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AMPHIBIAN EMBRYO AND PARENTAL DEFENSES AND A LARVAL PREDATOR REDUCE EGG MORTALITY FROM WATER MOLD

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Abstract. Water molds attack aquatic eggs worldwide and have been associated with major mortality events in some cases, but typically only in association with additional stressors. We combined field observations and laboratory experiments to study egg stage defenses against pathogenic water mold in three temperate amphibians. Spotted salamanders (*Ambystoma maculatum*) wrap their eggs in a protective jelly layer that prevents mold from reaching the embryos. Wood frog (*Rana sylvatica*) egg masses have less jelly but are laid while ponds are still cold and mold growth is slow. American toad (*Bufo americanus*) eggs experience the highest infection levels. They are surrounded by thin jelly and are laid when ponds have warmed and mold grows rapidly. Eggs of all three species hatched early when infected, yielding smaller and less developed hatchlings. This response was strongest in *B. americanus*. Precocious hatching increased vulnerability of wood frog hatchlings to invertebrate predators. Finally, despite being potential toad hatchling predators, *R. sylvatica* tadpoles can have a positive effect on *B. americanus* eggs. They eat water mold off infected toad clutches, increasing their hatching success.

Key words: Ambystoma; Bufo; *hatching; inducible defenses; Oomycete; pathogens; phenotypic plasticity; predation;* Rana; Saprolegnia; *vernal pools; water mold.*

Introduction

Many organisms experience high mortality rates in their early life stages, as a consequence of predation, infection, and harsh abiotic conditions (Wilbur 1980, Gosselin and Qian 1997). To reduce such mortality, a variety of organisms have evolved egg defenses through parental effects. Widespread parental defenses that reduce risk of predation or parasitism in both vertebrates and invertebrates include nesting site choice (Mappes et al. 1994, Edgerly et al. 1998, Stav et al. 1999, Marco et al. 2001, Rieger et al. 2004, Resetarits 2005) and egg-guarding behavior (Mappes et al. 1995, Asoh and Yoshikawa 1996, Winkelman 1996, Ryotaro Hara et al. 2003, Klug et al. 2005). Parents can also modulate hatching timing to improve offspring survival, as in spitting spiders (Li 2002), or provide eggs with maternally derived egg toxins, as in rough-skin newts (Hanifin et al. 2003). Parents also determine the physical structure of the clutch, which can protect the eggs from predators and abiotic stresses (Ward and Sexton 1981, Strathmann 2000, Warkentin et al. 2006), and may use external items such as leaves to cover and hide their eggs (Marco et al. 2001, Orizaola and Braña 2003, Perez-Santigosa et al. 2003). In contrast to the variety of parental defenses described, relatively few studies have examined embryonic defenses per se and only one type of embryonic defense has been demonstrated. In some species the embryos adjust their hatching time to balance egg and tadpole stage risks and improve survival (Sih and Moore 1993, Warkentin 1995, Moore et al. 1996, Warkentin 2000, Wedekind 2002, Kusch and Chivers 2004, Moreira and Barata 2005).

Water molds of the family Saprolegniaceae cause substantial mortality for aquatic eggs of a wide range of fish and amphibian species throughout the temperate world (Blaustein et al. 1994, Czeczuga et al. 1998, Kiesecker et al. 2001). Infections by *Saprolegnia ferax* have contributed to amphibian declines via embryo mortality in the Pacific northwestern United States (Blaustein et al. 1994, Kiesecker et al. 2001). Oomycete infections have also been observed in Britain on *Rana temporaria*, *Bufo calamita*, *B. bufo*, and *Triturus vulgaris* eggs (Banks and Beebee 1988, Beattie et al. 1991, Green 1999, Robinson et al. 2003) and in Spain on *B. bufo* and *B. calamita* eggs (I. Gomez-Mestre, *personal observation*). Major outbreaks of water mold infection of amphibian eggs have only been reported to occur when mold acted synergistically with other environmental stressors, such as UV-B radiation or unusually cool conditions (Kiesecker and Blaustein 1995, Beebee 1996, Robinson et al. 2003), suggesting that mechanisms exist to prevent massive infection under less stressful conditions. Interspecific differences in egg-laying behaviors could be important determinants of differential infection rate by water molds. For example, communal egg masses laid by *Rana cascadae* and *Bufo boreas* are at a greater infection risk than clutches laid singly (Kiesecker and Blaustein 1997, Green 1999), and water molds spread from egg to egg

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within *Triturus vulgaris* clutches more readily than among eggs laid singly (Green 1999). Early hatching in response to pathogens is known in embryos of five vertebrates (Warkentin et al. 2001, Wedekind 2002, Moreira and Barata 2005, Touchon et al. 2006), but it is unclear if such responses are common and widespread or isolated cases.

Here we combine a field survey with laboratory experiments to examine the incidence of pathogenic mold infection on embryos of three temperate amphibians and the potential defenses that eggs may have against infection. We studied spotted salamanders (*Ambystoma maculatum*), wood frogs (*Rana sylvatica*), and American toads (*Bufo americanus*), all of which breed in vernal pools at our study site in Lynn Woods, in northeastern Massachusetts, USA. Amphibian communities in vernal pools follow an annual sequence of recolonization. *Rana sylvatica* and *A. maculatum* breed soon after ponds thaw and refill with spring rainfall and melting snow, while *B. americanus* breeds weeks later (Petranka et al. 1994, Paton and Crouch 2002). Breeding phenology shapes many of the ecological interactions in amphibian communities through priority effects (Alford and Wilbur 1985, Dayton and Fitzgerald 2005). However, changes in the environment, such as increased water temperature and primary productivity, as well as concomitant shifts in predator abundance, also interact with the breeding sequence to alter interspecific interactions (Wilbur 1987, Skelly et al. 2002). Thus, despite the importance of breeding phenology in structuring amphibian communities, the intensity and nature of interactions can be affected by changes in the abiotic environment (Warner et al. 1993), predation (Morin 1981, Crossland 2000), or the presence of pathogens (Kiesecker and Blaustein 1999, Parris and Beaudoin 2004, Parris and Cornelius 2004). For example, by the time *B. americanus* eggs hatch, *R. sylvatica* tadpoles are approximately five-fold bigger than *B. americanus* hatchlings and actively prey on them, so that adult *B. americanus* tend to avoid ovipositing in ponds with abundant *R. sylvatica* (Petranka et al. 1994). However, we observed *R. sylvatica* tadpoles feeding on water mold growing on *B. americanus* clutches. By eating water mold off the toad eggs, *R. sylvatica* may reduce the spread of infection through the clutch.

