications were performed with the following steps: an initial denaturation at 94°C for 3 min followed by 35 cycles of 60 s at 94°C, 30 s at 50°C, and 1 min at 72°C. A final extension at 72°C for 7 min completed the amplifications. After amplification using PCR, the ITS region (primers ITS-1, 5′-TCC GTA GGT GAA CCT GCG G-3′; and ITS-4, 5′-TCC TCC GCT TAT TGA TAT GC-3′) of the water mold ribosomal DNA was sequenced on an Applied Biosystems® 377 Prism DNA Sequencer at the University of Virginia Biomolecular Research Facility.

Using Mega 4.0 (Tamura et al. 2007), I aligned 525 bp from the ITS region of the isolate collected from the *Rana catesbeiana* egg and 619 bp from the isolate collected from the *Pseudacris crucifer* egg with homologous ITS regions from reference samples acquired from Genbank. I compared my isolates to known water mold reference samples from the genera *Achlya*, *Leptolegnia* and *Saprolegnia* (see Petrisko et al. 2008). To determine the taxonomy of the isolates I collected, I performed a distance analysis of my isolates and the reference samples using the Jukes-Cantor model of Mega 4.0. A bootstrap analysis of the neighbor-joining tree was performed with 1000 replicates.

Zoospore experiments. This experiment was designed to determine if the presence of water mold zoospores would lead to higher mortality rates in *Rana catesbeiana* eggs. The design was a 2 × 4 factorial with 2 inoculation treatments, a water mold addition treatment and a sterile control, that were crossed with 4 density treatments consisting of 1, 2, 4 and 8 *R. catesbeiana* eggs. All treatments were placed in 70 ml plastic cups. A 1 × 1 cm piece of sterile cornmeal agar was added to each cup in the control treatments, and a 1 × 1 cm piece of cornmeal agar with 3 d old water mold growth was added to each cup in the water mold addition treatments. Three days before the eggs were added, the agar and 60 ml of pond water that had been boiled to remove pathogens were added to each cup. Adding the agar 3 d before the start of the experiment presumably allowed the water mold hyphae to release zoospores into the water before the eggs were added. On 10 July 2002 I collected *R. catesbeiana* eggs from a single egg mass that was laid the previous night in Riopel Pond. I separated the eggs individually using a disposable plastic pipette and a knife to cut through the jelly. In order to remove potential pathogens that had attached to the eggs prior to collection, I rinsed the eggs for approximately 10 sec in boiled pond water before adding them to the treatments.

The 8 treatments (2 inoculation treatments × 4 density treatments) were randomly distributed within blocks. There were a total of 63 blocks in a temperature-controlled room at MLBS. The temperature was set at a constant 20°C with a 12:12 h light:dark cycle. I checked the eggs daily for hatchlings and new infections. The experiment concluded after all of the eggs had either hatched or died. Dead eggs ceased to develop and were covered in white hyphae within 48 h.

On 15 May 2003, I tested the effect of water molds on the eggs of *Pseudacris crucifer*. The eggs were laid in plastic bins with pond water by the female of a pair of adults that were captured the previous night. Both the pond water and the adults were collected from Riopel Pond. In this experiment, I added only 1 egg to each cup. Three days prior to adding the eggs, I added a piece of agar to each cup with the water mold collected from an infected *P. crucifer* egg, or a sterile piece of agar as a control. I replicated each treatment 134 times and arranged the cups randomly on plastic trays in a temperature-controlled room at MLBS that was held at 20°C.

Statistics. I used Proc Genmod (SAS 9.1) with a binomial distribution and logit link function to test for differences in the number of infected eggs among the inoculation and density treatments in the *Rana catesbeiana* and *Pseudacris crucifer* experiments, and to test for an interaction between the effects of inoculation and density in the *R. catesbeiana* experiment.

RESULTS

The sequences from the samples collected from *Pseudacris crucifer* and *Rana catesbeiana* were submitted to Genbank (accession nos. EU071706 and EU071707, respectively). The phylogeny created in the distance analysis suggests that the water mold isolate collected from *R. catesbeiana* is a member of the genus *Saprolegnia* and the isolate collected from *P. crucifer* is a member of the genus *Leptolegnia*, with strong support (bootstrap = 100) in both cases (Fig. 1).

Significantly more *Rana catesbeiana* eggs died when exposed to *Saprolegnia* zoospores (41%) than when exposed to a pasteurized control (5%) (χ^2 = 228.33, df = 1, $p < 0.001$, Fig. 2). There was no significant effect of the density of eggs within a cup (χ^2= 1.24, df = 3, p = 0.74) and no interaction between the water mold treatment and density (χ^2= 7.24, df = 3, p = 0.06). Most new infections became apparent on the second day of development; the rate of new infections slowed after Day 2 (Fig. 3). After hatching, every individual survived until the end of the experiment on Day 8.