In this study we tested the following hypotheses: (1) Pathogenic water molds are present in vernal pools and cause mortality of amphibian eggs; (2) eggs of different species vary in their vulnerability to pathogenic infection due to clutch structure; (3) water mold growth is positively correlated with water temperature; (4) embryos respond to infection by hatching when younger and less developed; (5) early hatching increases vulnerability to hatchling predators; (6) *R. sylvatica* tadpoles increase hatching success of infected *B. americanus* clutches.

Methods

Study organisms

Our three study species are very distantly related amphibians. They vary in their breeding phenology, preferred oviposition microhabitats, and clutch structure (see Appendix A). *Ambystoma maculatum* lays oval-shaped clutches adhered to twigs, branches, or aquatic plants at mid-water column, each containing 120–200 eggs surrounded by a thick jelly coat. Amphibian eggs have several concentric gelatinous capsules surrounding each embryo, up to eight layers in ambystomatid salamanders (Duellman and Trueb 1986). In *A. maculatum* these individual capsules are surrounded by a tough outer layer of jelly that envelopes the entire clutch, leaving a 4–10 mm thick solid zone with no embryos (Pinder and Friet 1994). The compactness of the outer jelly matrix may be a barrier to oxygen diffusion, but symbiotic algae present in the jelly augment the oxygen supply for the embryos (Pinder and Friet 1994). *Rana sylvatica* lays both single and communal egg masses. Clutches are spherical, contain 1200–2000 eggs, and are adhered to branches a few centimeters below the surface. The egg mass is surrounded by a thin outer jelly layer (1–2 mm thick) and traversed by channels, allowing convective flow of water and oxygen (Pinder and Friet 1994). *Bufo americanus* also lays both single and communal masses, deposited in a thin gelatinous string (1–1.5 mm thick). Each clutch contains 4000–12 000 eggs and is laid draped over the substrate (emergent vegetation or leaf litter) in shallow, sunny areas of the pond.

Field survey

To assess the incidence and impact of water mold infection on eggs of our three study species, we surveyed nine vernal pools in Lynn Woods Reservation (Lynn, Massachusetts, USA) in spring 2005. We surveyed ponds twice weekly from 21 March to 7 June, flagging and monitoring all amphibian clutches found except for those few collected for laboratory experiments. Clutches were monitored until they had completely hatched. We visually estimated the degree of infection within each clutch as the percentage of eggs overgrown by water mold (see Plate 1). Eggs that are unfertilized or die due to developmental abnormalities are likely to become infected by mold that may be saprophytic rather than pathogenic. We considered clutches to be infected by pathogenic molds when hyphal growth extended over contiguous eggs over 5% or more of the clutch and increased over subsequent censuses, spreading to live embryos. Date of oviposition, hatching date, and proportion of the clutch infected was recorded for 61 *A. maculatum*, 86 *R. sylvatica*, and 72 *B. americanus* egg masses.

In order to determine the presence or absence of water molds in the ponds, we sank five tea bags filled with sterilized (boiled) hemp seeds in each pond as water

mold baits (Kiesecker and Blaustein 1995, Robinson et al. 2003). After 10 days, the bags were retrieved and plated on a sterilized (autoclaved) cornmeal agar medium on Petri dishes and grown in incubators at 11°C under a 10:14 light : dark cycle. Water molds cultured from these ponds belonged to the genera *Saprolegnia* and *Achlya* (J. Longcore, *personal communication*).

Temperature and water mold growth

To quantify the effect of temperature on water mold growth, we pooled the infected seeds collected from two ponds and plated two randomly selected seeds on cornmeal agar in each of 24 Petri dishes. We chose three temperature regimes to reflect the range of natural water temperatures experienced by *R. sylvatica* and *A. maculatum* eggs when laid (6.5°–8.5°C, night–day), by *B. americanus* eggs when laid (11°–15°C), and by *A. maculatum* eggs late in development (15°–18°C). Eight dishes were assigned to each temperature regime and were maintained in different incubators. We measured mold growth daily for five days, in increments of the surface area occupied by mold hyphae.

Water mold infection experiments

We quantified the effect of water mold infection on egg viability and hatching timing in *A. maculatum*, *R. sylvatica*, and *B. americanus* by inoculating eggs with field-collected water mold. All experiments were carried out in environmental chambers with controlled temperature and humidity. Groups of eggs were placed in carbon-filtered tap water and inoculated with infected hemp seeds with actively growing hyphae. All water mold used was field collected using previously sterilized hemp seed baits, grown on cornmeal agar in sterilized Petri dishes for at least one week, and transferred to clutches on newly infected hemp seeds. The same culture was used for all experimental infections. We did not isolate and purify the culture, and thus it remained a multispecies one, similar to samples retrieved from the study ponds. We used randomized block designs, with each incubator shelf as a block and all treatments replicated within each block. All experiments were checked daily until all eggs had either hatched or died, recording survival and hatching date.

Ambystoma *experiments*

In early April 2005, we collected eight newly laid *A. maculatum* clutches, with embryos at Harrison stage 11–12 (blastopore formation; Harrison 1969), and brought them to the laboratory. We split each clutch (120 eggs ± 12.7, mean ± SE) in half, placed each half in a different 500-mL glass beaker, and randomly assigned each beaker to either control or infection treatments. The infection treatment consisted of six mold-infected seeds deposited on the outer jelly of the clutch, while sterilized, noninfected seeds, were added to the controls. The beakers were held on two shelves of an incubator so that each shelf held four split clutches in both treatments. Temperature was set to 6.5°–8.5°C (night–day) and was gradually increased up to 15°–18°C as ponds in our field site grew warmer. All replicates were checked daily, recording egg survival and hatching.

Ward and Sexton (1981) showed that the jelly coating surrounding *A. maculatum* eggs protects them from predation. Previous observations by I. Gomez-Mestre and J. C. Touchon suggested that the thick outer jelly surrounding *A. maculatum* eggs may also be a barrier to mold growth. To test this hypothesis, we ran a complementary infection experiment on embryos removed from the outer jelly. We collected six more clutches and carefully extracted 16 eggs from each, removing them from the outer jelly cover with forceps, while leaving the thinner layer of jelly (~1.5–2 mm thick) surrounding the individual eggs intact. Each embryo was placed individually in a plastic cup filled with 115 mL of water. Eight embryos per clutch were held as controls, with two uninfected hemp seeds, and eight were inoculated with two infected seeds. All replicates were checked daily, recording egg survival and hatching. We preserved up to the first 10 hatchlings from each treatment in buffered 10% formalin. Preserved hatchlings were staged and digitally photographed through a dissecting microscope, then measured for total length using Image J version 1.33 (National Institutes of Health, Bethesda, Maryland, USA).

Rana *and* Bufo *experiments*

We collected six *R. sylvatica* clutches on 4 April and 5 April and six *B. americanus* clutches on 21 April. Embryos collected from both species were at Gosner stages 11–12 (mid-gastrula; Gosner 1960). We removed 20 groups of 10 eggs from each clutch and placed each group into a plastic cup with 115 mL of water. Cups were randomly assigned to either control or infection treatments, for a total of 10 replicates per treatment per clutch for each species. Water mold treatment eggs were inoculated with two infected hemp seeds with visible hyphal growth. The temperature was maintained at 6.5°–8.5°C (night–day) for *R. sylvatica* and at 11°–15°C for *B. americanus*. All replicates were checked daily, recording egg survival and hatching. In both *R. sylvatica* and *B. americanus*, the first hatchling from the first four replicates to hatch per clutch and treatment was preserved and later staged, photographed, and measured. Comparisons of the developmental stage and total length of hatchlings in the two treatments were based on these preserved animals. All other variables (survival, hatching time) were analyzed using one value per replicate (either proportion or mean) to avoid pseudoreplication (Hurlbert 1984).

Predation trials

To test for costs of mold-induced early hatching, we conducted predation trials on *R. sylvatica* and *B.*

americanus hatchlings of different ages. The only abundant tadpole predators in the ponds when these species hatched were backswimmers (Notonectidae), which we observed congregating around hatching clutches in the field. We collected 70 backswimmers from vernal pools and brought them to the laboratory. We ran predation trials in round, 21 cm diameter plastic containers containing 2 L of water and three oak leaves to provide substrate and refuge. The hatchlings tested came from the infection experiments described above. Each trial consisted of five synchronous hatchlings from the same clutch, pooled across replicates, and three backswimmers. We carried out 69 trials for *R. sylvatica* and 60 for *B. americanus*, using hatchlings from both infection and control treatments and throughout the range of hatching ages. Predation trials lasted for 24 h and predators were always starved for at least one day before a trial. Hatchling survival, age, and stage were recorded.

Effect of R. sylvatica *tadpoles on infected* B. americanus *eggs*

To test whether *R. sylvatica* tadpoles affect the hatching success of infected *B. americanus* clutches, we collected portions of four new *B. americanus* clutches (stages 13–14; Gosner 1960) and co-occurring *R. sylvatica* tadpoles (total length 19.2 ± 0.4 mm; mean ± SE) on 26 April. In an environmental chamber, we set up 40 plastic containers holding 6 L of carbon-filtered tap water plus 1 L of pond water and added 75 g of pond leaf litter covered in periphyton. The chamber was kept at a constant temperature of 13°C and with a 10:14 light : dark photoperiod. Ten strings of 20 *B. americanus* eggs were removed from each clutch. Each string was randomly placed in a container and assigned to one of four treatments in a full factorial design crossing presence/absence of *R. sylvatica* tadpoles with presence/absence of four heavily mold-infected hemp seeds. The containers were distributed on shelves so that each shelf held two replicates of each treatment. We monitored *B. americanus* embryo survival through four censuses over 16 days until all embryos had either died or hatched, and we made sporadic observations of *R. sylvatica* behavior.

Statistical analyses

All analyses were carried out with SAS version 8 (SAS Institute 1999). Clutch and block effects were considered random factors, and their contribution to model fit was assessed through the Akaike Information Criterion (AIC). We found no significant block effects, and its inclusion did not contribute to improve model fit, so block was therefore removed from the final analyses. Clutch effect was never significant but occasionally improved the model fit. Clutch was therefore included in the analyses when it lowered the AIC values.

Embryo survival data showed no significant deviations from a binomial distribution in any of the experiments and was thus analyzed fitting linear mixed models with an underlying binomial distribution and a logit link function, using the GLIMMIX macro. Hatching time and morphological data met parametric assumptions in all cases and were analyzed with PROC MIXED. Hatching time, developmental stage, and hatchling length are correlated variables. Therefore, we adjusted the significance level for tests on the preserved hatchlings for each species controlling the false discovery rate (FDR; Garcia 2004, Verhoeven et al. 2005) using the method described by Benjamini and Hochberg (1995). Differences in developmental stages across treatments were analyzed nonparametrically with Mann-Whitney *U* tests. No data transformations were necessary.

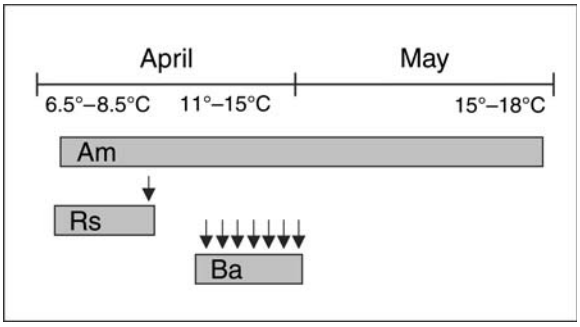

FIG. 1. Breeding phenology of *Ambystoma maculatum* (Am), *Rana sylvatica* (Rs), and *Bufo americanus* (Ba). Bars indicate the time span over which embryos of each of the species were present in the ponds. The arrowheads indicate when infected clutches were found. Temperatures given indicate the range of temperatures across the nine ponds experienced by eggs of each species when laid and by *A. maculatum* eggs late in development (15°–18°C). We surveyed nine vernal pools in Lynn Woods Reservation, Lynn, Massachusetts, USA.