The addition of water mold zoospores also increased mortality of eggs of *Pseudacris crucifer*. *P. crucifer* eggs that were exposed to zoospores of *Leptolegnia* had 12% mortality, which was significantly higher than the 3% that died in the pasteurized control treatment (χ^2 = 278.74, df = 1, p = 0.002).

DISCUSSION

This is the first study to show that water molds can be pathogenic to the eggs of *Rana catesbeiana* and *Pseudacris crucifer*. I also identified a genus of water mold, *Leptolegnia*, which has never been recognized as a pathogen of amphibians. Aquatic water molds are ubiquitous in freshwater systems, and in many cases they act as pathogens on amphibian eggs. Since they

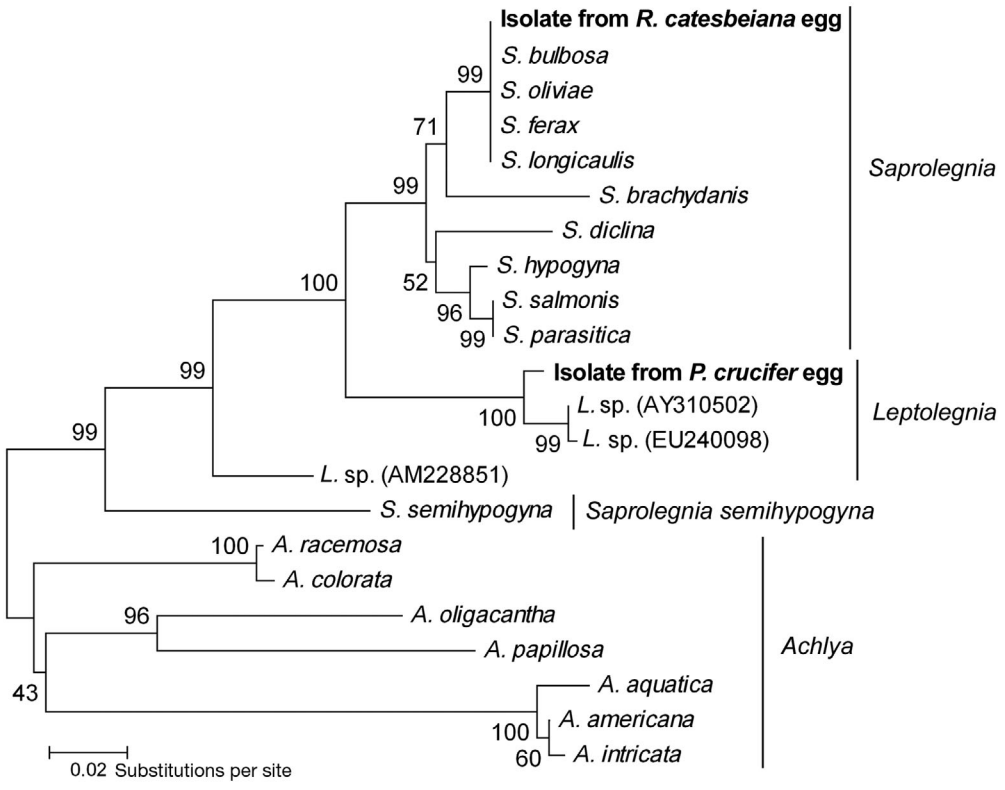

Fig. 1. *Rana catesbeiana* and *Pseudacris crucifer*. Jukes-Cantor neighbor-joining distance tree for Saprolegniaceae isolates from the 2 isolates collected from *R. catesbeiana* and *P. crucifer* eggs (noted in bold) and reference samples obtained from GenBank (n = 18). Species names for the *Leptolegnia* reference samples were not known so the GenBank accession numbers are noted in parentheses

may also act as saprobes, controlled studies that test the effect of water molds are necessary to elucidate their ecological role.

The experiments fulfilled the first 3 of Koch's 4 postulates for determining if a microorganism causes a disease (Koch 1932): (1) the organism is found on diseased organisms, but not healthy organisms; (2) the organism is isolated and grown in pure culture; (3) the organism causes disease when it is exposed to a healthy host; and (4) the organism can be reisolated from the experimentally infected host. Unfortunately, the fourth postulate was not fulfilled because the isolates from the experimentally infected eggs died before DNA was extracted from them, making it impossible to know if they were the same strain of water mold that killed the eggs. However, the overall appearance of infected eggs was identical to naturally infected eggs in the field. Also, the large difference in mortality between the water mold addition treatments and the control treatments suggests that the water molds acted as pathogens on the eggs of *Rana catesbeiana* and *Pseudacris crucifer*.