RESULTS

Field survey

Water mold (*Saprolegnia* sp. and *Achlya* sp.) was found in all ponds surveyed. Eight of the nine ponds had high infection rates, with 66–86% of seed baits infected, but seed baits from the largest pond with the thickest canopy and the lowest mean temperature showed only 16% infection rate. *Ambystoma maculatum* bred between 3 and 9 April, and its embryonic period lasted on average 49 ± 1.25 d (mean ± SE; Fig. 1). *Rana sylvatica* bred between 3 and 11 April and had an embryonic period of 14 ± 0.26 d. All *R. sylvatica* clutches hatched before *B. americanus* bred between 18 and 25 April (Fig. 1). The embryonic period for *B. americanus* was 11 ± 0.22 d.

We found no infected *A. maculatum* clutches in any of the ponds, and mean egg mortality was only 2% (range 0–25% across clutches), even though we found infected clutches of other species in the same ponds at the same time. We found water mold infection in 7% of *R. sylvatica* clutches. Mean mortality for those infected *R.*

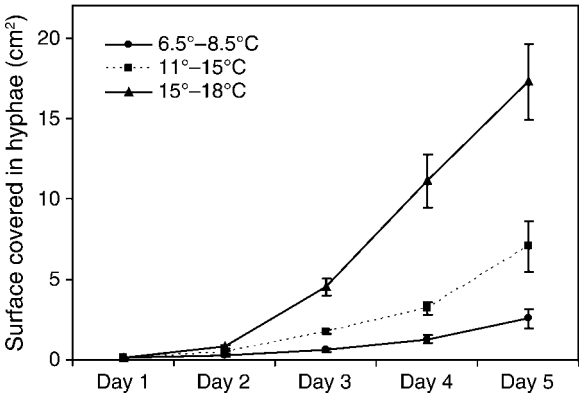

FIG. 2. Mold growth (mean ± SE) at each of three temperature regimes. The coldest temperature regime simulated pond temperatures experienced by *Rana sylvatica* and *Ambystoma maculatum* when they breed. The intermediate regime was set to temperatures experienced by *Bufo americanus* eggs. The warmest regime reflects temperatures that *A. maculatum* eggs experience later in the season due to their long embryonic period. Growth rates varied significantly with the temperature of incubation.

sylvatica clutches was 27.9%, but ranged from 5% to 80%. The one clutch with 80% mortality became infected early in development (at formation of the neural folds, Gosner stage 14). The other five infected clutches (5–20% mortality range) were among the latest *R. sylvatica* clutches to hatch (between 18 and 22 April). In *B. americanus* 62.5% of clutches were infected, and mean mortality of the infected clutches was 25% (range 5–90%). In contrast, survival in uninfected clutches was above 95% in all three species, except for two *A. maculatum* clutches that were partially predated, probably by mallards (*Anas platyrhynchos*), judging from the markings on the jelly. These predated clutches were not included in our survival estimates.

Temperature and water mold growth

Temperature significantly affected water mold growth (repeated measures ANOVA, $F_{2,19} = 35.72$, $P < 0.0001$). Water mold grew 2.7 times faster (measured in square centimeters per day) at 11°–15°C (night–day), the water temperature during the period when *B. americanus* breeds, than at cooler temperatures measured during *R. sylvatica* breeding (6.5°–8.5°C; Fig. 2). The higher temperatures experienced by *A. maculatum* eggs later in the season (15°–18°C) caused a 6.7-fold increase in mold growth rate compared to the coldest regime.

Ambystoma *experiments*

When *A. maculatum* eggs retained their protective external jelly coat, survival was very high and controls were statistically indistinguishable from the water mold infection treatment (0.99 ± 0.01, 0.98 ± 0.06 for control and infection treatments, respectively; Fig. 3). Mold grew slightly over the jelly surface, but the hyphae failed to penetrate it and in no case did it reach the embryos (see Appendix D). Hatching time, developmental stage at hatching, and hatchling size were not significantly different between treatments in the split-clutch design.

When individual eggs were removed from the clutch, with its jelly coat, mold infection increased mortality by 79% compared to uninfected eggs ($\chi^2_{1,88} = 45.49$, $P < 0.0001$; Fig. 3). Mold growing over eggs without a jelly coat easily penetrated the egg capsule, with hyphae reaching and killing the embryos without causing a general degradation of egg capsules. The only six surviving embryos in the infection treatment hatched significantly earlier than the controls (23.6% earlier on average; $F_{1,43} = 8.94$, $P < 0.005$). Infection treatment hatchlings had gill circulation but lacked forelimb buds (i.e., Harrison stages 36–37; Harrison 1969), while control hatchlings had forelimb buds and elongated gills (Harrison stage 38, see Fig. 4), although these differences in developmental stage were marginally nonsignificant (Mann-Whitney $U = 80$, $P = 0.035$; $\alpha' = 0.033$). In spite of differences in developmental stage, there was no difference in hatchling length between treatments ($F_{1,14} = 2.75$, $P = 0.12$).

Removal of the eggs from their external jelly coat did not alter development per se. Both uninfected treatments (controls for each experiment, with and without jelly coat) were very similar in their developmental stage (Harrison stage 38) and mean hatching times (36.2 ± 0.7 d and 34.2 ± 1.1 d, for eggs in jelly and eggs removed from jelly, respectively).

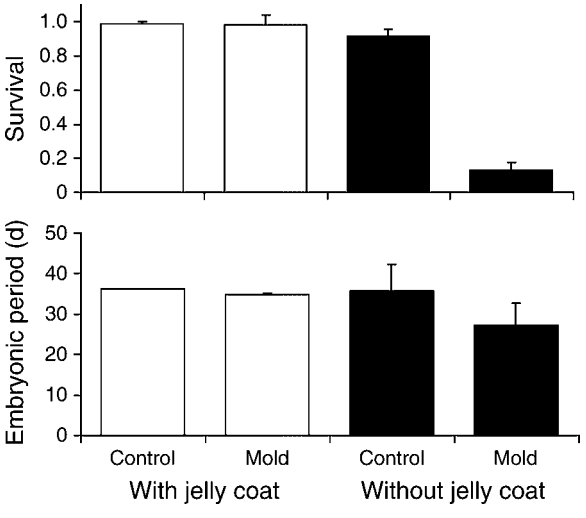

FIG. 3. Survival and embryonic period (mean ± SE) for split *Ambystoma maculatum* clutches exposed to water mold infection. Open bars represent clutches in which the outer jelly coat was left undisturbed, and solid bars represent unprotected embryos removed from their jelly coat. Embryos removed from the jelly coat and exposed to water mold had higher mortality, and hatched earlier, than both uninfected eggs and eggs exposed to water mold but protected by the natural clutch structure.

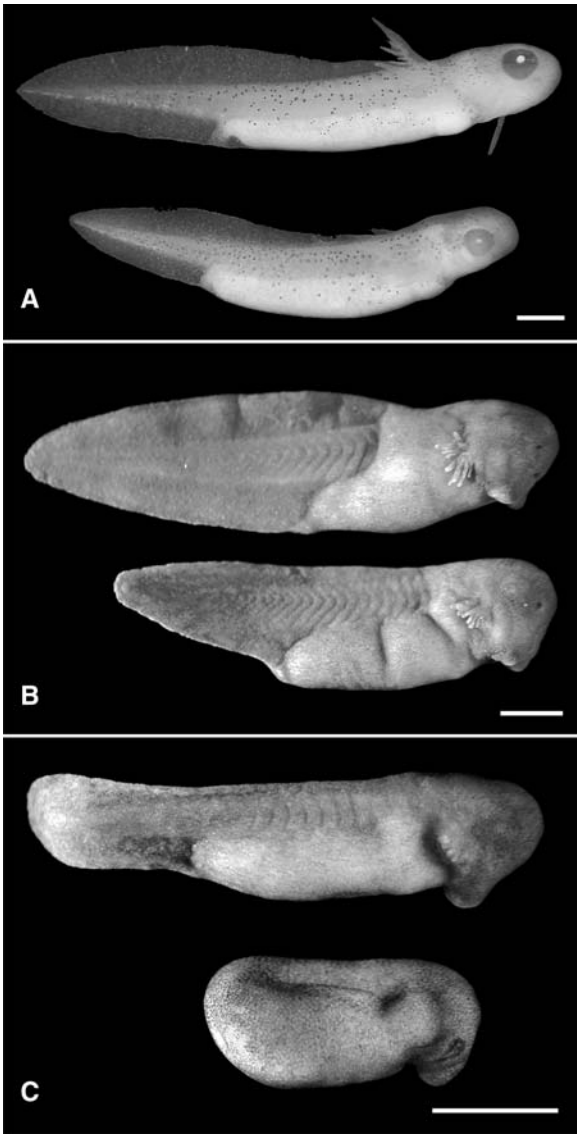

FIG. 4. Examples of spontaneous and mold-induced early hatchlings (upper and lower, respectively, in each frame) for (A) *Ambystoma maculatum*, (B) *Rana sylvatica*, and (C) *Bufo americanus*. Embryos were chosen to reflect the modal size and developmental stage of individuals in their respective treatments and do not represent the developmental extremes of hatching. Scales bars are 1 mm. The images were converted to grayscale, contrast was adjusted, and background was made uniform with Adobe Photoshop 8.0 (Adobe Systems, San Jose, California, USA).

Rana *and* Bufo *experiments*

Mold infection reduced *R. sylvatica* egg survival by 28.8% ($\chi^2_{1,104} = 35.44$, $P < 0.0001$), but had no significant effect on *B. americanus* survival in this experiment ($\chi^2_{1,93} = 0.79$, $P = 0.375$; Fig. 5). Infection induced early hatching in both species. *Rana sylvatica* embryos in the infection treatment hatched 13% earlier than controls ($F_{1,105} = 28.53$, $P < 0.0001$; Fig. 5). This shift in hatching time caused early hatchlings to be slightly but significantly less developed than controls (Mann-Whitney $U = 171$, $P = 0.046$, $\alpha' = 0.05$). The first hatchlings preserved from each replicate showed that infected treatment hatchlings had much less developed gills than control hatchlings (early stage 19 vs. early stage 20, Gosner 1960; Fig. 4) and were 19% shorter (6.73 ± 0.15 mm vs. 8.28 ± 0.16 mm total length, respectively, for mold-induced and control hatchlings; $F_{1,42} = 50.49$, $P < 0.0001$, $\alpha' = 0.033$). *Bufo americanus* eggs exposed to mold infection hatched on average 36% earlier than the controls ($F_{1,91} = 149.7$, $P < 0.0001$). First hatchlings from each replicate were significantly less developed in the infection treatment than in the control (Mann-Whitney $U = 80$, $P < 0.0001$, $\alpha' = 0.05$). Hatchlings from the infection treatment were early in the tailbud stage, lacking muscular response (Gosner stage 17), while the controls showed muscular response and had differentiated gill arches (Gosner stage 19; Fig. 4). Infection treatment hatchlings were on average 32% shorter in total length than the controls (2.82 ± 0.20 mm vs. 4.14 ± 0.20 mm, for mold-induced and control hatchlings, respectively; $F_{1,45} = 21.68$, $P < 0.0001$, $\alpha' = 0.033$).