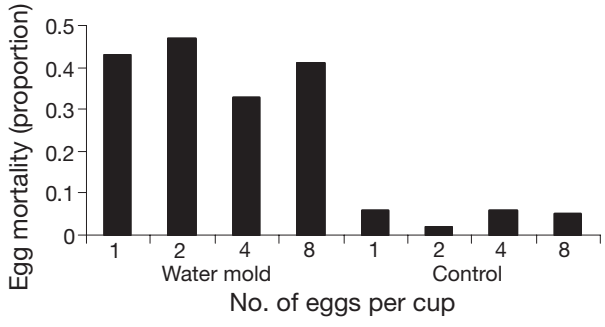

Fig. 2. *Rana catesbeiana*. Proportion of *R. catesbeiana* eggs that became infected before hatching in each treatment. Egg mortality represents the proportion of mortality over all of the eggs in a treatment (n = 63). Water mold treatments consisted of eggs in water containing a piece of cornmeal agar with the water mold *Saprolegnia* growing on it. Control treatments consisted of eggs in water containing a sterile piece of agar

The levels of mortality of the *Rana catesbeiana* eggs in the zoospore experiments were about 40%, which is high when compared to natural mortality (about 10%) (Ruthig 2006) and the mortality of *Pseudacris crucifer* eggs (12%); this may be due to the fact that the jelly layer around the eggs was disturbed. *R. catesbeiana* eggs within an egg mass share a continuous jelly, whereas *P. crucifer* eggs are laid individually. By sepa-

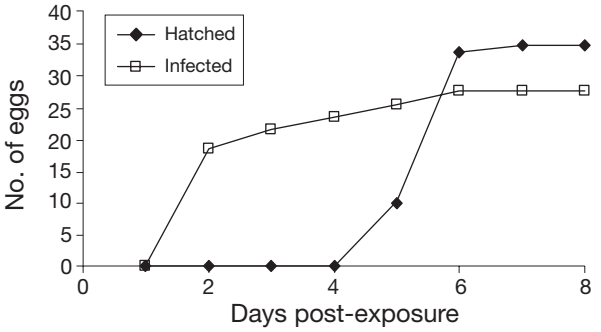

Fig. 3. *Rana catesbeiana*. Cumulative number of *R. catesbeiana* eggs that had hatched or had become infected in the single egg treatments when exposed to zoospores

rating the *R. catesbeiana* eggs I disrupted their protective jelly layer and likely made them more susceptible to infection (Gomez-Mestre et al. 2006). A potential way to test the impact of separating *R. catesbeiana* eggs on their susceptibility to pathogenic water molds would be to include an additional control with a known saprophytic water mold and compare the mortality of the eggs exposed to the saprophytic and pathogenic strains. In similarly conducted experiments, different strains of water molds had drastically different effects on *R. catesbeiana* eggs, ranging from being almost completely benign to killing up to 80% of the eggs that were exposed (K. Provost & G. Ruthig unpubl. data). The high survivorship of the eggs in the sterile control of this experiment also suggests that separating the eggs did little harm to the embryos and that the water molds were pathogenic.

The density of *Rana catesbeiana* eggs within a cup did not affect their likelihood of infection. At the temperature at which the experiment was conducted, all of the eggs were able to hatch within 4 to 6 d (Fig. 3). This may have not been enough time for hyphae from infected eggs to reach neighboring eggs (Ruthig 2006, 2008). Therefore, the infected eggs in the experiment may have been primarily infected by the water mold zoospores from the initial inoculation and host density would not have affected their probability of being infected.

Amphibians have several defense mechanisms against water mold infection. Parents may lay their eggs in locations where they are less likely to become infected (Ruthig 2006, 2008). The eggs also contain their own defense mechanisms that protect them (Sagvik et al. 2008a,b), including the jelly surrounding the eggs (Gomez-Mestre et al. 2006) and phenotypic plasticity in hatching time of eggs exposed to water molds (Touchon et al. 2006). These defenses can break down as the abiotic environment changes (Kiesecker et al. 2001a, Ruthig 2006, Touchon et al. 2006), leaving amphibian populations vulnerable to global climate change.

As amphibian populations suffer worldwide declines (Stuart et al. 2004), identifying the sources of amphibian mortality is important. Water molds represent a group of organisms that have likely coexisted with amphibian eggs for millennia, but their ecological relationship may change as the world's climate changes (Kiesecker et al. 2001a). Further research on the effect of water molds on amphibians is necessary to help predict their future impact on amphibian populations.

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