Predation trials

Survival of *R. sylvatica* hatchlings in the presence of notonectids was significantly affected by age and clutch infection by water mold ($\chi^2_{1,60} = 8.48$, $P < 0.004$; $\chi^2_{1,60} = 10.12$, $P < 0.002$, respectively). Nonetheless, the relationship with age was not linear, and the model required a quadratic term (age^2, $\chi^2_{1,60} = 9.07$, $P < 0.003$). Survival tended to be low for precocious hatchlings

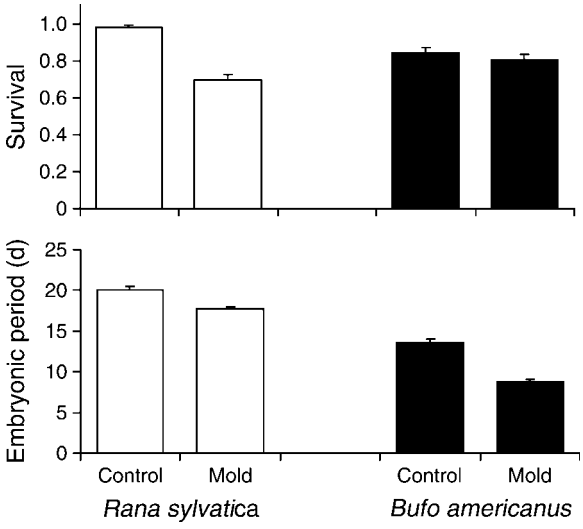

FIG. 5. Survival and embryonic period (mean ± SE) for *Rana sylvatica* (open bars) and *Bufo americanus* (solid bars) exposed to water mold infection. For both species, water mold infection caused significantly early hatching when compared to controls. Only for *R. sylvatica* did water mold infection cause significantly increased mortality in this experiment.

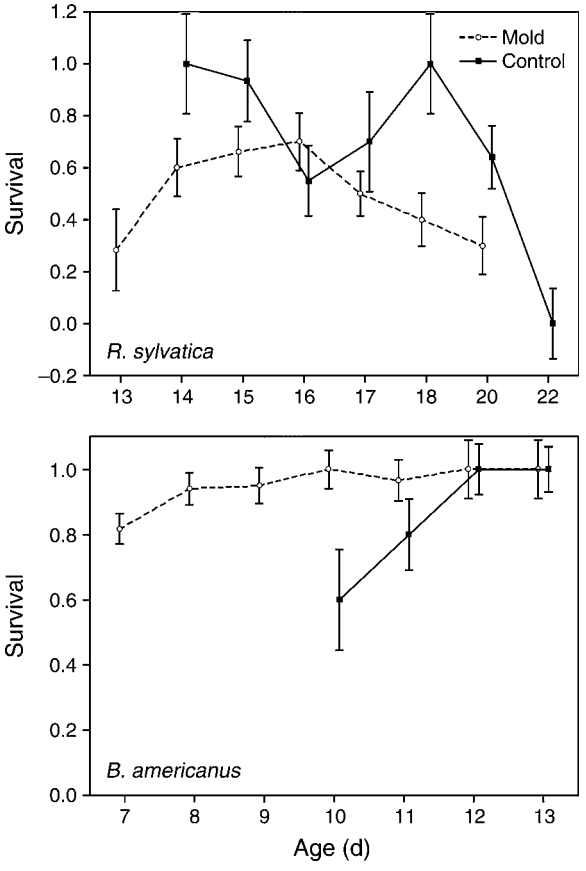

FIG. 6. Survival (mean ± SE) of (A) *Rana sylvatica* and (B) *Bufo americanus* hatchlings of different ages with backswimmers. Solid squares indicate control hatchlings; open circles indicate hatchlings from water mold infection treatments.

(more abundant in the infection treatment than in the control), higher for young spontaneous hatchlings, and even lower for older spontaneous hatchlings (Fig. 6). This pattern was similar for both treatments, but the infected treatment had consistently lower survival values.

Survival of *B. americanus* hatchlings was significantly affected by age ($\chi^2_{1,52} = 8.07$, $P < 0.005$), but survival increased linearly with age. Younger hatchlings seemed to experience lower survival than older ones (Fig. 6), but experimental treatment or its interaction with age had no significant effect on *B. americanus* survival.

Bufo *infection in the presence of* Rana *tadpoles*

Experimental treatments differed significantly in *B. americanus* hatching success ($\chi^2_{3,36} = 12.06$, $P = 0.007$; Fig. 7). Post-hoc tests revealed that only the infection treatment had significantly lower embryo survival than the control treatment (25% lower survival on average; Tukey test $P = 0.001$). The treatment with both water mold and *R. sylvatica* was not different from the control ($P = 0.237$) and was significantly higher than the treatment with water mold alone ($P < 0.04$). *Rana sylvatica* tadpoles were observed eating mold hyphae off the seeds and off infected *B. americanus* eggs, but were never observed eating toad eggs themselves (see Appendix E).

DISCUSSION

Despite the high prevalence of water mold across the vernal pools we studied, a combination of parental and embryonic defenses helped prevent infection and reduce egg mortality once infection had occurred. We also found experimental evidence for a beneficial effect of a hatchling predator on toad embryos at risk of mold infection.

Water mold infection of amphibian eggs

Saprolegnia and *Achlya* water molds were present in all vernal pools examined, but their effect on amphibian eggs varied across species. Although *A. maculatum* co-occurred in ponds with infected clutches of the other species, we found no sign of infection on *A. maculatum* clutches, despite their long embryonic period exposing them to water temperatures that facilitate rapid water mold growth (Figs. 1 and 2). The outer jelly coat surrounding *A. maculatum* eggs provided a physical barrier that effectively protected embryos from mold infection. When removed from the protective jelly coat, *A. maculatum* embryos suffered high mortality from water mold infection (Fig. 3). Interestingly, the few surviving embryos that had been removed from their jelly coat and exposed to the water mold hatched earlier and slightly less developed than uninfected ones (Fig. 4), although presence of water mold did not alter hatching time of eggs in normal jelly masses (Fig. 3).

Only 7% of *R. sylvatica* clutches observed in vernal pools were infected with water mold, and infection was apparent only towards the end of the embryonic period, coinciding with warmer temperatures. In addition to the thin capsules surrounding *R. sylvatica* eggs, the outer jelly layer is a rather loose matrix, featuring channels that traverse the clutch and allow water to penetrate and oxygenate the inner eggs within the clutch (Pinder and

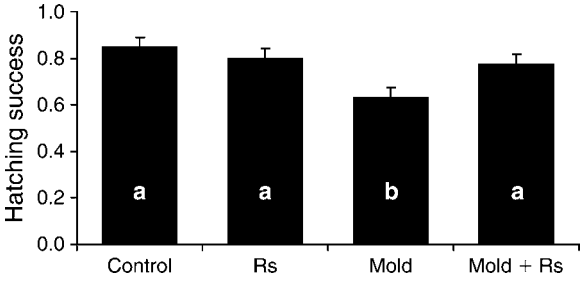

FIG. 7. Hatching success (mean ± SE) of *Bufo americanus* eggs in the presence of either *Rana sylvatica* tadpoles (Rs), water mold (Mold), or both (Mold + Rs). Treatments with the same letter were not significantly different from one another. *Bufo americanus* eggs infected with water mold and without *R. sylvatica* tadpoles had significantly diminished hatching success, whereas infected eggs paired with *R. sylvatica* tadpoles did not.

PLATE 1. *Bufo americanus* eggs infected with pathogenic water mold in a pond in Lynn, Massachusetts, USA. Photo credit: I. Gomez-Mestre.

Friet 1994). This potentially exposes even interior eggs to pathogens. However, *R. sylvatica* emerges from hibernation very early in the season and breeds soon after the ponds thaw, while temperatures are still very low. This early parental breeding behavior, combined with a short embryonic period, ensures that *R. sylvatica* embryos generally develop when water temperatures inhibit rapid mold growth (Figs. 1 and 2). Infected *R. sylvatica* clutches suffered varying degrees of mortality depending on the developmental stage when they were infected, but the mean mortality of infected clutches was below 30%. *Rana sylvatica* embryos hatched 13% early in response to experimental pathogen inoculation (Fig. 5), resulting in smaller and slightly less developed hatchlings. Early hatching seemed to come at a cost, as precocious hatchlings were more vulnerable to backswimmer predation than most spontaneous hatchlings. However, late hatchlings were also vulnerable to backswimmer predation (Fig. 6), presumably due to increased activity with age, which would draw the backswimmer's attention to them (Skelly 1994). There was also a significant effect of embryo treatment independent of age, suggesting that embryos exposed to mold may be at a greater overall risk of predation. This could be a synergistic effect of combined stresses, as has been reported for other amphibians at different life stages (Kiesecker and Blaustein 1995, Relyea and Mills 2001, Parris and Beaudoin 2004).

More than 60% of *B. americanus* clutches surveyed in ponds were infected with water mold (see Plate 1), but the mean mortality of infected clutches was only 25%, suggesting that most embryos were able to escape before being killed by the pathogen. In fact, water mold did not cause a significant increase in *B. americanus* mortality in one of the laboratory experiments, despite a relatively high water temperature that facilitates rapid mold growth (Figs. 2 and 5). *Bufo americanus* eggs hatched the most precociously of the three species (36% earlier than controls) in response to mold infection, resulting in very small hatchlings (Fig. 4). These early induced hatchlings are not yet capable of muscular contraction and are thus incapable of swimming. However, such early hatchlings were only slightly more vulnerable than later ones, suggesting that the cost of pathogen-induced early hatching for this species in the face of backswimmer predation is likely to be low. In fact, early hatchlings (10 d of age) from the control treatment experienced lower survival than equal-aged hatchlings from mold treatments, suggesting that the effect of mold exposure on hatchlings may be quite different for different species.

The use of a multispecies mold culture provided an inoculum representative of the mold present in the study area, but could also have caused a decrease in pathogenicity over time if competition displaced the most pathogenic strains. However, we observed no evidence that pathogenicity decreased over the course of our experiments. Due to the breeding phenologies of the three species, experiments on *A. maculatum* were conducted first, then those on *R. sylvatica*, and then those on *B. americanus*. Although mold killed fewer *B. americanus* than *R. sylvatica* embryos in the initial infection experiments, the same culture caused substantial *B. americanus* mortality in the last infection experiment, where presence of *R. sylvatica* was also manipulated. This suggests that the culture had not lost its pathogenicity, although there may have been hetero-

geneity in pathogenicity among different hemp seed batches.

Although some pathogens can degrade egg jellies (Halpern et al. 2003), early hatching in these three species was clearly an embryonic response to mold infection and not a side effect of mold degradation of the egg membranes. *Bufo japonicus* embryos develop hatching glands at Gosner stage 17 (Yamasaki et al. 1990), which was precisely the earliest developmental stage at which we observed precocious hatching in *B. americanus*. Embryos at developmental stages prior to development of hatching glands (Gosner stage 17) were never found out of their egg capsules over the course of mold infection and were killed confined within the egg capsules, with the capsules intact except for the micropunctures caused by hyphal penetration (see Appendices B and C).

In these three species, both parental traits (breeding phenology and egg mass structure, controlled by the ovipositing female) and embryo traits (developmental rate and inducible hatching) combine to reduce pathogen infection. *Ambystoma maculatum* has a long embryonic period (~50 d) and is exposed to temperatures that enable rapid mold growth, but the thick jelly coat provided by the mother prevents water mold from invading the clutch. *Rana sylvatica* eggs lack such a protective covering, but due to the early breeding phenology of the adults and their short embryonic period, they develop in cold water that inhibits water mold growth. Moreover, *R. sylvatica* embryos show a moderate acceleration of hatching in response to water mold infection. Like *R. sylvatica*, *B. americanus* eggs lack a thick protective jelly coating. In addition, *B. americanus* breeds when water temperatures have risen and mold grows quickly. However, *B. americanus* hatching is highly plastic and eggs hatch up to 36% early in response to infection.

The fate of early life stages is important for population dynamics and ultimately evolutionary history of a lineage (Chatterton and Speyer 1989, Eckman 1996, Gosselin and Qian 1997). However, embryos have traditionally been thought of as a defenseless life stage or defended solely by parental behavior or provisioning. We now have growing evidence that some embryos can actively respond to threats. Hatching plasticity constitutes a defensive mechanism in at least some frogs, salamanders, lizards, fishes, and spiders (Sih and Moore 1993, Warkentin 1995, 2000, Vonesh 2000, Chivers et al. 2001, Laurila et al. 2002, Li 2002, Johnson et al. 2003, Kusch and Chivers 2004, Moreira and Barata 2005). Adaptive hatching plasticity could be present in many more taxa, both vertebrates and invertebrates, if environmental conditions impose variable risks on the embryonic phase, the larval phase, or both. Emphasis has been placed on studying adaptive hatching plasticity in the context of anti-predator defenses, but it is also likely to be effective in response to environmental heterogeneity in other factors (abiotic, pathogens, competition). Pathogens and parasites affect the structure and functioning of ecological communities (Price et al. 1986, Poulin 1999, Thomas et al. 2000). However, there are only a few reported cases of pathogen-induced early hatching (Warkentin et al. 2001, Wedekind 2002, Moreira and Barata 2005, Touchou et al. 2006), and therefore it is still unclear how common it is.

Water mold is unlikely to impose selection for early hatching in *A. maculatum* in our study area, since the thick outer jelly coat prevents mold from reaching the embryos. Despite this, partially de-jellied *A. maculatum* eggs hatch early in response to infection. One explanation is that pathogen-induced hatching in *A. maculatum* reflects a response to a more general cue, such as oxygen stress, and that the plasticity evolved under some other selection pressure (nonspecific response hypothesis). Another possibility is that pathogen-induced early hatching evolved in a more vulnerable ancestor and has been retained in *A. maculatum* (phylogenetic inertia hypothesis). However, other *A. maculatum* populations experience higher vulnerability to mold infection (Tennessen and Zamudio 2003, Myers and Zamudio 2004). Therefore the selective pressure on hatching plasticity may vary geographically, and it may occur where it is not selected due to gene flow among populations (geographic mosaic hypothesis).

Induced early hatchlings of all three species appear perfectly viable and develop normally. In the laboratory, initial posthatching survival and development appeared similar for early induced and spontaneously hatching embryos. In the field, we observed early *B. americanus* hatchlings in ponds survive infection, develop to the free-feeding larval stage, and feed on the water mold hyphae that had consumed part of their own clutch. We also observed *R. sylvatica* feeding upon mold growing over *B. americanus* clutches in the ponds. Due to their phenological differences, *R. sylvatica* tadpoles are at least five-fold bigger than *B. americanus* hatchlings and become toad tadpole predators (Petranka et al. 1994). However, the presence of *R. sylvatica* did not significantly reduce *B. americanus*' hatching success in our experiment.

Pathogens may alter the outcome or intensity of interactions between tadpoles and their predators (Parris and Beaudoin 2004) or between competing amphibian species (Kiesecker and Blaustein 1999). The reverse may also be true, where the presence of a competitor/predator affects the likelihood of pathogenic infection. In our experiment, *B. americanus* embryo survival was reduced by mold unless *R. sylvatica* was also present, seemingly rescuing the toad eggs from the effects of mold (Fig. 7). Petranka et al. (1994) reported that *R. sylvatica* tadpoles in western North Carolina, USA, voraciously predated on *B. americanus* eggs, causing severe mortality in their experimental ponds, to the point that adult *B. americanus* avoided breeding in ponds containing *R. sylvatica* tadpoles. In our study, we found no evidence for predation by *R. sylvatica* on *B.*

americanus eggs in our laboratory experiments or field surveys (nor even in pilot experiments in which we housed toad eggs in bare containers with wood frog tadpoles). We have, however, observed *R. sylvatica* predation on toad hatchlings. The geographic differences in the interaction between these two species might have several causes (e.g., differences in toad egg palatability or in wood frog feeding habits) that require further study. At our study site, although they become predators later, *R. sylvatica* tadpoles appeared to play a beneficial role for infected *B. americanus* clutches, as they helped contain the spread of water mold by eating its hyphae. We confirmed the occurrence of this phenomenon in nature through direct observations of *R. sylvatica* tadpoles actively feeding on mold hyphae growing on infected *B. americanus* clutches (Appendix E). However, the magnitude of this beneficial effect under natural conditions and its balance with potential hatchling predation remains an open question that deserves further research.

Oomycetes are a common threat for aquatic embryos, but our study species have traits that function to reduce the risk of infection, as well as responses to infection that decrease mortality. Parental traits, such as breeding early in the year or providing eggs with a protective jelly, serve to decrease the risk of infection to a clutch. Hatching plasticity enables embryos to escape a clutch already infected with a pathogen.

These defenses appear to reduce the overall impact of water mold, so that massive egg mortality is normally only associated with a combination of multiple stressors. In addition, interactions with other species may further reduce the mortality of infected clutches. The effect of pathogenic water molds upon a given species will therefore depend not only on the effectiveness of the species' defenses but also on the community composition and the ecological interactions at work.

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APPENDIX A

Spotted salamander (*Ambystoma maculatum*), wood frog (*Rana sylvatica*), and American toad (*Bufo americanus*) adults and egg clutches, showing differences in clutch structure (*Ecological Archives* E087-154-A1).

APPENDIX B

Bufo americanus eggs infected with water mold, showing killed, prematurely hatched, and normally developing embryos (*Ecological Archives* E087-154-A2).

APPENDIX C

Close-up of a *Bufo americanus* embryo killed inside the egg capsule by water mold, and an embryo hatching precociously in response to water mold (*Ecological Archives* E087-154-A3).

APPENDIX D

Water mold growing on the surface of an *Ambystoma maculatum* clutch without penetrating the jelly to reach embryos (*Ecological Archives* E087-154-A4).

APPENDIX E

Rana sylvatica tadpoles feeding on water mold growing on *Bufo americanus* clutches in a pond in Lynn, Massachusetts, USA (*Ecological Archives* E087-154-A5).

APPENDIX F

Bufo americanus eggs on our laboratory experiment showing heavy mold infection in a replicate without *Rana sylvatica* tadpoles and healthy, mold-free eggs in two replicates in which *Rana sylvatica* tadpoles ate the mold (*Ecological Archives* E087-154-A6).